Drug target 5-lipoxygenase: A link between cellular enzyme regulation and molecular pharmacology

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

Vorgelegt beim Fachbereich für
Chemische und Pharmazeutische Wissenschaften
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
Lutz Fischer
aus Oberhausen/Rheinland

Frankfurt am Main (2005)
(DF1)

Vom Fachbereich Chemische und Pharmazeutische Wissenschaften der Johann-Wolfgang-Goethe Universität als Dissertation angenommen.

Dekan:	Prof. Dr. Harald Schwalbe
Gutachter:	Prof. Dr. Dieter Steinhilber
	PD Dr. Oliver Werz
Datum der Disputation:	13. Mai 2005

Meinen Eltern

Meiner Schwester Anne

Das schönste Glück des Menschen ist,
das Erforderliche erforscht zu haben und
das Unerforderliche ruhig zu verehren.

Johann Wolfgang von Goethe

TABLE OF CONTENTS

I. Abbreviations

AA	arachidonic acid
aa	amino acid
ADP	adenosine diphosphate
AKBA	3-O-acetyl-11-keto-**β**-boswellic acid
AM	alveolar macrophages
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BLT	LTB_4 receptors
C5a	complement C5a
CaMK	calcium/calmodulin-dependent kinase
cAMP	cyclic AMP
CLP	coactosin-like protein
COX	cyclooxygenase
cPLA$_2$	cytosolic phospholipase A_2
CTP	cytidine triphosphate
cysLT	cysteinyl leukotriene
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylendiaminetetraacetate
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
Grb2	growth factor receptor-bound protein 2
GSH	glutathione
H(p)ETE	hydro(pero)xyeicosatetraenoic acid
HeLa cells	epithelial cells derived from a human cervix carcinom

HL-60 cells	promyeloic leukemic cell line
HL-60TB	HL-60 cells negative for 5-LO
HpODE	hydroperoxyoctadecadienoic acid
Hsp27	heat shock protein 27
IL	interleukin
kDa	kilo Dalton
LO	lipoxygenase
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
LTA_4H	LTA_4-hydrolase
LTC_4S	LTC_4-synthase
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen-activated protein kinase-activated protein kinase
MBP	myelin basic protein
MEK	MAPK kinase
MEKK	MEK kinase
MGST	microsomal GSH-S-transferase
MK	MAPKAPK (mitogen-activated protein kinase-activated protein kinase)
MKK	MAPK kinase
MM6	Mono Mac 6
MNK	MAP kinase-interacting kinase
NFκB	nuclear factor **κ**B
NES	nuclear export signal
NIS	nuclear import sequence
NLS	nuclear localization sequence
NO	nitric oxide
OAG	1-oleoyl-2-acetylglycerol
PAF	platelet-activating factor
PC	phosphatidylcholine
PG	prostaglandin
PhGPx	phospholipid hydroperoxide glutathione peroxidase
PI 3-K	phosphatidyl inositol 3-kinase

PK	protein kinase
PL	phospholipase
PM	peritoneal macrophages
PMA	phorbol-12-myristate-13-acetate
PMNL	polymorphonuclear leukocytes
PPAR	peroxisome proliferator-activated receptor
RBL-1	rat basophilic leukemia
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	sodium arsenite
SH	src-homology
$sPLA_2$	secretory phospholipase A_2
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TRAP	TGFβ receptor-I-associated protein
U937 cells	lymphoblostoid cell line
UFA	unsaturated fatty acid
UTP	uridine triphosphate
$^{-/-}$	double knock out

II. Introduction

A. Lipoxygenases

1. Definition and nomenclature

Lipoxygenases (LOs) constitute a family of dioxygenases that catalyze the oxygenation of free and esterified polyunsaturated fatty acids containing a (1Z,4Z)-penta-1,4-diene system to produce the corresponding hydroperoxy derivatives [175]. They are widely expressed in plants, fungi, and animals but not in bacteria or yeast. In plants, they are involved in diverse aspects of plant physiology as growth and development, pest resistance and senescence or responses to wounding [346]. LOs are named according to the position in which molecular oxygen is incorporated in the main substrate arachidonic acid (AA). In some cases, also the stereoconfiguration and the tissue of occurrence are specified.

2. Family

When discovered in 1947 the first LO was referred to as lipoxidase, isolated and crystallised from soybean [336]. 12-(S)-hydroxyeicosatetraenoic acid (12-HETE) was the first product derived from a mammalian LO detected in 1974 when human platelets were treated with AA [129]. The first 5-LO was discovered in rabbit polymorphonuclear leukocytes (PMNL) [29]. To date, 18 different sequences of mammalian LOs are known and the enzymes are separated into four groups based on their enzymatic properties [176]. In humans, there are five enzymes, noted as reticulocyte-type 15(S)-LO [317], platelet-type 12(S)-LO [157], epidermis-type 12(R)-LO [27], epidermis-type 12(S)-LO [33], and 5(S)-LO (see figure 1) [216]. The whole family comprises enzymes with a molecular weight of 75-80 kDa in mammals and 94-104 kDa in plants.

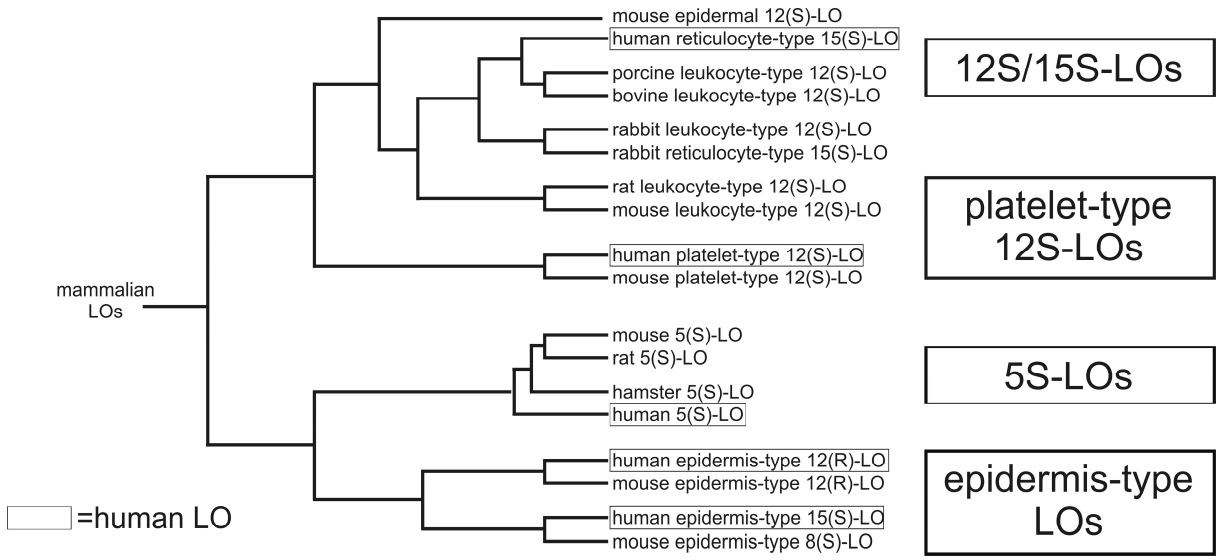


Figure 1: Phylogenetic tree of lipoxygenases

5-LOs consist of an N-terminal β-barrel domain plus a C-terminal catalytic domain containing a single atom of nonheme iron [32]. In general, LOs exert their biological functions via three different routes: First, mobilisation of lipids, second, peroxidation reactions resulting in structural and pathophysiological changes, and third, signalling pathways leading to formation of LTs and HETEs (see figure 2) (for detailed review see [32]).

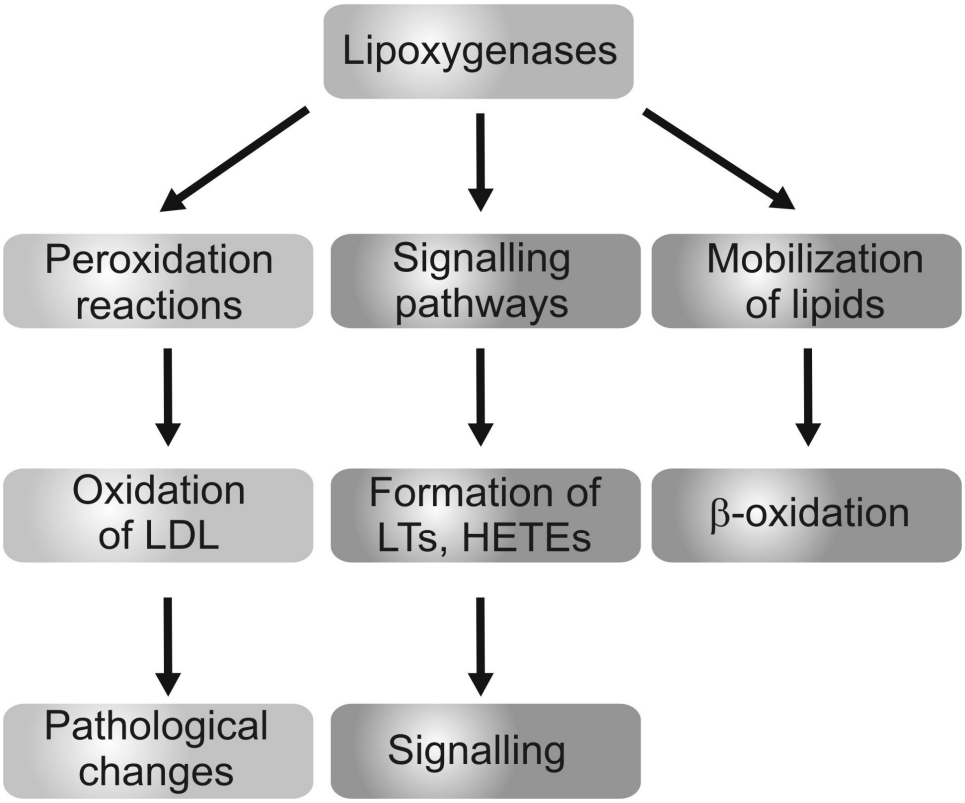


Figure 2: Biological roles of lipoxygenases

B. 5-lipoxygenase

1. Gene and protein expression

The human 5-LO protein is derived from a gene which is located on chromosome 10q11.2 whereas most other human LOs are located on 17p13 [329]. The 5-LO gene has a length of 82 kb separated into 14 exons and 13 introns [110]. Within the gene, numerous consensus binding sites are obvious including sp1/3, Egr–1/2, NFκB, GATA, myb, AP-2 [147] as well as response elements for vitamin D receptor, and TGFβ [326]. The 5-LO promoter shows no TATA or CCAAT sequence, is highly G+C rich and contains 10 sp1 binding sites. Five of them exist in overlapping tandem sites and are necessary for basal transcription [147]. There are promoter polymorphisms, consisiting of three to eight sp1 binding sites [88, 89]. These polymorphisms are connected to altered transcription efficacy of the gene [153] and to decreased response of asthma patients to 5-LO inhibitors [88]. The polymorphisms are no indication for the susceptibility of humans for asthma [308] but seems to be implicated in atherogenesis [89].

Expression of 5-LO is mainly limited to cells derived from bone marrow, but among granulocytes, monocytes, macrophages, mast cells, and B-lymphocytes [325], there are also human skin keratinocytes [162], and epidermal Langerhans cells that express 5-LO [324]. Differentiation of the myeloid cell lines MM6 and HL-60 with calcitriol and TGFβ yields an extensive increase of 5-LO mRNA, protein level, and enzyme activity [39, 40]. Also DMSO, phorbol-12-myristate-13-acetate (PMA), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-3, or LDL elevate the level of mRNA in several leukocyte cell lines [318].

DNA methylation and histone acetylation regulate 5-LO expression. Treatment of the 5-LO-negative cell lines U937 and HL-60TB with the demethylating agent 5-aza-2'-deoxycytidine upregulates expression of 5-LO primary transcripts and mature mRNA in a similar manner [340]. By means of reporter gene assays it was shown that the histone deacetylase inhibitor trichostatin A induces 5-LO promoter activity in MM6 cells [169].

2. Structure

So far only four crystal structures of LOs are available, three of them are from plants and the fourth is 15-LO from the rabbit reticulocyte. Using the latter as template, a model of human 5-LO was calculated [142]. As for all LOs, the secondary structure of 5-LO reveals a protein with two domains. A N-terminal β-barrel domain consisting of the first 120 amino acids (aa) and a C-terminal catalytic domain spanning from aa 121 to 673. Since LOs contain a nonheme iron within the catalytic domain there are residues important for iron binding in 5-LO, namely

His372, His550, and the terminal Ile673 [133, 377]. His367, Asn554, and a water molecule are supposed to be flexible ligands [130].

Different methods showed that Ca^{2+} binds to the β-barrel domain with a stoichiometry of 2 mol Ca^{2+} per mol protein with a K_d of 6 µM. Asn34, Asp44, and Glu46 are involved in Ca^{2+} binding [131, 132]. Recently, in a theoretical approach two Ca^{2+} binding sites were revealed, namely site 1 consisting of Phe14, Ala15, Gly16, Asp79, and site 2 consisiting of Asp18, Asp19, Leu76 [21].

The N-terminal region of 5-LO acts as a genuine C2-domain. C2-domains are conserved structural motifs which form an eight strand anti-parallel β-sandwich and mediate Ca^{2+}-dependent membrane association [151]. This applies for the β-barrel domain of 5-LO. Binding of Ca^{2+} to 5-LO leads to phosphatidylcholine (PC) selectivity of the protein in which the aromatic residues Trp13, 75, and 102 play an essential role [178]. Furthermore, the β-barrel domain of 5-LO is essential for nuclear membrane translocation and reveals similarity to the C2-domain of protein kinase (PK)C and cytosolic phospholipase (cPL)A_2 [53].

ATP stimulates 5-LO activity and the protein can be purified via ATP affinity chromatography. The DNA sequence shows no obvious NTP binding site [110] but affinity studies on ATP-agarose revealed binding of one mol ATP per mol 5-LO [92, 376]. Trp75 and Trp201 seem to be involved in this affinity to ATP and to other nucleotides [376].

5-LO is found in the cytosol but also in the nucleus. Also, the protein exerts (a) nuclear import sequence(s) (NIS). In 1998, a region of basic aa spanning residues 639-656 was found to be involved in 5-LO translocation [191]. In particular, Arg651 seemed to play an essential role in nuclear import of 5-LO [138]. However this residue is important for structural integrity and 5-LO activity and therefore the same authors rejected their assumption followed by a proposal for aa 518-530 as a "real" NIS [165]. Recently, Arg112 and Lys158 were identified as NISs [166]. One study showed that the N-terminal region mediates entering of 5-LO into the nucleus [55]. But this is controversial because the fusionprotein used for this study allows free diffusion between nucleus and cytosol [138].

Moreover, 5-LO contains a functional Src homology (SH)3-binding motif, a short, proline-rich region spanning residues 566-577. This suggests a role for 5-LO in tyrosin kinase signalling [192].

3. Catalysis

5-LO catalyses the first two steps in the biosynthesis of LTs from its main substrate AA (see figure 3). The nonheme iron of 5-LO cycles through different redox states and was shown to be necessary for the enzyme catalysis. Within the oxygenase reaction, 5-LO converts AA in a stereospecific manner to 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5(*S*)-

HpETE). The process starts with the oxidation of the iron from the ferrous state to the ferric form via lipid hydroperoxides (LOOH), followed by a homolytic cleavage of the pro-S hydrogen at C7 of AA. This results in the formation of a pentadienyl radical and the iron is again reduced to the ferrous form. Subsequently, molecular oxygen is inserted antarafacial at C5, yielding the intermediate product 5(S)-HpETE while iron is converted to the ferric form [63]. In the LTA_4 synthase reaction, 5-HpETE is converted to the unstable epoxide LTA_4. After the homolytic cleavage of the pro-R hydrogen at C10 of 5-HpETE and the reduction of the iron followed by allylic shifts, an electron and a proton are transferred to the cleared hydroxyl moiety, resulting in H_2O, LTA_4, and oxidized iron of 5-LO [316]. The mechanism indicates the necessity of LOOH and a correct redox cycling of the iron during the catalytic reaction. The sequence of the reactions is made up of the initial phase where 5-LO is converted to the active state, followed by a high conversion rate, and finally an irreversible inactivation phase [103]. The latter phase has to be considered as an activity of the enzyme and is also referred to as suicide inactivation. LOOH are discussed to be responsible for this inactivation [3].

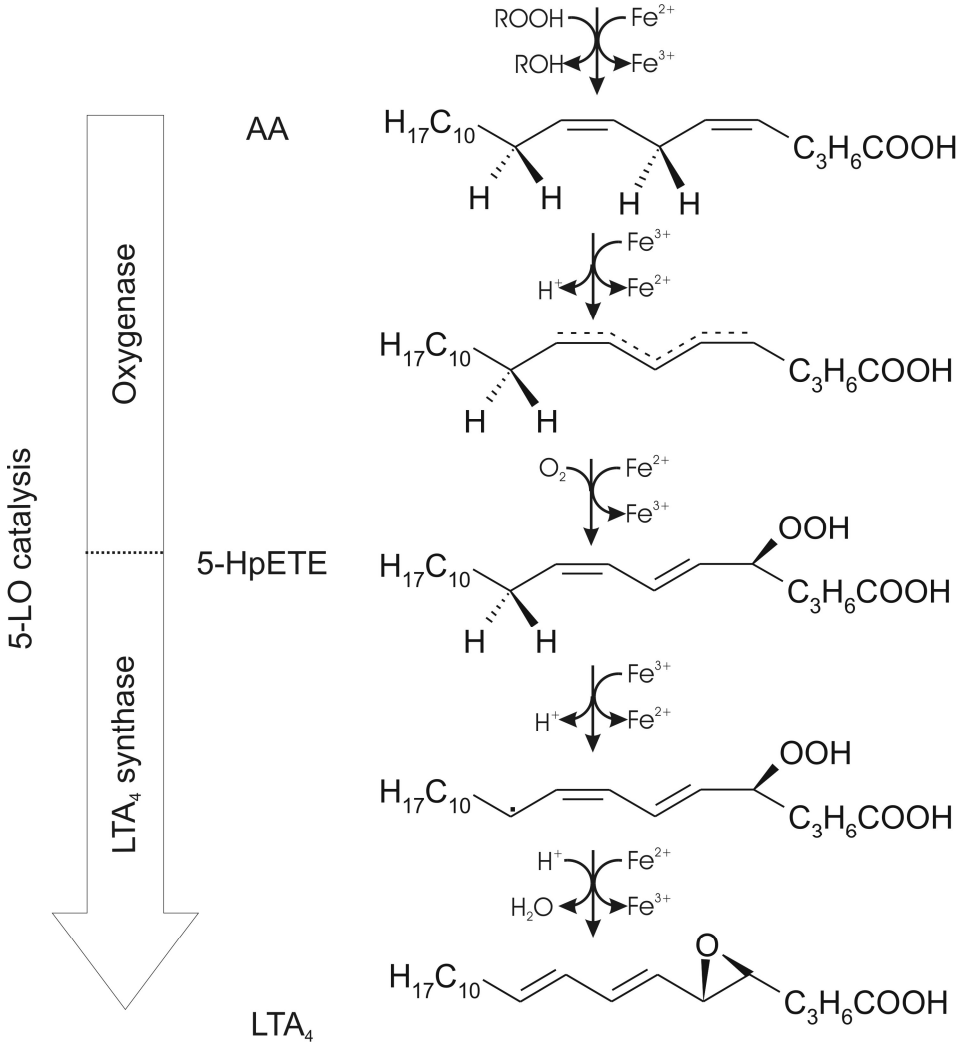


Figure 3: 5-LO catalysis

It was shown that AA and 5-HpETE compete with each other, suggesting that both reactions, oxygenase and LTA_4 synthase, take place at the same catalytic site. The rate of produced 5-HpETE to LTA_4 depends on several conditions, e.g. presence of FLAP and membrane association of 5-LO, amount of 5-LO, concentration of AA or 5-HpETE.

Another enzyme property is described as a pseudoperoxidase activity. Reducing agents, like N-hydroxyurea-derivatives or hydroxamic acids, are oxidized via fatty acid hydroperoxides, e.g. 5-HpETE. This reaction, catalyzed by 5-LO, is a one electron transfer and therefore termed as a pseudoperoxidase activity. The reducing agents are redox-type inhibitors of 5-LO, and those which exhibit a better substrate ability for the pseudoperoxidase reaction are more potent inhibitors. Thus, the compounds attain their potency from reducing the ferric enzyme to the ferrous form and as an alternative substrate for 5-LO. For an extensive overview of 5-LO kinetics see [103].

4. Stimulating factors

a) Calcium

Effects of Ca^{2+} on LT formation were first reported when human PMNL were treated with the ionophore A23187 [30]. A variety of other stimuli, such as thapsigargin (TG), N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), leukotriene B_4 (LTB_4), or antigen-stimulation, also lead to an increase of free intracellular Ca^{2+}-levels and subsequently to activation of 5-LO [309, 365, 366]. Although other LOs do not require Ca^{2+} and Ca^{2+} is not absolute essential for 5-LO activity, it strongly stimulates LT formation [242, 269]. For purified 5-LO, half maximal activity is obtained at concentrations of 1 - 2 μM Ca^{2+} [259], maximal activity at 4 - 10 μM [242]. In intact cells, 150 - 350 nM intracellular free Ca^{2+} are sufficient for activation of 5-LO [309]. The enzyme is capable of binding two Ca^{2+} ions with a $K_d = 6$ μM whereas mutation of Asn43, Asp44, and Glu46 impairs the affinity of 5-LO to Ca^{2+} [131, 132].

Ca^{2+} promotes all three described enzyme activities of 5-LO [279], increases the hydrophobicity [132], and triggers binding of 5-LO to phosphatidylcholine [242]. Moreover, binding of Ca^{2+} to the N-terminal β-barrel domain facilitates translocation of cytosolic 5-LO to the nuclear envelope as well as membrane binding [178, 286, 290]. Also Ca^{2+} confers 5-LO resistant against inhibition by glutathione peroxidase (GPx)-1, suggesting an increased affinity of 5-LO to LOOH at a regulatory fatty acid binding site in presence of Ca^{2+} [42, 44]. Regarding kinetics, Ca^{2+} shortens the initial lag-phase, enhances the steady state velocity, and decreases the K_M for AA [4].

The environment of 5-LO dictates the necessity of Ca^{2+} for enzyme activity. In cell free systems, high concentrations of PC or AA render 5-LO activity Ca^{2+} independent [275, 320]. Mg^{2+} as a bivalent cation can also bind to 5-LO and activates the enzyme within a range of 0.1 – 1 mM. To raise hydrophobicity at least 4 mM Mg^{2+} are required. Additionally, Mg^{2+} and Ca^{2+} bind to the same site of 5-LO [21, 274], indicating that Mg^{2+} substitutes for Ca^{2+} in a less efficient manner.
In a model, using the mast cell line PT-18, it was shown that Ca^{2+} is not required for LT synthesis [348]. In human PMNL, 5-LO shows only a partial dependence on extracellular Ca^{2+} [309] and AA-induced LT formation is mediated by Ca^{2+} and/or phosphorylation [45, 330, 360, paper I].
Recent studies revealed that there are two independent ways of 5-LO activation, namely by Ca^{2+} and by phosphorylation [45]. Hyperosmotic, stress-induced 5-LO activation is completely Ca^{2+}-independent and can be blocked by specific kinase inhibitors, implying that phosphorylation is operative [353].

b) ATP

ATP is a well-known stimulation factor which is able to potentiate the enzymatic activity of crude 5-LO up to 6-fold. Also other nucleotides including AMP, ADP, cAMP, CTP, UTP, and the nonhydrolyzable γ-*S*-ATP stimulate 5-LO activity [92, 241, 244]. The effect of ATP is Ca^{2+} independent and has a maximum impact at 0.1 mM ATP [103, 241, 320]. Within intact cells, ATP occurs in complex with Mg^{2+} as $MgATP^{2-}$, suggesting the latter as physiological stimulus [274]. ATP has K_a values of 30 - 100 μM for stimulating and binding to 5-LO is estimated one mol ATP per one mol enzyme (for detailed review see [272]).

c) Membrane fractions

Since the ratio of AA to phospholipids is an important factor for the rate of 5-LO reaction, there is basic evidence that the enzymatic reaction of purified 5-LO takes place at a lipid-water interface [280]. Thus, it is reasonable that microsomal membrane fractions stimulate the catalytic activity of 5-LO [289, 291]. They can be replaced by synthetic lipids like PC or by Tween 20 but not by phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, or diacylgycerol [240, 269]. The stimulating effect can be observed both in the presence and absence of Ca^{2+} [274, 320]. Nevertheless, the C2-like domain of 5-LO is a genuine Ca^{2+}-dependent membrane-targeting domain, which directs the enzyme selectively to PC rich membranes, conferred by the tryptophan residues Trp13, Trp75, and Trp102 [178].

d) Lipid hydroperoxides

Initial oxidation of the active site iron within the centre of 5-LO by LOOH is essential for the catalytic activity of the enzyme. Consequently, LOOH like 5-HpETE, 12-HpETE, 15-HpETE, or 13(S)-HpODE shorten the lag-phase and stimulate 5-LO, whereas H_2O_2 and other peroxides without long chain fatty acyl moiety fail [278, 288]. In PMNL homogenates, dithiothreitol (DTT) counteracts this effect [278, 288], and in intact cells, GPx-1 reduces LOOH, thus suppressing 5-LO activity [350].

Of considerable interest, addition of 13(S)-HpODE or inhibition of GPx by iodoacetate or selenium-deficiency, leads to impaired efficacy of nonredox-type 5-LO inhibitors [359, paper IV].

5. Subcellular distribution and translocation of 5-LO

The subcellular distribution of 5-LO in resting cells is a puzzling issue, since 5-LO is a rather mobile enzyme. In resting cells, 5-LO resides in the cytosol or the soluble compartment of the nucleus. The distribution depends on the cell-type: in peripheral blood neutrophils and monocytes, differentiated HL-60 and MM6 cells, and in peritoneal macrophages (PM), the enzyme is located in the cytosolic fraction. However, in alveolar macrophages (AM), rat basophilic leukaemia cells (RBL-1), mouse bone marrow-derived mast cells, and in Langerhans cells of human skin, 5-LO is mainly in the soluble intranuclear region [352]. For transfected cells like RAW macrophages, HEK 293, COS, CHO and NIH-3T3 cells, nuclear localization was reported [352]. A dynamic regulation seems to be responsible for compartmentalization of 5-LO. During the course of monocyte differentiation into PM, 5-LO is localized in the cytoplasm, but migration of the PM into the alveolar compartment is linked to an import of 5-LO into the nucleus [64]. Nuclear import of cytosolic 5-LO was also shown for PMNL after *in vitro* adherence [37]. With respect to the molecular mass of 5-LO, the ability for a nuclear import of the enzyme should depend on nuclear localization sequences (NLS) [36, 202]. Moreover, mutation of the phosphorylation site Ser271 results in reduced 5-LO nuclear localization [202]. In contrast, Leptomycin B, a specific inhibitor of nuclear export signal (NES)-dependent transport, diminished the cytosolic localization of 5-LO in HL-60 and GFP-5-LO in CHO-K1 cells [134].

Nuclear import of 5-LO is not automatically associated with an increase in LT formation. For adherent PMNL an increased ability for 5-LO activity is reported [37], whereas adherent eosinophils show a decreased LT formation because of failed translocation to the membrane [35].

LT formation from endogenous AA serving as substrate implies translocation of either cytosolic or nuclear 5-LO to the outer or inner nuclear envelope. Upon stimulation of intact cells, an increase of intracellular Ca^{2+} leads to an association of soluble 5-LO with cellular membranes [286, 290, 365]. In addition, phosphorylation of 5-LO is an alternative impulse for translocation, shown in PMNL and differentiated MM6 cells [194, 353, 356].

Factors like protein-protein interactions via the SH3 domain-binding region of 5-LO and tyrosine kinase signalling [192, 194, paper VII] as well as cellular interacting proteins [265, 267] may play an important role in 5-LO translocation. Unlike earlier reports, translocation seems to be independent of FLAP [167].

6. Regulation of cellular 5-LO activity

There are some critical steps during activation of cellular 5-LO. (i) Oxidation of the ferrous, resting state to the ferric, active state of 5-LO via LOOH. (ii) Mobilisation of intracellular Ca^{2+} and/or phosphorylation of 5-LO. (iii) Redistribution of activated 5-LO from cytoplasm to the NE/ER accompanied with translocation of activated $cPLA_2$. (iiii) Release of endogenous AA via $cPLA_2$ and transfer of the substrate via FLAP to 5-LO for production of 5-HpETE and LTA_4, respectively. These individual schematic steps can be carried out upon different stimuli and activation pathways. Depending on the experimental setup or the conditions *in vivo*, respectively, the importance of the different steps is weightened variable.

a) Ca^{2+} mobilisation

Treatment of cells with ionophore A23187 [30] or TG [249] results in Ca^{2+} mobilisation and subsequently in LT formation. But also natural ligands such as fMLP, complement C5a (C5a), LTB_4, PAF, IL-8 or particles like zymosan and phosphate crystals cause an elevation of intracellular Ca^{2+} and LT formation [352]. The resulting degree of 5-LO activity corresponds to the extent of Ca^{2+} mobilisation. Thus, Ca^{2+}-ionophore A23187 leads to a prominent LT formation. In contrast, fMLP, PAF, or LTB_4, which interact with specific G protein coupled receptors (GPCRs), cause moderate 5-LO activity. Since LT formation depends on the availability of free AA, $cPLA_2$ activity is a determinant for 5-LO product synthesis [306]. For this reason, minor LT formation upon stimulation with natural ligands could be due to low amounts of free AA. Supporting evidence is given by the observation that co-addition of exogenous AA to cells stimulated by natural agents yields in increased LT synthesis [57, 309].

Growth factors, cytokines, phorbol esters or lipopolysaccharide (LPS) do not cause 5-LO activity by themselves. However, priming of cells with these agents leads to an augmented LT formation after stimulation with natural ligands [68, 84, 310].

Whether these priming effects are based on increased supply of AA, a pronounced mobilisation of Ca^{2+} or a higher degree of phosphorylated 5-LO depends on the priming agent or is still unclear. For detailed review see [352].

b) Phosphorylation of 5-LO

Mammals exhibit a well-characterized family of mitogen-activated protein kinases (MAPK). This family consists of four subgroups including extracellular signal-regulated kinases (ERK 1/2), c-Jun amino-terminal kinases (JNKs), p38 MAPKs, and ERK5. They all control a huge variety of physiological processes. In general, these enzymes are regulated by an upstream relay of two protein kinases which phosphorylate and subsequently activate one another [163]. Especially ERKs and p38 MAPK are in the focus of 5-LO research (see figure 4).

p38 MAPK is activated in immune cells in response to inflammatory cytokines but also by hormones, ligands for GPCRs, cell stress or heat shock. ERKs are mainly activated by growth factors, cytokines, virus infection, transforming agents, and also by activators of GPCRs [163, 180]. p38 MAPK has been shown to phosphorylate several cellular targets including MAPKAPK-2/3 (MK2/3). p38 activity is involved in macrophage and neutrophil responses, including adhesion, degranulation, oxidative burst, chemotaxis, and also cytokine production [188, 285]. Activated ERKs phosphorylate numerous substrates in all cellular compartments and ERK1/2 signalling has been implicated as a key regulator of cell survival and proliferation [285].

In the primary sequence of human 5-LO several phosphorylation motifs including motifs for PKA, PKC, Ca^{2+}/calmodulin-dependent kinase (CaMKII), MAPKAPK-2 (MK2) and -3, S6 kinase, MAPK1/2, and Cdc2 are obvious [352]. Already in 1996, phosphorylated 5-LO was discovered in the nucleus of Ca^{2+}-ionophore-stimulated HL-60 cells. However, the group failed to phosphorylate 5-LO by MAP kinase, Cdc-2 or Lyn *in vitro* [194]. The same group showed that PD098059, a potent and selective inhibitor of MAP kinase kinase (MEK), inhibits 5-LO translocation as well as 5-LO activity, independent of $cPLA_2$ [193].

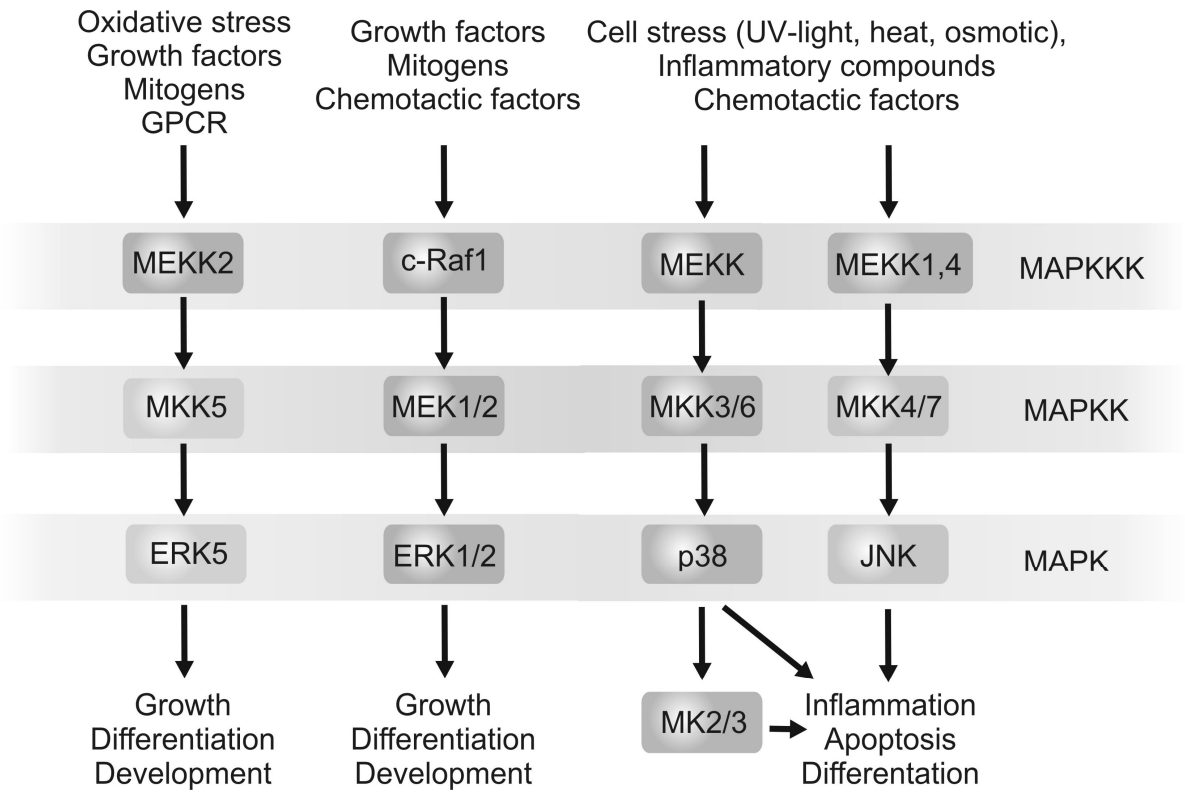


Figure 4: MAPK phosphorelay system

By means of an in-gel kinase assay, MK-2 was found to phosphorylate 5-LO [355]. Stimuli like Ca^{2+}-ionophore, TG, fMLP, PMA, TNFα, or sodium arsenite (SA) lead to activation of MK-2 and finally also to activation of 5-LO [354]. Specific inhibitors, targeting designated 5-LO kinases, block redistribution and activation of 5-LO. If PKC, which is upstream of ERKs, is inhibited by calphostin C, PMA-induced 5-LO translocation and activation is potently blocked [356]. Also SB203580, a specific p38 MAPK inhibitor, diminishes LT formation upon stimulation of PMNL with Ca^{2+}-ionophore [355]. Because cell stress is capable of activating the p38 MAPK cascade, treatment with hyperosmotic stress (NaCl, sorbitol) or genotoxic stress (SA) leads to 5-LO translocation and activation in B-lymphocytes and PMNL [353, 354]. Moreover, the p38 MAPK inhibitor SB203580 blocked stress-induced 5-LO product formation efficiently. Intriguingly, cell stress activates 5-LO in isolated PMNLs independent of Ca^{2+} [353]. However, if stimuli that increase intracellular Ca^{2+} like Ca^{2+}-ionophore are combined with stimuli that cause cell stress, then 5-LO activity is strongly suppressed [43].

Ser271 of 5-LO is a phosphorylation site of several Ser/Thr-kinases. Thus, *in vitro* kinase assays revealed that not only MK2 but also CaMKII and PKA are capable of phosphorylating 5-LO [203, 355, 360]. Mutation of Ser271 to Ala in 5-LO sequence showed the necessity of this residue for phosphorylation *in vitro*. In gel kinase assays prove that the enzyme is phosphorylated at Ser271 [360]. HeLa cells transfected either with wt 5-LO or with a Ser271Ala mutant showed the same LT formation when cells were treated with Ca^{2+}-ionophore plus

exogenous AA. However, when cells were stimulated with AA alone, conditions which promote phosphorylation of 5-LO (see below), 5-LO activity of the Ser271Ala mutant was significantly lower as compared to wt-5-LO [360].

Compared to heat shock protein (Hsp)27, 5-LO is a weak substrate for MK2. However, addition of unsaturated fatty acids up to 50 μM results in similar phosphorylation by MK-2 as for Hsp27 [360]. In contrast, this was not observed for CaMKII and PKA. The role of ERKs regarding 5-LO activation are addressed in papers I, II. Paper III-VI show the consequences of phosphorylated 5-LO for inhibition of LT formation by pharmacological inhibitors.

c) Induction of 5-LO activity by 1-oleoyl-2-acetylglycerol (OAG)

OAG was found to function as a direct agonist for PMNL stimulating 5-LO product formation. OAG (i) does not increase the release of endogenous AA, (ii) does not promote the redistribution of 5-LO from the cytosol to the nuclear envelope (NE), (iii) and does not alter the mobilisation of intracellular Ca^{2+}-level. Moreover, specific kinase inhibitors for p38 MAPK and ERKs fail to inhibit OAG induced 5-LO activity [7]. In addition, OAG mimics the stimulatory effects of Ca^{2+} on 5-LO catalysis. Thus, under optimized assay conditions where Ca^{2+} is present, OAG caused no stimulation of 5-LO. However, after omission of Ca^{2+} by chelation using EDTA, OAG strongly and concentration-dependently stimulated crude 5-LO. In contrast, phospholipids or cellular membranes abolished the effects of OAG. In analogy to Ca^{2+}, OAG could render 5-LO activity resistant against inhibition by GPx activity, which again was reversed by phospholipids [14].

7. Proteins binding to 5-LO

Until today, there are four proteins in total known to bind 5-LO. Three of them had been detected by the yeast two-hybrid system using a human lung cDNA as screening system [267]. FLAP, often assumed to serve as an anchor for 5-LO, was not demonstrated to bind to the enzyme.

a) Growth factor bound receptor protein 2 (Grb2)

As mentioned above, 5-LO contains a short proline-rich region which is able to serve as a binding motif for SH3 domains. There is a high specificity for interaction of 5-LO with Grb2, a protein involved in tyrosine kinase-mediated cell signalling. Purified 5-LO binds to a GST-Grb2 fusion protein and a corresponding peptide to the SH3-binding motif of 5-LO inhibits the formation of a 5-LO/Grb2 complex [192]. The peptide inhibited also the translocation of 5-LO from the cytosol to the NE after activation by Ca^{2+} ionophore A23187. Furthermore, it was shown that 5-LO associates to actin-sepharose, and α-actinin-sepharose respectively [192]. These

associations can also be revoked by the mentioned peptide. The data imply a role of 5-LO in tyrosine kinase signalling as well as a link to dynamics of the cytoskeleton.

b) TGFβ receptor-I-associated protein-1 (TRAP-1)

TRAP-1, a 96-kDa cytoplasmic protein, binds to the activated TGFβ receptor. It plays a role in the Smad-mediated signal transduction pathway, interacting with the common mediator, Smad4, in a ligand-dependent fashion [272]. Binding of 5-LO to the C-terminal end of TRAP-1 was shown by the yeast two-hybrid system [267]. Based on the fact that TGFβ and calcitriol increase 5-LO expression and activity [39], a connection of 5-LO and TRAP-1 seems to be reasonable.

c) Coactosin like protein (CLP)

CLP, similar to coactosin, is a 17 kDa actin-binding protein from *Dictyostelium discoidium*. CLP itself binds to F-actin and has no effect on actin polymerization [266]. The yeast two-hybrid system revealed association of 5-LO with CLP [267], confirmed by coimmunoprecipitation experiments. CLP binds 5-LO in a 1:1 molar stoichiometry in a Ca^{2+}-independent but pH-dependent manner [265]. Lys131 is involved in 5-LO binding [265] whereas Lys75 is linked to the association with actin [266]. No ternary F-actin/CLP/5-LO complex could be detected but 5-LO competes with F-actin for binding to CLP. Moreover, 5-LO was found to interfere with actin polymerization [265].

d) Ribonuclease (RNase) III, dicer

Long dsRNA is processed by a RNase III enzyme called dicer into small interference RNAs (siRNAs), which subsequently serve as the sequence determinants of the RNAi pathway by directing cleavage of homologoues mRNA via an RNA-induced silencing complex (RISC) [338]. A protein containing a RNase III motif and a dsRNA binding domain was identified via two-hybrid system to associate with 5-LO [267]. A functional connection between dicer and 5-LO could not be displayed so far.

8. Targeted disruption of the 5-LO gene

Studies were performed using 5-LO knockout mice to determine how LTs contribute to different physiological and pathophysiological actions.

In general, 5-LO $^{-/-}$ mice develop normally and are healthy [54]. Only slight differences with respect to bone constitution are described [107]. After treating isolated cells, levels produced of prostaglandin(PG)s are equal to wild type mice [108].

5-LO knockout mice displayed reduced signs of inflammation in AA-induced ear oedema, zymosan-induced peritonitis, or ovalbumin-induced airway inflammation [47, 54, 117, 154]. 5-LO deficient mice are also able to recover from PAF-induced shock [54]. In contrast, inflammation induced by PMA did not differ in the absence of 5-LO products [107].
In an allergic airway inflammation model, 5-LO deficient mice displayed a decreased airway hyperresponsiveness and also a reduced amount of eosinophils in the airway after treatment [109].

C. Proteins involved in LT formation

1. Phospholipase A_2

The numerous members of the phospholipase(PL)A_2 family are defined by their ability to catalyze the hydrolysis of the centre (sn-2) ester bond of the phospholipid substrate. PLA_2 products such as AA or oleic acid (OA) operate as stores of energy, but AA mainly acts as a second messenger [113] and as a precursor of eicosanoids [22]. Lysophospholipids are also products of PLA_2 and can be acetylated to PAF. They are also important in cell signalling, phospholipid remodelling and membrane perturbation [226].
Up to now there are 11 groups of PLA_2, subdivided into further subgroups, disposed dependent on the enzyme activity, sequence, homology, and splice variants. For detailed review see [319].
Mammalian cells contain a variety of PLA_2 enzymes which can be found together in the same cell. Among these enzymes, the low-molecular-weight 14 kDa secreted PLA_2 ($sPLA_2$) requires millimolar levels of Ca^{2+} and has no fatty acid specificity. $sPLA_2$ are involved in digestion of lipids (group I), chronic inflammatory diseases (group IIa), and are implicated in AA release (group V) (more details in [113]). The second family in mammals belongs to the Ca^{2+}-independent PLA_2 with a molecular mass of 85 kDa. They are expressed ubiquitously, exhibit also no sn-2 specificity and might be involved in phospholipid remodelling [18].
In 1986 the first member of a cytosolic PLA_2 family was reported from human neutrophils [11] and platelets [171]. After sequencing [59], [315], the 85 kDa enzyme was classified into group IVA. The structure is based on two domains, a C2 domain and an α/β hydrolase PLA_2 domain [77]. This $cPLA_2$ is the only member with specificity to sn-2 AA [80] but has also a strong lysophospholipase [195] and weak transacylase activity [276]. Human $cPLA_2$ is expressed in a variety of tissues and its levels are regulated by cytokines and growth factors [198]. Posttranslational events like binding of Ca^{2+} to the C2 domain and phosphorylation of the enzyme dominate the regulation of $cPLA_2$ activity. Thus, Ca^{2+}-mobilizing agents like ionophore A23187 and zymosan induce AA release by promoting translocation of $cPLA_2$ from the cytosol

to the nuclear envelope and endoplasmatic reticulum, respectively [311]. Phosphorylation of $cPLA_2$ on Ser505 and Ser727 by MAPK and MNKs, respectively, is also important for activation of the enzyme activity [75, 199]. However it is not clear how phosphorylation affects $cPLA_2$ function. $cPLA_2^{-/-}$ mice develop normally but females have reproductive defects leading to reduced fertility [28]. A more rapid recovery from allergen-induced bronchoconstriction and no airway hyperresponsiveness was observed. PM of $cPLA_2^{-/-}$ mice fail to produce PGs, LTB_4 and cysLTs after stimulation. Bone marrow-derived mast cells also fail to generate eicosanoids (for detailed review see [306]). Targeted disruption of $cPLA_2$ revealed the importance of the enzyme in inflammation and tissue injury. Therefore, the control of AA production by inhibiting PLA_2 could be beneficial for treatment of diseases caused by eicosanoids.

2. 5-lipoxygenase activating protein

5-lipoxygenase activating protein (FLAP) is an 18 kDa membrane protein which belongs to the MAPEG-family (see below) and therefore it is no surprise that there is a sequence identity of 31 % to LTC_4S. ALOX5AP encoding FLAP is located on chromosome 13q12 including 5 exons and 4 introns with a size of 31 kbp [168].

FLAP is co-expressed with 5-LO in myeloid cells like PMNL, monocytes/macrophages and B-lymphocytes. However, some cell lines negative for 5-LO protein express FLAP, e.g. T-cells, undifferentiated MM6 or U937 cells, indicating substantial differences in gene activation [351].

The observation that MK886 blocks only LT formation in intact cells but not in the corresponding homogenates [114] led to the discovery of FLAP. It was shown that MK886 sticks to FLAP [225] and co-transfection of osteosarcoma cells with 5-LO/FLAP demonstrates that both proteins are necessary for LT formation [83]. ^{125}I-labelled AA binds to the protein [208], and AA and other cis-unsaturated fatty acids compete with MK886 for binding to FLAP [272]. Further studies revealed that FLAP resides at the nuclear envelope and the neighbouring perinuclear endoplasmatic reticulum [261, 367].

The precise function of FLAP has still to be elucidated but so far it is known that the protein stimulates the utilization of AA by 5-LO and promotes the conversion of 5-HpETE to LTA_4 [1]. FLAP can also facilitate the conversion of 12-HETE to 5, 12-diHETE by 5-LO [211].

Lately the presence of two distinct multimeric complexes that organize the biosynthesis of LTC_4 and LTA_4 in RBL-2H3 cells was demonstrated by using fluorescence lifetime imaging microscopy and immunoprecipitation. One complex including LTC_4S and FLAP is responsible for the formation of LTC_4. The other one consists of multimers of FLAP, providing LTA_4 for LTB_4 formation [212]. Regarding the FLAP gene it was reported that variants of ALOX5AP are associated with greater risk of myocardial infarction and stroke [141]. FLAP-binding inhibitor

MK886 induces apoptosis in U937 cells and in cultured malignant cells from patients with chronic myelogenous leukaemia [12]. In addition MK886 significantly increased caspase-3 activity [74] but acts independent of the FLAP protein [73]. Moreover, FLAP seems to be involved in cell division [368].

Targeted disruption of FLAP in mice results in reduced responses to AA, an increased resistance to PAF-induced shock, less edema in zymosan-induced peritonitis [46] as well as collagen-induced arthritis [118]. Thus, FLAP $^{-/-}$ resembles the 5-LO $^{-/-}$ phenotype.

3. Leukotriene A_4 hydrolase

LTB_4 is generated by hydrolyzing the unstable epoxide LTA_4, catalysed by leukotriene A_4-hydrolase (LTA_4H) [123]. LTA_4H has been purified from a variety of species as a soluble monomeric enzyme [273]. LTA_4H, consisting of 610 aa with a calculated molecular weight of 69 kDa, have been cloned and sequenced from human, mouse, rat, and guinea pig [123]. The corresponding gene is located on chromosome 12q22 with a size of >35 kbp and contains 19 exons. The promoter contains a phorbol ester response element (AP2) and two xenobiotic response elements (XRE), but no definitive TATA box [209]. From sequence comparisons with certain zinc proteases and exopeptidases, a zinc-binding motif was discovered which lies in the catalytic domain [207, 345]. One mol of zinc per mol of protein binds to the three binding ligands His295, His299, and Glu318 [124, 221]. Beside the epoxide hydrolase activity LTA_4H shows a peptide-cleaving activity towards the synthetic substrates alanine-4-nitroanilide and leucine-4-nitroanilide [125]. This aminopeptidase properties can be activated by monovalent anions like thiocyanate and chloride as well as albumin [361].

A characteristic feature of LTA_4H is the inactivation of both enzyme activities by covalent binding of its substrate LTA_4 [91, 250] in the active site [229]. Also divalent cations inhibit LTA_4H activity with different potency and specificity for the two enzyme activities [362].

The crystal structure of LTA_4H in complex with the inhibitor bestatin was recently solved and revealed a protein folded into three domains, N-terminal, catalytic and C-terminal. Together these three domains form a zinc-binding cleft from where a deep hydrophobic pocket leads into the enzyme where LTA_4 fits in via a L-shaped binding conformation [337].

In addition to bestatin, other aminopeptidase and angiotensin-converting enzyme inhibitors such as captopril were found to suppress LTA_4H activity [252]. The LTA_4H inhibitor SC-57461, *N*-methyl-*N*-[3-[4-(phenylmethyl)-phenoxy]propyl]-b-alanine, blocked ionophore-induced LTB_4 production in human whole blood with an IC_{50} of only 49 nM [375], is orally active, and showed promising results in an animal model of colitis.

LTA_4H is widely spread in mammalian species and found in almost all tissues and blood cells. Basophils express only small levels of LTA_4H and platelets seem to be virtually devoid of the enzyme. Many of the cells expressing LTA_4-hydrolase lack 5-LO, thus giving evidence for transcellular metabolism [123]. The enzyme is generally thought to reside in the cytosol and has been reported only once with a membrane bound activity in liver cells [122].

Mice deficient in LTA_4H means to lose the ability to convert LTA_4 into LTB_4. They develop normally and are healthy. Treating mice with proinflammatory stimuli revealed that LTA_4H is required for LTB_4 formation. $LTA_4H^{-/-}$ mice are resistant against the lethal effects of systemic shock induced by PAF [47], as found for 5-$LO^{-/-}$ mice.

4. Leukotriene C_4 synthase

Leukotriene C_4-synthase (LTC_4S) conjugates LTA_4 with glutathione (GSH) to form LTC_4, the parent compound of the cysteinyl LTs [373]. Sequential cleavage of glutamatic acid and glycine from the GSH moiety of LTC_4 yields LTD_4 and LTE_4 respectively [251]. The enzyme is expressed in eosinophils, mast cells, basophils, monocytes/macrophages, and some leukemic cell lines (THP-1, KG-1) as well as platelets which lack 5-LO [183]. The 150 aa of the LTC_4S gives an 18 kDa membrane protein, existing as a homodimer [236]. The enzyme activity is augmented by Mg^{2+} and inhibited by Co^{2+} and MK-886 [183]. 5 Exons plus 4 introns, in conjunction 2.5 kbp, constitute the human LTC_4S gene located on chromosome 5q35. LTC_4S and FLAP exhibit similar genomic organization [257]. The deduced amino acid sequence demonstrated that FLAP and LTC_4S are homologs with 31 % amino acid sequence identity. Also the LTC_4S polypeptide displayed a similar hydropathy pattern compared to FLAP [161]. $LTC_4S^{-/-}$ mice grow normally and fail native and adaptive immune inflammatory permeability responses [183].

5. MAPEG-family

FLAP and LTC_4S belong to the widespread MAPEG (**m**embrane **a**ssociated **p**roteins in **e**icosanoid and **G**SH metabolism)-superfamily which is defined according to enzymatic activity, sequence motifs, and structural properties [160]. The six human proteins FLAP, LTC_4S, microsomal glutathione S transferase (MGST)1–3, and MGST1-L1 as well as several homologues from different sources can be subdivided into four groups, based on sequence alignment. FLAP, LTC_4S, and MGST2 are part of the first subfamily. The second consists of MGST3 together with the members found in plants and fungi. Subfamily three and four include proteins identified in bacteria and MGST1/MGST1-L1 [160].

Properties of FLAP are described in section II.C.2. LTC_4S conjugates LTA_4 with GSH, but also from MGST2 and 3, LTC_4S activity is reported. Additionally, for both enzymes a peroxidase

activity was discovered [158, 159]. They are sequence homologues of FLAP and LTC_4S but possess a broader substrate specificity. It is discussed that both, MGST2 and 3, are involved in detoxification and oxidative stress because of their occurrence in liver and their enzyme activities.

Also MGST1 has a wide substrate specificity and is broadly expressed, with the highest concentration in liver [227]. In addition to MGST2 and 3, the substrates for MGST1 include halogenated arenes like CDNB and various polyhalogenated hydrocarbons whereas LTA_4 and other epoxides are poor substrates for the enzyme. Interestingly, LTC_4 is a binding inhibitor of MGST1 but the function is not known so far [161]. MGST1-L1 as well is involved in redox regulation and has been reported to be controlled by p53 [263].

D. Eicosanoids and their functions

1. Oxoeicosanoids

Oxoeicosanoids are a family of biologically active AA derivates. Among other compounds, 5-HETE and its corresponding keto derivate 5-oxo-ETE form this group as well as oxoeicosanoids from the 12- and 15-LO pathway (12- and 15-HETE, hepoxilins). Formation and release of 5-oxo-ETE has been shown in platelets [105], eosinophils [264], and neutrophils [246]. 5-oxo-ETE is capable of inducing chemotaxis in vitro [116] and *in vivo* via mobilisation of Ca^{2+} [247]. It also provokes degranulation [247] and stimulates oxygen radical formation after GM-CSF treatment [245]. Recently, a G-protein-coupled receptor was isolated which is activated by 5-oxo-ETE and to a lesser extent by the related 5-HpETE and 5-HETE. [148, 164].

An increase in glucagon release from isolated pancreatic islets and an augmented occurrence in early diabetic processes are impacts of 12-HETE. It also plays a role in regulating cell growth and apoptosis, is enhanced in hypertensive rats and in patients with essential hypertension [374]. 15-HETE is involved in cell differentiation and maturation, and in humans, superoxid anion formation, migration of PMNL, and LTB_4 formation is inhibited. Contraction of human bronchial smooth muscle cells and pro-athereogenic properties has also been shown [177]. Hepoxilins, constituting epoxy-hydroxy eicosanoids, are biosynthesized via the 12(S)-LO pathway. Recently, it was shown that rat leukocyte-type 12(S)-LO exhibits an intrinsic hepoxilin A_3 synthase activity [239]. This eicosanoid causes strong chemotaxis of human neutrophils at submicromolar concentrations and leads to a prominent Ca^{2+} release [331]. Furthermore, hepoxilin A_3 is required in neutrophil migration across intestinal epithelia [228].

2. Leukotriene B_4

LTB_4 (5(S), 12(R)-dihydroxy-6, 14-*cis*-8, 10-*trans*-eicosatetraenoic acid, LTB_4) is a potent lipid mediator biosynthesized by metabolizing the precursor LTA_4 via LTA_4H to LTB_4 (see figure 5). Its all-trans isomers are produced by non-enzymatic hydrolysis of the unstable epoxide LTA_4 [303]. In contrast to the ubiquitous expression of LTA_4H, 5-LO is limited to haematopoietic cells. Therefore, LTB_4 is thought to be produced mainly by leukocytes under normal conditions. During inflammation, LTB_4 production occurs also in other cell types. Transcellular metabolism of the precursor LTA_4 seems to be prerequisite [299]. Recent data suggest that also FLAP has an influence on the extent of LTB_4 generation [212]. Several metabolizing mechanisms for LTB_4 are known including ω-oxidation by cytochrome P450 4F3, dehydrogenation, and β-oxidation. All reactions lead to inactivation of the biological function of LTB_4 [370]. Most actions of LTB_4 are mediated by cell surface GPCRs, namely BLT1 and BLT2 (see below, [369]). It is discussed that LTB_4 is a ligand of the orphan nuclear receptor PPARα implying a role in gene expression [78].

Initially, LTB_4 was identified as an activator of granulocytes [305] with biological characteristics comparable to classical chemoattractants such as IL-8, C5a, and fMLP. In CHO cells transfected with BLT1, LTB_4 increases the intracellular Ca^{2+}-level [369] and upon stimulation, leukocytes show potent chemotactic and chemokinetic activities. Leukocyte rolling and adhesion to the venous endothelium [71] is as much increased as secretion of superoxid anion and release of lysosomal enzymes [126]. Moreover, LTB_4 contracts the guinea pig aorta via an indirect mechanism involving the release of histamine and thromboxane [17]. Experiments using bone marrow cultures suggest that LTB_4 stimulates bone resorption by enhancing the formation of osteoclasts [111]. LTB_4 has been also implicated in the development of atherosclerosis [76] and is discussed to be involved in cell-survival signalling [248]. A release of anti-HIV mediators after administration of LTB_4 to humans has been revealed recently [100].

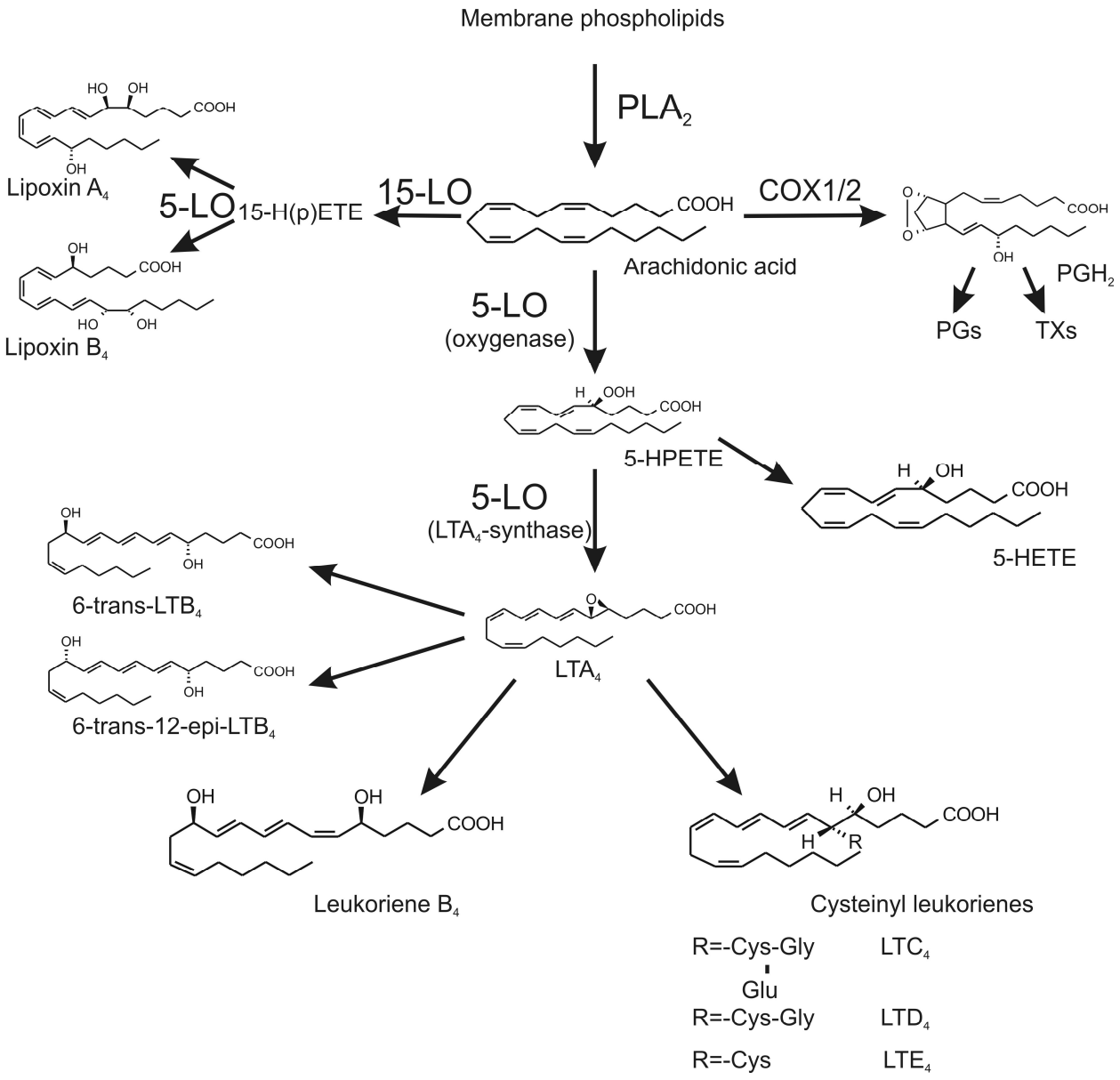


Figure 5: The AA cascade with emphasis on the 5-LO pathway

3. Cysteinyl leukotrienes

Slow-reacting substance of anaphylaxis (SRS-A) was the first name given to the cysteinyl LTs. In 1979 the SRS-A was identified as LTC_4, D_4, and E_4 [304]. Conjugation of reduced GSH with LTA_4 via LTC_4S leads to LTC_4 which is metabolized to LTD_4 and E_4 by cleavage of glutamic acid and glycine, respectively. Also MGST-2 is capable of catalyzing this conjugation step. Thus, formation of cysLTs is controlled by both enzymes [322].

CysLTs are inactivated by formation of the corresponding sulfoxides or, similar to LTB_4, by ω-hydroxylation followed by β-oxidation [238]. CysLTs are formed by eosinophils, mast cells, monocytes, macrophages but not by PMNL [322]. In contrast to LTB_4, cysLTs primarily affect smooth muscle and other cells with contractile capacity. In analogy to LTB_4, cysLTs exert their

biological effects via GPCRs, namely cysLT1 and 2. CysLTs are potent inducers of bronchoconstriction in guinea pig airways with a 100-1000 fold higher potency than histamine [85, 139] and also cause contraction of isolated human bronchi [72]. Depending on the concentrations, they contract or relax vascular smooth muscle cells [10]. CysLTs also promote plasma exudation [71], stimulate mucus secretion [215], and possess a high capacity to promote eosinophil recruitment [341]. In addition they are reported to reduce myocardial contractibility and coronary blood flow [224].

4. Lipoxins

Lipoxins (LX) are trihydroxytetraene-containing eicosanoids and may act as endogenous anti-inflammatory lipid mediators [217]. LXA_4 and LXB_4 are the principal molecules formed in mammals [312, 313]. The 15R-enantiomers of LXA_4 and LXB_4 are referred to as aspirin-triggered LXs (ATLs) [58]. The LXs are generated via cell-cell interactions. Enzyme activity of 5-LO combined with activity of 12-LO ($\rightarrow LXA_4$), 15-LO ($\rightarrow LXA_4/B_4$), or COX-2 ($\rightarrow$ ATLs) is necessary. There is a variety of effects mediated by LXs *in vitro* dependent on the cell type. In human PMNL, they inhibit chemotaxis, Ca^{2+} mobilisation [189] as well as cell adhesion, and PMNL-mediated vascular permeability [255, 334]. In contrast, chemotaxis and adhesion are stimulated in monocytes [206]. In nonmyeloid cells like endothelium, proliferation, chemotaxis, and P-selectin mobilisation is inhibited. For detailed review see [217]. Thus, lipoxins exhibit anti-inflammatory effects through their own GPCR but also via antagonizing LTD_4 effects on $cysLT_1$ [218].

E. Receptors

The actions of eicosanoids are mediated mainly by specific receptors. All known LT receptors belong to the family of the seven transmembrane-spanning GPCRs. They are classified into two main groups: The LTB_4-receptors BLT1 and 2 which mediate the actions of LTB_4, as well as the cysLT1 and 2 receptor group which is mainly responsible for the effects of LTC_4, D_4 and E_4.

BLT1 was first cloned from differentiated HL-60 cells in 1997 [369] and from mouse in 1998 [150]. The human gene is located on chromosome 14q11.2-q12 and encodes for a 352 aa protein with an approximate mass of 43 kDa [253]. The human BLT1 shares a 78 % homology with the corresponding receptor from mice and 45.2 % to BLT2 which was identified in 2000. Surprisingly, the open reading frame of human BLT2 was located within the promoter of the BLT1 gene suggesting a common transcriptional regulation of these two receptors [372]. BLT2 is expressed ubiquitously in contrast to BLT1 which is mainly expressed in leukocytes [333]. LTB_4 is the main ligand of these receptors with a 20 fold higher affinity to BLT1 than to BLT2

[372]. Other ligands such as 12-H(p)ETE or 15-HETE compete with LTB_4 binding to BLT2, but not BLT1 [371], suggesting that BLT2 functions as a low affinity receptor with broader ligand specificity for various eicosanoids. Generation of a BLT1 $^{-/-}$ mice strain revealed that the major activities of LTB_4 are mediated by BLT1 [136, 332]. The BLT1 $^{-/-}$ mice are viable, fertile and develop normally. In contrast to BLT1 positive mice, recruitment of neutrophils and PM failed as well as LTB_4-induced Ca^{2+} flux and chemotaxis. In addition, oedema and protein extravasation in response to AA is reduced and also BLT1 $^{-/-}$ mice show a reduced mortality towards PAF induced shock [136, 332].

In 1999 two groups cloned the cysLT1, a GPCR consisting of a 337 aa protein yielding a molecular mass of 38 kDa [204, 307]. CysLT1 is expressed in leukocytes, mast cells, endothelial cells as well as in lung and nasal mucosa [90]. Among the cysLTs, binding of LTD_4 and LTC_4 to cysLT1 follows the much strongest contraction of human bronchial smooth muscle. LTE_4 is less potent [16]. Radio binding studies revealed that selective antagonists, such as montelukast and zafirlukast, potently block LTD_4 binding to cysLT1, but not to cysLT2. The solubilized receptor is associated with a PTX-sensitive G-protein (for detailed review see [238]).

Less is known about the cysLT2 receptor which was cloned in 2000. Located on chromosome 13q14, a region linked to atopic asthma, the gene encodes for a protein consisting of 346 aa with a 38% homology to the cysLT1 receptor [140]. CysLT2 mRNA shows a strong expression in the human heart, adrenals, peripheral blood leukocytes, spleen, lymph nodes and a weak expression in smooth muscle cells [140]. The rank order of agonist potency on human pulmonary venous smooth muscle preparations is $LTC_4 = LTD_4 > LTE_4$, with LTE_4 as a partial agonist [181]. In contrast for cysLT1-receptor, there is no selective inhibitor but with BAY u9773 a dual antagonist towards both cysLT-receptors. A further class of cysLT-receptor is discussed [16, 238].

Human cDNA encoding for LXA_4R was identified on basis of high affinity to LXA_4. Like all LT receptors, it is a seven transmembrane-spanning protein with PTX-sensitivity [97]. Binding of LXA_4 to LXA_4R results in a subsequently activation of phospholipase D [98]. LXA_4 competes with LTD_4 for binding with cysLT1 on endothelial cells [218].

F. Clinical implications of 5-LO products

Asthma is a chronic inflammatory disorder of the airways in which the inflammatory response contributes to the airway hyperresponsiveness (AHR) and the episodic, widespread, reversible airway narrowing that is characteristic of the disease [13]. Symptoms of an asthma attack include coughing, wheezing, shortness of breath, chest tightness. Asthma occurs in people who are

predisposed to develop asthma because of genetic and environmental factors that determine susceptibility. A variety of "triggers" may start or worsen an asthma attack, including, exposure to allergens, viral respiratory infections, airway irritants, such as tobacco smoke and certain environmental pollutants, and exercise (National Institute of Allergy and Infectious Diseases, NIH). The inflammatory response is characterized by an increased number of leukocytes such as eosinophils, basophils, neutrophils, mast cells and lymphocytes in the epithelium, submucosa or bronchoalveolar lavage fluid (BALF). Pronounced mucus secretion and mast cell degranulation are other clinical symptoms [13].

The role of LTs in asthma contributes to the observed actions of LTs *in vivo* and *in vitro* [305]. The amount of LTs is increased in BALF and also the urinary LTE_4 levels are higher in asthma patients in contrast to healthy subjects [87, 185]. Moreover, anti-LT therapy leads to an increased bronchodilatation, reduced asthmatic response to triggering factors as well as a decreased AHR [70, 155, 156, 190, 234].

The properties of LTs imply also a significant role in other inflammatory diseases like arthritis, psoriasis, inflammatory bowel disease or allergic rhinitis [352]. Another clinical implication of 5-LO products is the involvement in atherogenesis. Atherosclerosis is associated with hypercholesterinaemia combined with an increased oxidation of low-density lipoproteins (LDL) which stick to the subendothelial layer of the artery wall, finally resulting in inflammation of the vessel wall [284]. 5-LO was identified as a gene contributing to atherosclerosis susceptibility in LDL receptor $^{-/-}$ mice [223]. There is also evidence that variations of 5-LO genotypes may be responsible for an increased atherosclerotic vulnerability [89]. 5-LO expressing cells, such as monocytes and dentritic cells, are markedly increased in advanced atherosclerotic lesions [323]. LTB_4 is responsible for monocyte infiltration in these lesions, and this can be counteracted by specific LTB_4 receptor antagonists [5]. In contrast to non-atherosclerotic coronary arteries, challenging of atherosclerotic coronary arteries with LTC_4 or LTD_4 results in contraction [9, 10]. Recent data show that 5-LO contributes to the formation of aortic aneurysms induced by an atherosclerotic diet in Apo $^{-/-}$ mice and that 5-LO links hyperlipidemia and the production of systemic inflammatory chemokines in several mouse models of hyperlipidemia [378]. For detailed review see [271].

In addition to these diseases 5-LO products may contribute to carcinogenesis and cell survival [15, 82, 96, 121] as well as bone metabolism [54, 111, 186]. Therefore, anti-LT therapy is salutary in the treatment of inflammatory diseases [325] but may also be beneficial in the therapy of cancer [82, 112, 282], osteoporosis [6, 186] and atherosclerosis [89, 222].

G. 5-LO inhibitors

1. Endogenous Inhibitors

a) Glutathione peroxidase

A certain level of LOOH is required for 5-LO to enter the redox cycle which is necessary for its activation [103]. The cellular peroxide level is mainly regulated by GPx, particularly in cells devoid of catalase [343]. The family of GPx consists of three tetrameric GPx (1-3) and the monomeric phospholipid (Ph)GPx (GPx-4) [344]. Their biosynthesis strictly depends on the availability of selenium. They become inactivated if their selenocysteins are carboxymethylated by iodoacetate or if the selenium is eliminated [343]. Reducing agents like GSH, DTT, or β-mercaptoethanol act as cofactors of GPx [102].

Phospholipid hydroperoxide glutathione peroxidase (PhGPx) activity is primarily responsible for the reduction of 5-HpETE and therefore governs the actual activity of leukocyte 5-LO via regulating the tone of endogenous hydroperoxides [350, 358]. PhGPx suppresses 5-LO activity in A431 and RBL2H3 cells [149, 152]. After differentiation of MM6 cells by TGFβ and calcitriol, the induction of 5-LO protein is obvious but an increase in activity is not prominent [40]. Later it was discovered that GPx-1, not only PhGPx, is an efficient inhibitor of 5-LO and is involved in the regulation of cellular 5-LO activity in various cell types [328]. Moreover, GPx-1 confers 5-LO activity Ca^{2+}-dependent and further data suggest that interaction of Ca^{2+} at the C2-like domain of 5-LO protects the enzyme against the effect of GPx-1 [42, 45].

b) Adenosine

Adenosine plays an important role in biochemical processes, such as energy transfer - as ATP and ADP - but also in signal transduction as cAMP. Effects of adenosine on leukocytes are inhibition of phagocytosis, generation of toxic oxygen metabolites, and adhesion without inhibiting degranulation or chemotaxis [66]. Concerning LT formation, fMLP-induced synthesis of LTB_4 in whole blood pretreated with LPS and TNFα is concentration-dependently inhibited by adenosine [172]. The removal of endogenous adenosine with either adenosine deaminase or the blockade of its action by an adenosine A2a receptor antagonist, markedly reversed the effect on LTB_4 synthesis upon ligand stimulation [173]. Adenosine inhibits AA release and LTB_4 synthesis via cAMP [101]. cAMP-elevating agents such as isoproterenol, PGE_2, adenosine A2a receptor agonist, type IV phosphodiesterase inhibitors, and forskolin, all inhibit LT biosynthesis and 5-LO translocation to the nucleus in human PMNL stimulated with TG [101]. These effects are mediated by direct activation of PKA by cAMP. It was shown that the catalytic subunit of

activated PKA directly phosphorylates 5-LO *in vivo* and *in vitro* and inhibits 5-LO activity in intact cells and in cell free systems by phosphorylation of Ser523 [203].

c) Nitric oxide (NO)

NO is a radical produced by NO synthase and has pleiotrophic activities in health and disease. In a model using soybean LO, NO-releasing agents inactivated the enzyme by reducing the catalytic iron to the inactive ferrous form and counteracted the H_2O_2-mediated activation of the LO-catalyzed dioxygenase reaction. [205]. Prolonged exposure of AM to LPS impairs LT production via NO-mediated suppression of 5-LO [61]. Moreover, NO and reactive oxygen species (ROS) are known to react with each other forming peroxynitrite ($ONOO^-$). $ONOO^-$ suppressed LT synthesis in intact AM which was reversed by an $ONOO^-$ scavenger [60].

2. Pharmacological inhibitors

Based on the pathophysiological profile of LTs, anti-LT therapy is a reasonable pharmacological strategy for treatment of a variety of inflammatory diseases as well as atherosclerosis, osteoarthritis or cancer. Therefore, development of compounds which intervene in the 5-LO pathway is attempted.

In principle there are two different ways of anti-LT therapy, namely (i) repression of LT formation and (ii) blocking LT action at its specific receptors. LT receptor antagonists are discussed in detail in [34].

Blocking LT formation can be achieved by suppression of substrate availability, direct inhibition of 5-LO and inhibition of FLAP. A simplified illustration is given in figure 6.

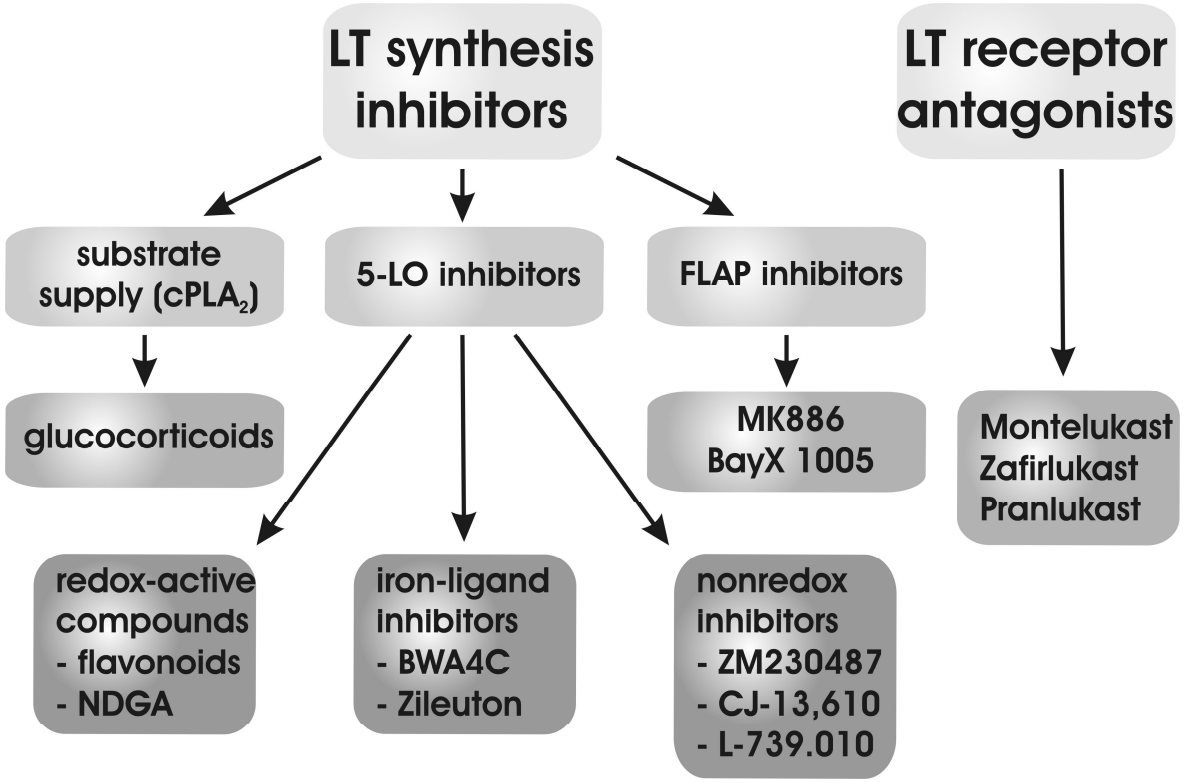


Figure 6: Pharmacological strategies of anti-LT therapy

Experiments with $cPLA_2$ deficient mice revealed that lack of $cPLA_2$ leads to a complete repression of eicosanoid formation [306, 342]. Since glucocorticosteroids suppress expression of PLA_2, glucocorticosteroids have been assumed to inhibit formation of all eicosanoids. This has been confirmed in various settings *in vitro*, but in urine, gained from patients treated with high doses of glucocorticosteroids, significant amounts of eicosanoids are still detectable [56]. Moreover, dexamethasone increases the expression of 5-LO and FLAP in human monocytes [277].

Direct inhibitors of 5-LO can be separated into three groups: (i) redox-active compounds, (ii) iron-ligand inhibitors and (iii) nonredox inhibitors.

Phenols like nordihydroguaretic acid, caffeic acid, flavonoids, or coumarins are potent redox-active compounds. They act by reducing the active site iron causing an interruption of the catalytic cycle of the enzyme. However, the reducing properties and the poor selectivity result in severe side effects such as methemoglobin formation [220]. A synthetic approach led to potent and orally active compounds such as BW755C or AA-861. But they failed to enter the market due to several side effects [103].

Derivatives of hydroxamic acid and N-hydroxyurea form the group of iron-ligand inhibitors. BWA4C, a hydroxamic acid, is a potent 5-LO inhibitor with an IC_{50} of 40 nM in stimulated granulocytes. Since rapid hydrolysis of this substance leads to formation of toxic nitroxide radicals, the development of alternative substances was required [335]. Zileuton (Zyflo®) is the

only direct 5-LO inhibitor which was licensed for the treatment of asthma, solely in the USA. It is a N-hydroxyurea derivate with a short oral half life of 3 h. The compound revealed an IC_{50} between 0.5 to 1 μM in a variety of cell types, independent of the origin [20, 49]. Chronic administration of zileuton is associated with improved airway function, decreased asthma symptoms, and a decreased need for alternative medication in patients with mild to moderate asthma [86]. However, in diseases like allergic rhinitis, rheumatoid arthritis, and ulcerative colitis, zileuton showed no significant benefit [349]. A refinement of the potency and oral half life of zileuton led to the development of ABT-761. It exhibits an improved metabolic stability with $t_{½}$=15 h and the IC_{50} was decreased up to 150 nM in whole blood [38]. Another new approach is to connect benzhydryl piperazine, the H_1 pharmacophore of the antihistamines cetirizine, with a N-hydroxyurea moiety. First results showed an IC_{50} for 5-LO and H_1 receptors of 150 nM and 420 nM, respectively, in human whole blood [196].

The handicap of redox-active compounds and iron-ligand inhibitors are their poor selectivity and low bioavailability which led to the development of nonredox-type 5-LO inhibitors. They were suggested to act by competing with AA for binding to 5-LO. Almost all inhibitors of this class contain a methoxyalkylthiazol or a methoxytetrahydropyran moiety. Improvements of lead structures resulted in ZD2138 or its ethyl analogue ZM230487 which exhibit IC_{50} of 20 – 50 nM in whole blood [65]. ZD2138 inhibited antigen-induced release of LTD_4 and LTB_4 with IC_{50} values of 0.3 μM and 0.4 μM, respectively [179]. In contrast, treatment with ZD2138 did not prevent pulmonary inflammation or the development of AHR. Moreover, ZD2138 did not inhibit more chronic responses following multiple antigen exposure [339]. In asthmatic subjects the inhibitor did not protect against allergen-induced asthmatic responses [234]. Thus, it seems that treatment of acute inflammatory diseases with nonredox-type 5-LO inhibitors is succeessful, but inhibition of chronic inflammatory processes fails in this respect.

Additionally, ZM 230487 or the Merck compound L-739,010, suppress cellular LT synthesis of ionophore stimulated granulocytes with IC_{50} values of about 50 nM. However, in cell homogenates or in preparations of purified enzyme as well as when LOOH are added to intact cells, up to 150-fold higher concentrations are required for similar inhibition of 5-LO activity. Addition of reducing agents such as GSH or DTT reversed the inhibitory potency of ZM230487 or L-739,010 so that 5-LO inhibition was comparable with that of intact cells [359]. These data suggest that physiological conditions associated with oxidative stress and increased peroxide levels lead to impaired efficacy of nonredox-type 5-LO inhibitors like ZM230487 or L-739,010.

Replacement of the dihydroquinolinone pharmacophore of Zeneca's ZD2138 by ionisable imidazolylphenyl moiety has led to the discovery of a novel series of potent and orally active

nonredox-type 5-LO inhibitors [214]. Structural optimization led to metabolic stability as well as improved bioavailability and a good toxicological profile [213]. In this study, the molecular pharmacology of CJ-13,610, a recently developed analogue of these compounds, was tested in various *in vitro* test systems with relevance for the *in vivo* pharmacology [paper IV].

A new approach for treating inflammatory diseases is the development of dual inhibitors of the 5-LO/COX pathways which posses a wider spectrum than that of classical nonsteroidal anti-inflammatory drugs (NSAIDs) [99]. Among dual inhibitors, a novel class of non-antioxidant compounds has been described; the most potent and well balanced dual inhibitor is ML3000 ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid, presently in clinical phase III [99, 187]. The compound inhibits 5-LO in bovine and human granulocytes with IC_{50} of 0.18 and 0.23 μM [187].

Hyperforin represents a substance of plant origin within the dual inhibitors. In freshly isolated human PMNL stimulated with Ca^{2+}-ionophore A23187, hyperforin inhibited 5-LO product formation with IC_{50} values of about 1-2 μM. Experiments with purified human 5-LO demonstrate that hyperforin is a direct 5-LO inhibitor (IC_{50} approximately 90 nM), acting in a noncompetitive fashion. In ionophore-stimulated human platelets, hyperforin suppressed COX-1 product formation with an IC_{50} of 3 μM. Hyperforin could not interfere with COX-2 product formation and did not significantly inhibit 12- or 15-LO in platelets or leukocytes, respectively [8].

Other potent non synthetic substances which inhibit LT formation are 11-keto-beta-BA (KBA) and their acetyl derivative 3-O-acetyl-11-keto-beta-BA (AKBA). They are contents of the gum resin of *Boswellia serrata*. Among the BAs, AKBA induced the most pronounced inhibition of 5-LO product formation with an IC_{50} of 1.5 μM [294]. Effects of BAs on the MAPK cascade, cellular Ca^{2+} mobilisation as well as formation of ROS, release of AA, and LT biosynthesis are depicted in this study [paper V,VI].

An additional target for inhibition of LT synthesis is the development of FLAP inhibitors. The indole derivate MK886 potently inhibits LT formation in intact cells (IC_{50}=2.5 nM) but shows an impaired efficacy in whole blood (IC_{50}=1.1 μM) and failed to directly inhibit 5-LO [114]. Bay x1005, a quinoline derivate, as well as MK0591, a indole/quinolines hybrid, are also found to be FLAP inhibitors exhibiting potent LT synthesis-inhibitor properties [104, 210]. For Bay x1005 inhibits allergen-induced asthma and bronchoconstriction in asthma subjects [69]. But nevertheless it shows only a weak activity in whole human blood (IC_{50}=11.6 μM) [104]. The impaired efficacy of FLAP inhibitors in whole blood assays is conceivable since FLAP is not absolutely required for cellular LT synthesis if exogenous AA is present [287, 327, 357]. Also

high affinity of FLAP inhibitors to plasma proteins may impair the efficacy [52]. Moreover, since AA triggers apoptosis [258] it is not surprising that inhibitors of FLAP, like MK886, have the same effect [94]. It is obvious that also other members of the MAPEG-family are potential targets of these compounds [184]. Since these drugs compete with AA and other unsaturated fatty acids (UFA)s for binding to fatty acid binding sites it should be also considered that they may possess several additional properties. A 5-LO inhibitor overview gives figure 7.

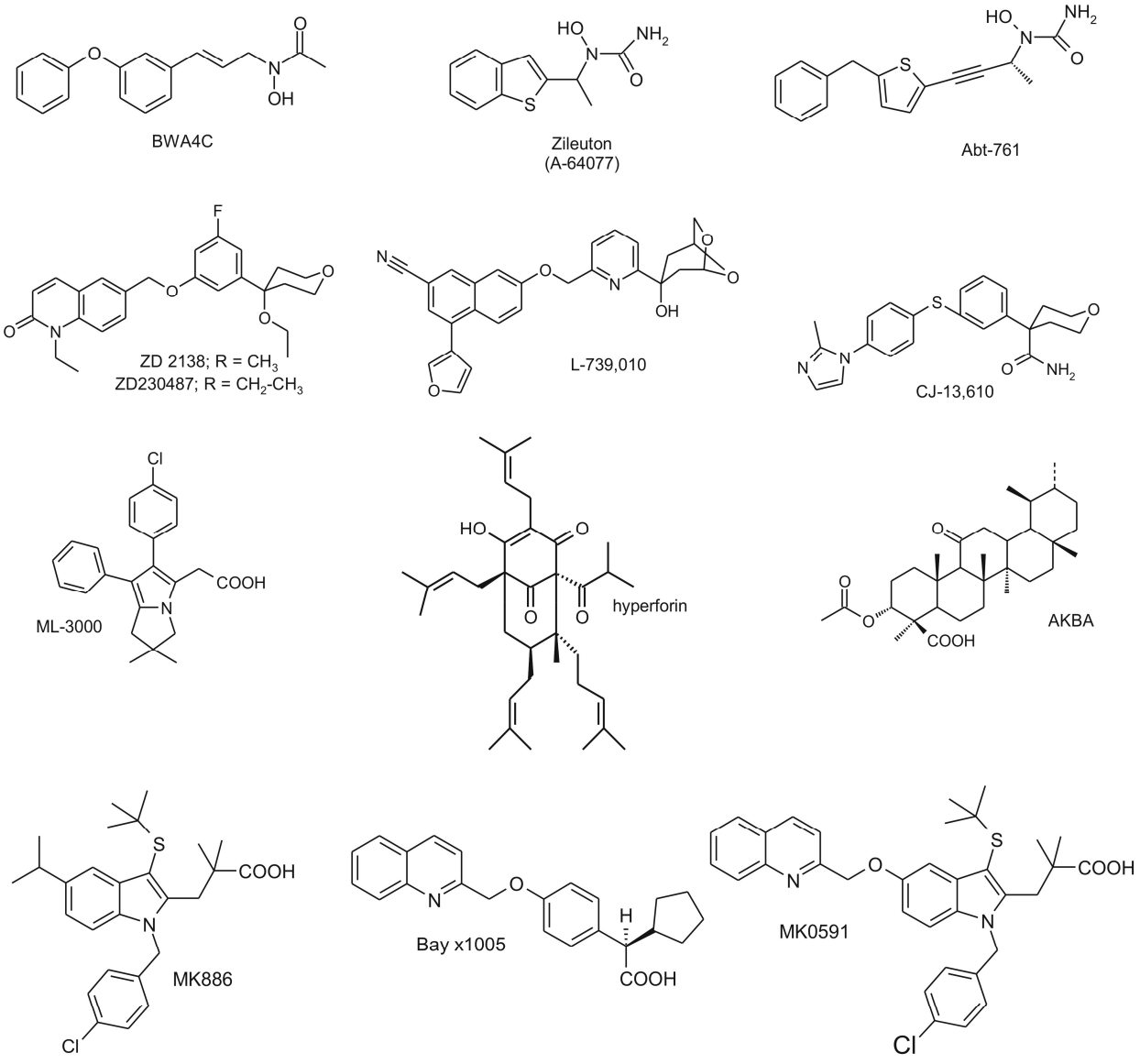


Figure 7: 5-LO inhibitors

III. Aims of the present studies

LTs are key mediators involved in a variety of inflammatory and allergic disorders but also in diseases like cancer, osteoporosis and atherosclerosis. Therefore, anti-LT therapy might be beneficial for the treatment of such diseases [106, 305, 325, 352]. Due to the fact that 5-LO initializes the biosynthesis of LTs, detailed knowledge about the cellular activation of 5-LO is required for the development of drugs targeting this enzyme.

Ca^{2+}, ATP, phosphatidylcholine, LOOH and leukocyte factors are involved in the enzymatic regulation *in vitro* [103, 272]. Recent data showed that phosphorylation of 5-LO by p38 MAP kinase dependent MK-2 is a novel pathway which leads to a Ca^{2+}-independent cellular LT formation [353, 355]. However, there are indications that ERK1/2 are involved in regulation of cellular 5-LO activity [25, 193, 380].

One main goal of this study was to elucidate if ERK1/2 phosphorylate 5-LO and if there is evidence for ERK1/2 signalling in the activation of 5-LO in intact cells.

The classical nonredox-type 5-LO inhibitors failed in clinical trials evaluating the efficacy in chronic inflammatory diseases [234, 339]. Some diseases, which are associated with an increased level of 5-LO metabolites, revealed an inreased phosphorylation status of the cells. Inflammatory reactions, cancer, or atherosclerosis are some examples [127, 163]. In this respect, the second main goal was to investigate if the activation pathway of 5-LO determines the efficacy of nonredox-type 5-LO inhibitors and if the novel inhibitor CJ-13,610 exhibits advantages over former compounds.

Further purposes were to address the effects of the 5-LO inhibitors boswellic acids on various signalling pathways important for inflammatory responses of neutrophils.

Finally, the aim of this study was to enlighten the link between the cytoskeleton and cellular LT formation.

IV. Methods

Method	Paper
isolation of human polymorphonuclear leukocytes (PMNL)	I-VII
5-LO activity assay	I-IV, VI, VII
cellular $cPLA_2$ activity ([^{3}H]AA release)	I, VI, VII
subcellular fractionation, detergent lysis	IV, VII
gel electrophoresis, Western blot	I, II, IV-VII
immunoprecipitation	I
MAPK activation	I, III, V-VII
in-gel kinase assay	III, IV
in vitro protein kinase assay	I, II, III
transient transfection of HeLa cells	I-IV
site directed mutagenesis	I-IV
protein expression using E.coli	I, III, IV
partial 5-LO purification	I, III, IV
measurement of intracellular Ca^{2+} levels	V, VI, VII
determination of cellular peroxide formation	III, VI
detection of superoxide anion	VI

The following cells and cell lines have been used:

PMNL, freshly isolated from human healthy donors	I-VII
Mono Mac 6 cells (monocytes/macrophages)	I, VI
HeLa cells (human cervix carcinoma cells)	I-IV
HL-60 cells(human acute myeloid leukaemia cells)	VI

V. Results

Paper I: Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2002) Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.* 16, 1441-1443.

Paper II: Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2003) 5-Lipoxygenase activation by MAPKAPK-2 and ERKs. *Adv Exp Med Biol* 525, 129-132.

Paper III: Fischer, L., Szellas, D., Rådmark, O., Steinhilber, D., and Werz, O. (2003) Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB J.* 17, 949-951.

Paper IV: Fischer, L., Steinhilber, D., and Werz, O. (2004) Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610. *Br. J. Pharmacol.* 142, 861-868. Epub 2004 Jun 2014.

Paper V: Altmann, A., Fischer, L., Schubert-Zsilavecz, M., Steinhilber, D., and Werz, O. (2002) Boswellic acids activate p42 MAPK and p38 MAPK and stimulate Ca^{2+} mobilisation. *Biochem. Biophys. Res. Commun.* 290, 185-190.

Paper VI: Altmann, A., Pöckel, D., Fischer, L., Schubert-Zsilavecz, M., Steinhilber, D., and Werz, O. (2004) Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leukocytes. *Br. J. Pharmacol.* 141, 223-232. Epub 2003 Dec 2022.

Paper VII: Fischer, L., Bürkert, E., Steinhilber, D., and Werz, O. (2005) Inhibitors of actin polymerization upregulate arachidonic acid release and 5-lipoxygenase activation by upregulation of Ca^{2+} mobilisation in polymorphonuclear leukocytes involving Src family kinases. *Manuscript.*

A. Paper I: Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes
Paper II: 5-lipoxygenase activation by MAPKAP-2 and ERKs

Background and rationale:

Upon stimulation of intact cells, LT formation is an interplay of various enzymes including $cPLA_2$, FLAP and 5-LO. Besides elevation of intracellular Ca^{2+}, phosphorylation of $cPLA_2$ by MAP kinase as well as phosphorylation of 5-LO by MK2 at Ser271 leads to an increased AA release and LT formation respectively [113, 355, 360]. Based on inhibitor studies, there are several reports suggesting also a role for ERK1/2 in agonist induced 5-LO translocation and product formation in HL-60 cells [193] and leukocytes [2, 19, 25, 380]. Priming of eosinophils with IL-5 or GM-CSF and subsequent stimulation with fMLP leads to ERK activation concomitant with LTC_4 formation [19]. Moreover, inhibition of MEK1/2 by U0126 prevents 5-LO translocation in fMLP-challenged human neutrophils [25]. Finally, U0126 completely inhibits both PAF-induced ERK phosphorylation and LTC_4 generation in PAFR cells [2]. Although, 5-LO contains the minimal consensus sequence Ser/Thr-Pro for phosphorylation by ERK1/2, so far phosphorylation by these kinases could not be proved [194].

The aim of this study was to identify 5-LO as a substrate for ERK1/2 and to provide the molecular basis for the involvement of ERK1/2 signalling in the activation of 5-LO in intact cells. A second aim was to point out the parallels of MK2 to ERK1/2 regarding phosphorylation and activation of 5-LO.

Results:

By *in vitro* kinase assays using activated recombinant ERK2 kinase and purified 5-LO as substrate, a concentration dependent phosphorylation of 5-LO by ERK2 could be demonstrated. In contrast to myelin basic protein (MBP) 5-LO was a weak substrate for ERK2. Addition of AA or other UFAs up to 50 µM promoted 5-LO phosphorylation. However, saturated fatty acids had no effect on 5-LO phosphorylation. After substitution of Ser663 to Ala in 5-LO enzyme, ERK2 failed to strongly phosphorylate 5-LO, independent of added UFAs. Using specific inhibitors for PKC (GF109203x) and for MEK1/2 (U0126), we could demonstrate the involvement of these enzymes in ERK1/2 activation by PMA in MM6 cells. Furthermore, ERK1/2, immunoprecipitated from PMA-primed or Ca^{2+} ionophore stimulated MM6 cells, phosphorylated 5-LO or MBP in *in vitro* kinase assays.

Interestingly, ERKs are involved in AA release and 5-LO product formation in MM6 cells. Thus, inhibition of PKC or MEK1/2 suppressed PMA-induced [^{3}H]AA release and also 5-LO product formation. Also for human PMNL, an involvement of ERKs in 5-LO product formation could be demonstrated. Addition of increasing amounts of exogenous AA to PMNL in absence of extracellular Ca^{2+} resulted in an increased activation of ERK1/2 correlating with an elevated level of 5-LO products. U0126 (3 μM) reduced residual 5-LO activity to 40% in contrast to 5-LO activity induced by Ca^{2+} or cell stress, which was not affected by U0126. Also addition of chemotactic fMLP to PMNL resulted in ERK1/2 dependent 5-LO product formation.

Transient transfection of HeLa cells with wt 5-LO or Ser663Ala-5-LO and subsequent stimulation with increasing amounts of AA in presence of Ca^{2+} ionophore led to a similar product formation. However, stimulation of 5-LO activity by AA alone resulted in a distinct reduced LT formation for mutated 5-LO in contrast to wt 5-LO, implicating a prominent role for ERKs in cellular 5-LO activation. Further experiments revealed a conjugated role of MK2 and ERKs regarding activation of cellular 5-LO product formation. Thus, preincubation of human PMNL with SB203580 or U0126 followed by challenging with AA in absence of extracellular Ca^{2+} led to a markedly reduced LT formation with 60% and 40% residual activity, respectively. Combining both inhibitors gave an additive effect resulting in 17% residual activity. These results could be supported by experiments using HeLa cells transfected with wt 5-LO, Ser663Ala-5-LO or a 5-LO mutant (Ser271Ala/Ser663Ala-5-LO) where both phosphorylation sites were removed. For wt 5-LO, the ratio of AA- to AA plus ionophore-induced 5-LO product formation at 10-60 μM AA was 24-43%, for 5-LO-Ser663Ala 11-22%, and for the 5-LO-Ser271/663Ala 5-14%. Thus, phosphorylation at both, Ser663 and Ser271, appears to be required for 5-LO product formation in intact cells at low Ca^{2+} levels.

In analogy to ERK2, 5-LO was also a weak substrate for MK2 and dose dependently phosphorylated by both kinases up to 30 mU of kinase. Moreover, according to phosphorylation by ERK2 also addition of up to 50 μM AA as a cofactor to MK2 led to an increased phosphorylation of 5-LO. This UFA-promoted phosphorylation correlated with a raised 5-LO activity in intact human PMNL. Adding increasing amounts of exogenous AA gave maximum 5-LO kinase activity associated with maximum LT formation. Specific inhibition of kinases upstream of both 5-LO kinases by SB203580 (p38 MAPK) or/and U0126 (MEK1/2) pointed out the involvement of MK2 and ERK1/2 in 5-LO activation in intact cells. 5-LO reveals putative phosphorylation motifs for MK2 (aa 266-271) and ERK2 (aa 661-664) with Ser271 and Ser663 as potential phosphorylation sites. Finally, after mutation of these residues to alanine and transfection of HeLa cells with wt and mutated 5-LO, cellular 5-LO activity was determined.

Nearly identical amounts of 5-LO products were accumulated when cells were treated with AA plus Ca^{2+} ionophore, whereas clearly less LTs were produced by mutated 5-LO as compared to wt 5-LO when cells were stimulated with AA alone. Lastly, removing of both potential phosphorylation sites resulted in an even more pronounced reduction of AA-induced 5-LO activity.

The author of this thesis contributes the results of studies regarding the influence of saturated and unsaturated fatty acids, determined by *in vitro* kinase assay.

B. Paper III: Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors

Background and rationale:

The bioactive lipids have pivotal functions in pathophysiology of inflammatory diseases such as allergy or arthritis [303] and are involved in carcinogenesis [121, 281], metabolism of bone [111, 186] and atherogenesis [271, 323]. Suppression of 5-LO mediated LT formation via specific 5-LO inhibitors confirmed the therapeutic value of antileukotriene therapy in asthma and other inflammatory diseases [325]. Furthermore, based on recently discovered clinical implications of LTs in cancer [15, 82, 112], osteoporosis [6, 186], and vascular diseases [271, 378], 5-LO inhibitors may also posses considerable potency in the treatment of such diseases.

In addition to activation by Ca^{2+}, 5-LO can also be activated via phosphorylation by MK2 and/or ERKs using genotoxic (SA) or osmotic (NaCl) stress, or even AA alone as stimulus [paper I, II, 45, 354-356, 360]. In particular, activation of 5-LO by phosphorylation is Ca^{2+}-independent in human PMNL [353].

The nonredox-type 5-LO inhibitors, such as ZM230487 and L-739.010, are potent, orally active inhibitors of LT biosynthesis in a variety of *ex vivo* and *in vitro* models which reduce acute inflammatory responses [219, 220, 321], but failed to suppress more chronic inflammatory processes [234]. It was found that nonredox-type 5-LO inhibitors require GPx for efficient inhibition of 5-LO activity [359].

The aim of this study was to reveal if the activation pathway of 5-LO contributes to the efficacy of nonredox-type 5-LO inhibitors.

Results:

In a 5-LO activity assay under standard assay conditions using Ca^{2+} ionophore as stimulus, ZM230487 and L-739.010 were potent nonredox-type 5-LO inhibitors in isolated human PMNL with IC_{50} values of 20 nM and 10 nM, respectively. In contrast, 10- to 100-fold higher concentrations were required when 5-LO was activated by phosphorylation using genotoxic

(10 µM SA) or osmotic (300 mM NaCl) stress as stimulus. In an analogous manner, activation of 5-LO by 60 µM AA alone caused a shift of the IC_{50} value up to 0.4 µM and 0.8 µM for ZM230487 and L-739.010. Also, upon treatment of PMNL with natural ligands such as PAF or fMLP the potency of nonredox-type 5-LO inhibitors was significantly impaired. In contrast, the potency of the iron ligand inhibitor BWA4C was stimulus-independent with a consistent IC_{50} of 70-90 nM.

Further experiments showed that Ca^{2+}-depletion impaired the efficacy of ZM230487 in SA-activated PMNL. Removal of extracellular Ca^{2+} by adding 1 mM ETDA further reduced the efficacy of ZM230487 up to fivefold compared to conditions when extracellular Ca^{2+} was present. Again, almost no difference could be shown for BWA4C in this respect. To check if Ca^{2+} is required for potent 5-LO inhibition by nonredox-type 5-LO inhibitors, PMNL were exposed to SA 3 min before synchronic addition of AA and Ca^{2+} ionophore. The results revealed that SA counteracted efficiently 5-LO inhibition by ZM230487 in ionophore-stimulated PMNL and that Ca^{2+} was not needed for effective 5-LO inhibition. This fact could be underlined by using purified 5-LO enzyme.

Although treating PMNL with Ca^{2+} ionophore leads to activation of MK2, phosphorylation of 5-LO is not responsible for an impaired potency of nonredox-type 5-LO inhibitors in this case. This can be explained by comparison of kinetic data. In ionophore-induced 5-LO product formation 80% of the maximal product amount were achieved after 1 min. However, significant activation of MK2 occurred first after 1 min, with a maximum after 2 min in ionophore-challenged PMNL. Consequently, 5-LO phosphorylation events should not counteract inhibition of ionophore-induced 5-LO activity by nonredox inhibitors.

Transfection of HeLa cells with wt 5-LO or non-phosphorylatable mutant 5-LO (Ser271Ala/Ser663Ala) showed that deletion of the phosphorylation sites increases the efficacy of nonredox-type 5-LO inhibitors. In HeLa cells expressing wt 5-LO, stimulation with 40 µM AA, ZM230487 and L-739.010 suppressed 5-LO product formation with IC_{50} values of 630 nM and 510 nM, respectively. Interestingly, for Hela cells transfected with mutant 5-LO, 6- to 10-fold lower concentrations were needed. In contrast, the iron ligand inhibitor BWA4C showed no such differences.

To verify if phosphate incorporation alone protects 5-LO towards nonredox-type inhibitors, ZM230487 was tested against phosphorylated and non-phosphorylated purified enzyme. However, there was no significant shift of the IC_{50} values of ZM230487 for phosphorylated compared to unphosphorylated enzyme in cell free assays.

The author of this thesis is responsible for the following studies, published in this paper:

- Efficacy of nonredox-type 5-LO inhibitors in human PMNL
- Ca^{2+} depletion impairs the efficacy of nonredox-type 5-LO inhibitors in SA-activated PMNL
- SA counteracts efficient 5-LO inhibition by ZM230487 in ionophore-stimulated PMNL
- Ca^{2+} has no influence on inhibition of purified 5-LO by ZM230487
- Effects of N-acetylcysteine on 5-LO inhibition by ZM230487
- Effects of *in vitro* phosphorylation of 5-LO on the susceptibility toward ZM230487

C. Paper IV: Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610

Background and rationale:

Although a huge number of 5-LO inhibitors have been developed within the last 20 years, only one of the compounds could make it to the market. This is due to the fact that these inihibitors showed poor efficacy *in vivo*, only a short half life, and a number of side effects. However, with respect to the biological properties of LTs, further development of 5-LO inhibitors is still beneficial for the treatment of a variety of diseases (see above and [352]).

5-LO contains a nonheme iron in the active site, which is essential for catalytic activity in its oxidized form. Reducing compounds (such as polyphenols) or iron-ligands (such as zileuton) are potential inhibitors of 5-LO, but despite their high potency *in vitro*, these compounds exhibit only poor bioavailability, only modest selectivity for 5-LO and thus a high potential for side effects [325].

Another approach for efficient and selective 5-LO inhibition is the development of nonredox-type inhibitors. Promising data revealed a high potency in several *ex vivo* and *in vitro* studies of such compounds but they failed to inhibit more chronic inflammatory processes [234, 339]. It was found that the efficacy of nonredox-type inhibitors depends on the peroxide level [359] as well as on the activation pathway of 5-LO [paper III]. In contrast to the known inhibitors, the new compound CJ-13,610 exhibits an enhanced metabolic stability which results in fewer *in vivo* metabolites, as well as improved bioavailability and a better toxicological profile.

The aim of this study was to characterize the molecular pharmacological profile of the novel ionizable imidazolylphenyl compound CJ-13,610.

Results:

When freshly isolated human PMNL were incubated with CJ-13,610 and stimulated with Ca^{2+} ionophore in the absence of exogenous AA, the IC_{50} value was 70 nM. Supplementation of exogenous AA impaired the efficacy of CJ-13,610 and shifted the IC_{50} to higher values. In detail, adding 2 µM AA resulted in an IC_{50} of about 280 nM and further increase of substrate concentration up to 100 µM diminished the potency of CJ-13,610. Kinetic analysis emphasizes competitive inhibition of 5-LO in intact human PMNL. Moreover, at low substrate availability CJ-13,610 completely blocked 5-LO activity whereas at high concentrations of AA, some basal activity still remained.

In order to check if CJ-13,610 is a direct 5-LO inhibitor, the efficacy of this compound in broken cell preparations was examined. Likewise for classic nonredox-type inhibitors, also CJ-13,610 failed in homogenates at high peroxide levels, independent of the substrate concentration. However, restoring peroxidase activity by inclusion of thiols, the inhibitor regained its potency. At low AA levels (4 µM) the IC_{50} value was about 280 nM. When the AA concentration was increased up to 40 µM, the IC_{50} shifted to 700 nM, indicating a competitive mode of action. This could also be observed in the corresponding Lineweaver-Burk plot. Regarding inhibition of purified recombinant 5-LO, CJ-13,610 failed again under non-reducing conditions. Again, when 30 mU GPx-1 was added, CJ-13,610 showed an IC_{50} of about 5 µM at 4 µM AA. Using 40 µM AA as substrate, the compound failed completely to inhibit 5-LO.

To confirm the hypothesis that the efficacy of CJ-13,610 depends on the peroxide level, exogenous LOOH were added to intact cells before challenging the cells with Ca^{2+} ionophore and AA. When 3 µM 13(S)-HpODE was added to the cells, the IC_{50} shifted from 0.55 µM to 3.1 µM. Further increase of LOOH to 10 µM completely abolished 5-LO inhibition by CJ-13,610. Interestingly, in the absence of exogenous AA, 3 µM 13(S)-HpODE reduced the efficacy to the same extent.

In contrast to ZM230487 and L-739.010, the potency of CJ-13,610 was not affected by cell stress. Stimulating human PMNL with 10 µM SA, 300 mM NaCl, or 2.5 µM Ca^{2+} ionophore led to similar IC_{50} values.

In order to confirm that inhibition of 5-LO by CJ-13,610 is independent of the phosphorylation status of the enzyme, HeLa cells were transfected with wt 5-LO or mutant 5-LO where the phosphorylation sites were removed. Interestingly, in contrast to PMNL, low concentrations of CJ-13,610 led to an increased 5-LO product synthesis in HeLa cells transfected with wt 5-LO. The IC_{50} values for mutant and wt enzyme were approximately the same but were 10-fold higher than in human PMNL.

Finally, the effect of CJ-13,610 on 5-LO translocation was examined using a subcellular fractionation protocol. In resting cells, 5-LO was detected mainly in the non-nuclear fraction. However, challenging cells with Ca^{2+} ionophore caused enrichment in the nuclear fraction which was not affected by increasing amounts of CJ-13,610 up to 10 μM.

All experiments and results, published in this paper, were performed by the author of this thesis.

D. Paper V: Boswellic acids activate p42 MAPK and p38 MAPK and stimulate Ca^{2+} mobilization

Background and rationale:

Boswellic acids (BAs) are pentacyclic triterpenes which have been identified as the active principles of Frankincense, the gum resin of *Boswellia* species. *Boswellia serrata* (BS) extracts have been traditionally used as folk medicine to cure inflammatory and arthritic diseases [296]. Moreover, BAs were pointed out to suppress the formation of LTs by targeting 5-LO [294, 297] and to inhibit human leukocyte elastase [295]. In addition, they have been reported to affect the growth and differentiation of tumour cells [115, 146, 200, 256, 364]. Activation of caspase-8 [200] and ERK1/2 [256], or inhibition of topoisomerases [146] may contribute to these effects.

Intriguingly, the potency of AKBA to inhibit LT formation strongly depends on the cell type and differs between intact cells (~12 μM) and homogenates (~50 μM) [357]. Furthermore, low concentrations of BS extract [293] and 3-oxo-tirucallic acid [26], isolated from BS extracts, enhanced 5-LO product formation. For this reason, the aim of this study was to address the effects of BAs on various signalling pathways regulating cellular 5-LO product synthesis in PMNL such as MAPK and Ca^{2+}.

Results:

After treatment of human PMNL with increasing amounts of BS extract up to 13 μg/ml, activation of MAPK by extracts of *B.serrata* and isolated BAs was analyzed using phospho-specific antibodies. BS extract (0.38 to 13 μg/ml) led to a dose dependent activation of p38 and ERK2 and also its ingredients AKBA (30 μM) and KBA (30 μM) showed similar effects for both MAPKs. In contrast, the corresponding keto-free BAs β-BA and A-β-BA showed no activation of ERK2 and only slight activation of p38 MAPK. The pentacyclic triterpenes α-amyrin and ursolic acid failed to activate MAPKs. These effects could be underlined also by in-gel kinase assay with MBP as substrate for ERK2.

Concentration response experiments with AKBA revealed that p38 and ERK2 became clearly activated when cells were treated with 30 μM. In contrast, 100 μM KBA were required for substantial activation of ERK2 whereas 10 to 30 μM was sufficient for activation of p38. The

failure of β-BA and A-β-BA indicated that the 11-keto group is a structural requirement for activation of MAPKs. Kinetic analysis exhibited a rapid activation after 30 s with a maximum after 1 to 2.5 min.

In order to check if BAs induce Ca^{2+} mobilisation, isolated PMNL were loaded with the dye FURA-2 and subsequently the fluorescence was analyzed and $[Ca^{2+}]_i$ determined. In unstimulated cells the basal $[Ca^{2+}]_i$ was about 40-50 nM. After stimulation with 30 μM AKBA, the $[Ca^{2+}]_i$ raised up to ~230 nM, similar to treatment with 1 μM fMLP (~240 nM). Challenging cells with 30 μM KBA gave Ca^{2+} concentrations up to 145 nM. Other pentacyclic triterpenes were virtually not effective.

To elucidate the activation pathways upstream of MAPK, the dependency on Ca^{2+} mobilisation was verified. Depletion of extracellular and/or intracellular Ca^{2+} only partially suppressed AKBA-induced ERK2 activation. Finally, the involvement of PKC and phosphatidyl inositol 3-kinase (PI 3-K) was investigated by Western blot analysis and in-gel kinase assay. Inhibition of PKC by RO-31-8425 slightly reduced ERK2 and p38 activation by 30 μM AKBA, whereas GF109203x rather enhanced ERK2 activation. Inhibition of PI 3-K by wortmannin strongly decreased ERK2 and p38 activation by AKBA.

The following experiments of this paper were performed by the author of this thesis:

- Activation of MAPK by extracts of *B. serrata* and isolated BAs
- Effects of PKC and PI 3-K inhibitors on MAPK activation induced by AKBA

All results were determined by in-gel kinase assay.

E. Paper VI: Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leukocytes

Background and rationale:

In paper V it was shown that KBA and AKBA activate MAPKs and stimulate Ca^{2+} mobilisation in human PMNL. In this study we attempted to connect these observed effects to functional responses of PMNL including formation of ROS, release of AA, and LT biosynthesis.

Results:

Human PMNL were loaded with DCF-DA and subsequently the oxidation of the dye was measured via fluorescence spectoscopy. fMLP (1 μM), Ca^{2+} ionophore (2.5 μM) as well as PMA (100 nM) caused a rapid formation of ROS. Also, challenging cells with AKBA and KBA (each 30 μM) led to prominent ROS formation, whereas BAs without keto-moiety, namely β-BA and A-β-BA, had only moderate effects. DPI, a direct inhibitor of NADPH oxidase, almost

completely blocked PMA- and also AKBA-induced ROS formation. Depletion of extra- and intracellular Ca^{2+} had similar effects. Further inhibitor studies revealed an involvement of PI 3-K (using 50 nM wortmannin or 30 µM LY-294002) and ERK1/2 (3 µM U0126) in AKBA-induced ROS formation. This differed clearly from the activation pathway of PMA-induced ROS formation since only the PKC inhibitor RO-318425 had a pronounced effect. Pertussis toxin, a potent inhibitor of $G_{\alpha i/0}$-proteins, attenuated BA-induced mobilisation of Ca^{2+} and MAPK activation in PMNL.

The action of BAs on the liberation of AA was tested and both, AKBA and KBA, considerably enhanced the release of [^{3}H]-labelled AA, whereas β-BA and A-β-BA showed only a weak impact. Chelation of Ca^{2+} again completely abolished the effects of keto-BAs.

Although BAs had been reported to inhibit 5-LO product formation, the later results suggested a feasible activation of 5-LO due to the ability of BAs to mobilize Ca^{2+} and activate ERK1/2. Interestingly, simultaneous addition of AA and keto-BA in absence of Ca^{2+} resulted in an increase of 5-LO products up to 3.8 fold as compared to cells stimulated with AA alone, whereas β-BA and A-β-BA had no effects. Concentration response analysis indicated that AKBA at higher concentrations (100 µM) failed to stimulate 5-LO. Surprisingly, adding MAPKs inhibitors did not influence AKBA-induced LT formation. Since prolonged exposure of 5-LO to Ca^{2+} or ROS leads to inactivation of the enzyme [103], extended treatment of cells with AKBA could explain its inhibitiory potency. Therefore, cells were preincubated in the absence of Ca^{2+} with AKBA and finally stimulated with Ca^{2+} ionophore and AA in the presence of Ca^{2+}. This experiment showed a strongly impaired efficacy of AKBA when cells were preincubated in the absence of Ca^{2+}.

Finally, the impact of BAs was tested towards different haematopoietic cell lines, such as differentiated MM6 and granulocytic HL-60 cell lines. None of the BAs had any influence regarding MAPKs activation, Ca^{2+} mobilisation or ROS formation in MM6 cells, whereas effects of BAs in granulocytic HL-60 cells equated those in PMNL.

The testings of BAs regarding their ability to elevate the liberation of AA from PMNL were carried out by the author of this thesis.

F. Paper VII: Inhibitors of actin polymerization upregulate arachidonic acid release and 5-lipoxygenase activation by upregulation of Ca^{2+} mobilisation in polymorphonuclear leukocytes involving Src family kinases

Background and rationale:

Previous studies proposed a regulatory role of the cytoskeleton for the capacity of leukocytes to form LTs. Inhibition of actin polymerization by cytochalasin (Cyt) potentiates LT formation induced by fMLP [128, 254, 300]. Additionally, 5-LO is able to bind to actin [192] and interacts with CLP, an actin-binding protein [265, 266]. Consequently, there are several implications for an involvement of the cytoskeleton in matters of regulation of LT biosynthesis.

The aim of this study was to enlighten the link between the cytoskeleton and cellular LT formation and to elucidate the signalling molecules modulating the effector enzymes 5-LO and $cPLA_2$.

Results:

Freshly isolated PMNL were incubated in presence or absence of 3 μM latrunculin B (LB), a potent inhibitor of actin polymerization, with increasing amounts of TG. Determination of synthesized 5-LO products revealed an increasing signal starting at a concentration of 1 μM TG in absence of LB. Inhibition of actin polymerisation by 3 μM LB already led to a prominent signal using 0.01 μM TG as stimulus, and increased LT formation up to 20-fold. Dose response experiments showed an EC_{50} of 0.2 μM LB with a maximal effect at 3 μM. Furthermore, synchronic stimulation with LB and TG gave the strongest effect, whereas prolonged preincubation reduced 5-LO stimulatory effects of LB. In contrast to the TG signal, LB only marginally potentiated ionophore- or fMLP-induced LT formation. Finally, kinetic analysis showed a lag-phase of 70 sec when TG was coincubated with LB, as compared to TG alone (150 sec).

In accordance with 5-LO product formation, LB multiplied also the release of AA from PMNL induced by TG, but not by Ca^{2+} ionophore or fMLP. Again, the EC_{50} of LB for the AA release was about 0.2 μM, and pronounced effects were obtained at suboptimal concentrations of TG (0.01 to 1 μM).

In order to determine if LB also promotes 5-LO translocation, PMNL were stimulated with TG or ionophore with or without LB. Subsequently, subcellular localisation of 5-LO was assessed. Whereas TG caused only weak 5-LO translocation to the nucleus, addition of LB caused a significant enrichment of 5-LO at the NE. In contrast, the innate prominent effect of ionophore was not further promoted.

Based on these results, elevation of intracellular Ca^{2+} might be the reason for the observed LB effects. PMNL were loaded with FURA-2 and subsequently Ca^{2+} mobilisation was determined using fluorescence spectrometry. TG caused a moderate Ca^{2+} influx (from 100 to about 200 nM) as compared to ionophore (from 100 to about 800 nM). Intriguingly, LB alone gave a delayed but prominent signal up to 300 nM $[Ca^{2+}]_i$. An additive effect was observed when cells were simultaneously stimulated with LB and TG (500 nM). Similar effects were also detected for ionophore and fMLP. Concentration-response studies showed an EC_{50} of ~0.2 µM LB regarding augmentation of TG-induced elevation of $[Ca^{2+}]_i$. Parallel to 5-LO activity and AA release, there were also concentration-dependent effects of TG on $[Ca^{2+}]_i$, whereas LB consequently potentiated Ca^{2+} mobilisation on top of TG signalling. U73122, a PLC inhibitor, suppressed LB-induced elevation of $[Ca^{2+}]_i$ as well as the upregulatory effects of LB but not the TG signal alone.

Activation of $cPLA_2$ and 5-LO is also regulated by p38 MAPK and ERK1/2 [113, 360, paper I, II]. LB alone caused only weak but rapid MAPK activation. Also, treating cells with TG induced a low signal as compared to fMLP and Ca^{2+} ionophore. Costimulation of PMNL with LB and TG did not result in an increased activation of p38 MAPK or ERK1/2.

Inhibitor studies revealed that Src family kinases mediate the stimulatory effects of LB on Ca^{2+} mobilisation, AA release and 5-LO activity. The specific Src kinase inhibitors PP2 and SU6656 suppressed LB-induced and -upregulatory effects of intracellular Ca^{2+} level but failed to reduce TG mediated signalling. The inactive analogue PP3 showed no influence. Similar results were shown for $cPLA_2$-mediated AA release and 5-LO activity. Finally, inhibition of Src family kinases by PP2 and SU6656 also blocked the upregulatory effects of LB and 5-LO translocation in PMNL treated with TG.

All experiments and results, published in this manusscript, were performed by the author of this thesis.

VI. Discussion

A. Phosphorylation of 5-LO related to physiological LT formation

Phosphorylation of proteins is a covalent modification of certain amino acid residues and a highly effective means of controlling the activity of specific proteins in structural, thermodynamic, kinetic, and regulatory manners. The 5-LO sequence contains a variety of phosphorylation motifs for kinases including PKA, PKC, CaMKII, MK-2/3, S6 kinase, ERK1/2, and Cdc2 [352]. However, residues distant to a designated motif, can contribute to a potential phosphorylation. The protein conformation may alter the accessibility of a possible phosphorylation site for a certain kinase.

In 1996, Lepley et al. were the first who demonstrated that ionophore-activated HL-60 cells contain a phosphorylated and a non-phosphorylated form of 5-LO [194]. Moreover, inhibitor studies indicated that MAPKK-1 participates in the molecular processes governing activation and translocation of 5-LO from the cytosol to the nuclear membrane [193]. Furthermore, the specific MEK1/2 inhibitor U0126 blocked LTC_4 production in IL-5 primed eosinophils [19] and fMLP challenged neutrophils [25, 194]. Release of AA by $cPLA_2$ is required for biosynthesis of LTs. Activation of $cPLA_2$ is mediated by phosphorylation via MAPKs [113, 199]. Inhibition of MAPK activity by U0126 or SB203580 suppressed AA and LTC_4 release in fMLP stimulated eosinophils [380]. But also in presence of exogenous AA, 5-LO activity was blocked by the specific MEK1/2 inhibitor PD98059 [193], implicating a more direct effect of ERKs in respect to 5-LO regulation. However, phosphorylating of 5-LO *in vitro*, using purified MAPK, could not be shown.

We could demonstrate that 5-LO is phosphorylated by MK-2/3 and ERK1/2 [355, paper I, II]. In gel kinase assay identified 5-LO as a substrate for MK-2/3 and a as poor substrate for ERK1/2. However, addition of UFAs such as AA increased the potency for both kinases, MK-2/3 and ERK1/2, to phosphorylate 5-LO [paper I, II, 360]. This effect was restricted to 5-LO as substrate, but did not occur on phosphorylation of MBP (ERKs) or Hsp27 (MK-2/3), suggesting a regulatory role of AA/UFAs regarding 5-LO conformation [paper I, II, 360].

By site directed mutagenesis studies, Ser271 was pointed out as potential phosphorylation site on 5-LO for MK-2/3 [360]. On the other hand, ERK1/2 failed to phosphorylate 5-LO, when Ser663 was substituted by Ala in the 5-LO sequence. HeLa cells transfected with 5-LO Ser663Ala produced only about 20% of 5-LO activity in contrast to cells transfected with wt 5-LO when cells were treated with 60 µM AA [paper I]. Thus, the results support a strong evidence for Ser663 as a putative ERK phosphorylation site in 5-LO.

We failed to demonstrate *in vivo* phosphorylation of 5-LO after stimulation of PMNL or differentiated MM6 and subsequent immunoprecipitation of 5-LO [paper I]. Experimental settings, rapid dephosphorylation, or inefficient ^{32}P-labelling of cells might be the reason. Furthermore, only a small amount of phosphorylated 5-LO may appear in stimulated cells, sufficient to induce substantial LT biosynthesis. However, stimulus-dependent differences in 5-LO product formation of the 5-LO Ser663Ala compared to wt 5-LO suggests that phosphorylation by ERKs plays a role for 5-LO activation in intact cells.

For cPLA$_2$ it is known that both, mobilisation of intracellular Ca^{2+} and phosphorylation, lead to activation of the enzyme [113, 270]. Our studies regarding cellular activation of 5-LO showed a similar pattern. HeLa cells, expressing the 5-LO Ser663Ala mutant, revealed equal LT formation compared to cells expressing wt 5-LO when the enzyme was activated by Ca^{2+}. However, the 5-LO Ser663Ala mutant, lacking the ERK1/2 phosphorylation site, produced significantly lower amounts of 5-LO metabolites when an ERK-dependent stimulus such as AA was used [paper II]. It was shown for AA, that it causes a pronounced ERK activation in various cell types [48, 145], but only a moderate Ca^{2+} influx [231, 353].

Almost all natural ligands that induce LT formation in human neutrophils, such as fMLP, PAF, C5a, or AA, activate ERKs [41, 95, 120] without inducing a pronounced mobilisation of Ca^{2+} [231, 309, 363]. In this context, inhibition of ERK activation by specific inhibitors could not prevent 5-LO activity induced by Ca^{2+} ionophore or by cell stress (mediated by phosphorylation of 5-LO via MK-2), whereas AA-induced LT formation correlated with ERK activity [paper I]. Interestingly, autoregulatory loops for AA-induced 5-LO activity may occur in cells since addition of exogenous 5-H(p)ETE to neutrophils stimulated ERKs, and conversion of AA by 5-LO was required for ERK activity [48, 50].

ERK and p38 MAP kinase activation are equally required for activation and maintenance of cPLA$_2$ activity [137]. A similar activation pathway can be supposed also for 5-LO (see figure 8). Using conditions where 5-LO phosphorylation is markedly promoted, we observed a gradually reduced enzyme activity when one or both phosphorylation sites (Ser271 and Ser663) were removed [paper I, II]. Moreover, blocking 5-LO kinase activation by specific inhibitors (SB203580 and/or U0126), we also observed effects implying that p38 and ERK pathway act in conjunction to stimulate 5-LO product formation [paper I, II].

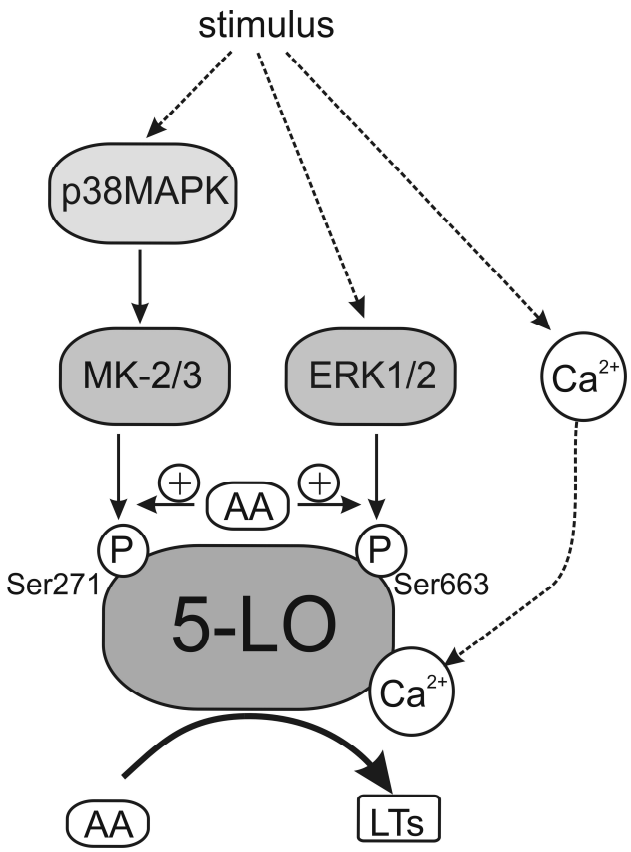


Figure 8: Phosphorylation of 5-LO

Translocation of a variety of enzymes (e.g. PKC, PI 3-K, or $cPLA_2$) to cellular membranes depends on their phosphorylation status [352]. There is clear evidence that also phosphorylation of 5-LO contributes to translocation of the enzyme. The PKC activator PMA up-regulates capacities for phosphorylation and translocation of 5-LO in MM6 cells and human PMNL [356]. Rapid nuclear translocation was also observed after activation of p38 MAPK by SA in human neutrophils, independent of the intra- or extracellular Ca^{2+} level [353]. Moreover, mutation of Ser271 reduced ionophore-induced LTB_4 synthesis in parallel with reduced nuclear localization of 5-LO [202]. Also the ERK pathway plays an essential role in 5-LO redistribution since inhibition of MEK1/2 blocked translocation in HL-60 cells and neutrophils [25, 193].

In intact cells, concentrations of 150 - 350 nM Ca^{2+} are sufficient for activating 5-LO [309]. Incorporation of anionic phosphate into a molecule alters the netto charge of the whole molecule and adds a negative charge to the protein. Therefore it might be reasonable that phosphorylated 5-LO shows a higher affinity to Ca^{2+}. For this reason the necessity of Ca^{2+} for activation of 5-LO would be impaired when the enzyme is phosphorylated. However preliminary results did not confirm this hypothesis (unpublished data).

B. Cell stress and phosphorylation alter the efficacy of nonredox-type 5-LO inhibitors

LTs participate in host defense reactions and pathophysiological conditions such as immediate hypersensitivity and inflammation [143, 305]. In view of these properties, inhibitors of 5-LO have therapeutic potential in a range of diseases including inflammatory disorders, cancer, osteoporosis, and atherosclerosis [325, 352].

In search of potential orally active and specific inhibitors, nonredox-type 5-LO inhibitors came in the focus of anti-LT research. In several *in vitro* and *ex vivo* systems they exhibited high efficacy [65, 219, 321]. However, significantly higher doses were required for *in vivo* studies [321], and they failed in respect to suppression of chronic inflammatory diseases [339].

The results of paper III provide strong evidence that the efficacy of the nonredox-type 5-LO inhibitors depends on the 5-LO activation pathway. 10- to 100-fold higher concentrations are required for cell stress-induced 5-LO product formation, including 5-LO kinase activity, in contrast to ionophore-induced LT formation, initiated by elevation of intracellular Ca^{2+}. BWA4C, an iron ligand inhibitor, revealed a potent efficacy, independent of the activation pathway. Studies using mutant 5-LO, lacking the phosphorylation sites, underlined the hypothesis that phosphorylation of 5-LO strongly impairs the potency of nonredox 5-LO inhibitors [paper III]. Further experiments showed that inhibition of both 5-LO kinases via SB203580 and U0126 and subsequent stimulation of 5-LO via SA in presence of exogenous AA restored the potency of the classical nonredox-type 5-LO inhibitors (unpublished data).

In general, 5-LO can be activated by two different pathways. First, by mobilisation of intracellular Ca^{2+} and second, by p38 MAPK- and/or ERK-dependend phosphorylation of 5-LO which is Ca^{2+} independent [352]. p38 is activated in response to cell stress and chemotactic factors such as LPS and ILs [144, 180]. Activation of ERKs is observed after priming cells with GM-CSF, PMA, fMLP or PAF which also all lead to an increased 5-LO activity [paper I]. Therefore, it seems reasonable that, under physiological conditions, LT formation occurs after activation of 5-LO kinases associated with cell stress (heat shock, osmotic or genotoxic stress), with the release of inflammatory cytokines (TNFα, IL-1) or chemotactic factors (C5a, PAF, fMLP). As a result, phosphorylation-dependent 5-LO catalysis showed an impaired sensitivity towards nonredox-type inhibitors which may explain the loss of effectivity of these inhibitors in the treatment of chronic inflammatory diseases [234, 339].

More detailed investigations showed that Ca^{2+} is not required for efficient 5-LO inhibition. Treating cells with genotoxic stress via SA 3 min before stimulation with ionophore showed also a loss of potency of the nonredox inihibitor ZM230487 [paper III]. Oxidative stress and

increased peroxide levels lead to an impaired efficacy of nonredox-type 5-LO inhibitors, too [359]. However, NaCl or SA did not increase the cellular peroxide level, and addition of reducing agents such as N-acetylcysteine (NAC) to cells stimulated by cell stress did not restore the potency of ZM230487 [paper III], implying a mechanism independent of the peroxide level.

Transfection experiments support the hypothesis that in intact cells phosphorylation of 5-LO alters the susceptibility to nonredox-type 5-LO inhibitors. Mutation of 5-LO resulted in a different sensitivity towards ZM230487 or L739.010 as compared to wt 5-LO when the enzyme was activated via p38 MAPK/ERK-mediated pathways. In detail, the non-phosphorylatable mutant 5-LO (Ser271Ala/Ser663Ala) activity was significantly more suppressed by ZM230487/L-739.010 than the phosphorylatable wt 5-LO [paper III]. However, *in vitro* studies using purified enzyme revealed that phosphate incorporation alone is not the sole reason for this effect [paper III], implicating that cellular integrity or cellular components are required. In previous *in vitro* studies it was found that deletion of the phosphorylation sites did not alter the enzymatic activity of 5-LO *in vitro* [paper I, 360]. However, deletion of phosphorylation sites caused significant loss of 5-LO product formation in intact HeLa cells stimulated by MAPK pathways [paper I, II, 360], supporting the finding that phosphorylation of 5-LO affects catalytic properties of the enzyme in intact cells only. Indeed, 5-LO interacts with several proteins [265, 267] and these interactions might be affected by the phosphorylation status of 5-LO.

Initially, for nonredox-type 5-LO inhibitors such as ZM230487 and L-739.010 a competitive mode of action was assumed by binding to a fatty acid substrate binding site at the active site. However, only under non-reducing conditions this type of inhibitors revealed such competitive properties [359]. Instead, under reducing conditions the compounds propably bind to a regulatory domain distal of the catalytic site, presumably a fatty acid hydroperoxide binding site. Since phosphorylation of 5-LO is promoted by UFA [paper I, II] it is reasonable that incorporation of phosphate in close proximity to such a regulatory fatty acid binding site enables the enzyme to interfere with a cellular protein as outlined before. Such protein/protein interactions could interefere with the affinity of ZM230487/L739.010 to the potential binding site by promoting the binding of LOOH to 5-LO which in turn compete with the nonredox-type 5-LO inhibitors at this regulatory site. This model would be compatible with the evidence that the efficacy of BWA4C does not differ between the phosphorylated and non-phosphorylated 5-LO.

So far, potential nonredox-type 5-LO inhibitors were tested by functional test-systems using leukocytes stimulated by the unphysiological stimulus Ca^{2+} ionophore [219, 321, 357]. Although Ca^{2+} ionophore causes not only a rapid mobilisation of Ca^{2+} but also activates MAP kinases

[paper I, III], kinetic studies revealed that Ca^{2+} is the predominant mediator of 5-LO activity under these conditions [paper III].

Based on these facts, it is not astonishing that ZM230487 or L-739.010 showed high activity in several *ex vivo* and *in vitro* test-systems, but failed in clinical studies. Thus, the development of new nonredox-type 5-LO inhibitors should lead to compounds which show a high inhibition efficacy, independent of the 5-LO activation pathway. For this reason, the use of phosphorylation-based test-systems as described should be taken into account.

C. CJ-13,610 as novel approach for potent 5-LO inhibition

The initial idea for the development of nonredox-type 5-LO inhibitors was to design molecules which compete with AA at the catalytic binding site of 5-LO [220]. In fact, their precise mode of action has not been elucidated so far. Under reducing conditions, ZM230487 or L-739.010 showed an uncompetitive mode of inhibition. However, elevated peroxide levels changed the mode of action to a competitive manner [359]. Moreover, detailed studies revealed, that LOOH compete with the inhibitors at a potential regulatory binding site of 5-LO, since oxidative stress and increased peroxide levels lead to impaired efficacy of ZM230487 or L-739.010 [359]. Also, the new nonredox-type inhibitor CJ-13,610 requires a low peroxide tone for potential 5-LO inhibition. Again, addition of LOOH impaired the efficacy of the compound [paper IV].

Experimental data support that 5-LO has two fatty acid binding sites, a regulatory LOOH binding site and an catalytic AA binding cleft at the active site [45, 298]. In contrast to ZM230487, the potency of CJ-13,610 is impaired by AA also under reducing conditions, implying that the compound interferes not only with a regulatory site but also with the catalytic AA binding site. This could be shown by several experimental setups: In intact PMNL, addition of exogenous AA clearly impaired the potency of CJ-13,610. Also in broken cell preparations, supplemented with DTT, elevated levels of AA significantly decreased the efficacy of the compound. Finally, by using purified enzyme in the presence of GPx and GSH, CJ-13,610 strongly suppressed 5-LO activity at 4 µM AA but failed at 40 µM AA [paper IV]. These data revealed a completely different inhibitory profile for ZM230487 or L-739.010 whose high affinity to 5-LO was not affected by AA under reducing conditions [359]. Intriguingly, LOOH is superior to AA in competing with CJ-13,610, since addition of 3 µM HpODE results in a shift of the IC_{50} value independent of the present amount of AA [paper IV]. For this reason it should be considered that CJ-13,610 may have a reduced potency in chronic inflammation which are associated with high LOOH levels and free AA [51, 62]. In fact, the classical nonredox-type 5-LO inhibitors failed in the treatment of chronic inflammatory diseases [234, 339].

In HeLa cells, transiently transfected with 5-LO, the IC_{50} of CJ-13,610 was about 10-fold higher than in PMNL [paper IV]. Differences in LOOH and Ca^{2+} levels, expression levels of GPx or the absence of FLAP may be the cause of the impaired potency of CJ-13,610 in HeLa cells. However, cotransfection of 5-LO and FLAP had no influence regarding inhibition of LT formation by CJ-13,610 [paper IV]. These findings were supported by the fact that CJ-13,610 does not inhibit the translocation of 5-LO, which may be FLAP dependent [262]. Therefore, the reasons for the difference in the efficacy of CJ-13,610 in HeLa cells and PMNL remain to be elucidated.

In response to external stimuli, cellular LT formation occurs after redistribution of cytosolic 5-LO to the nuclear envelope or cellular membranes [262]. This is mediated by elevation of intracellular Ca^{2+} and/or phosphorylation of 5-LO by MK-2/3 and ERK1/2, respectively [30, paper I, II]. Inhibition of phosphorylation-mediated activation of 5-LO required 10- to 100-fold higher concentrations of ZM230487 and L-739.010 than for Ca^{2+}-mediated 5-LO activity [paper III]. In contrast, the efficacy of CJ-13,610 is independent of the 5-LO activation pathway in PMNL. Also, CJ-13,610 exhibits no pronounced differences in the potencies towards phosphorylatable wt- or non-phosphorylatable mutant 5-LO in HeLa cells [paper IV]. It is noteworthy that several diseases, which are associated with an increased level of 5-LO metabolites, display also an increased cellular phosphorylation status. Inflammatory reactions, cancer, or atherosclerosis are some examples [127, 163].

Based on the fact that the potency of CJ-13,610 does not depend on the activation pathway of 5-LO, treatment of LT-associated diseases with CJ-13,610 might be beneficial. Although the therapeutic potential of CJ-13,610 could be limited under conditions of elevated peroxide levels, the novel compound exhibits advantages over former members of this class of inhibitors, which failed in clinical trials.

D. Boswellic acids activate proinflammatory functions in PMNL

Frankincense extracts and BAs, biologically active pentacyclic triterpenes from frankincense, block LT biosynthesis and exert anti-inflammatory effects [295, 357]. However, low concentrations of BAs [293] and 3-oxo-tirucallic acid [26] enhance LT formation. In this study we could demonstrate that BAs, namely 11-keto-BAs, stimulate activation of ERK2 as well as p38 MAPK. Also, mobilisation of intracellular Ca^{2+} by (A)KBAs was detected [paper V]. Consequently, BAs were able to induce the production of ROS, liberation of AA and, finally, induction of LT formation in PMNL and differentiated HL-60 cells [paper VI]. Notably, only 11-keto-BAs contributed to the later effects. In detail, the presence of the 11-keto group is

essential for these effects since β-BAs, lacking the keto group, failed in this respect. In parallel, the 11-keto group is necessary for suppression of the synthesis of DNA, RNA and protein in HL-60 cells [314]. It is remarkable that the 11-keto group was essential for inhibition of 5-LO activity [298], but on the other hand necessary for induction of LT formation [paper VI].

Isoforms of PKC are associated with the activation of ERK1/2 as well as p38 MAPK in PMNL [197]. However, specific inhibitors of PKC only partially inhibited (A)KBA-induced MAPK activation [paper V], which was also shown for fMLP-induced MAPK activation [174]. Inhibitor studies revealed that PI 3-K, which is partially involved in MAPK activation [174], plays a role in keto-BA-induced ERK2 activity [paper V]. In addition, treating of PMNL with (A)KBA resulted in a prominent increase of ROS which was abolished by the specific NADPH oxidase inhibitor DPI [paper VI]. Activation of NADPH oxidase requires Ca^{2+} and phosphorylation by certain kinases [79]. Pharmacological targeting of the proximal signalling pathways revealed that (A)KBA-induced ROS formation seemingly depends on PI 3-K and ERK2 activation as well as on Ca^{2+} but not on p38 MAPK activation [paper VI]. Similarly, fMLP-induced activation of NADPH oxidase was suppressed by inhibition of ERK2 [79] and PI 3-K [81] but not by inhibition of p38 MAPK [79].

Certain signalling pathways, including Ca^{2+} mobilisation and phosphorylation via MAPK, can lead to $cPLA_2$ activation and AA release [113]. 11-keto-BAs, but not β-BA and A-β-BA, induced the release of considerable amounts of AA, with a similar efficacy as fMLP. Like ligands acting via GPCR, AKBA-induced AA liberation also depends on Ca^{2+} and ERK2 activity [paper VI]. Interestingly, AA can induce apoptosis of macrophages [230] and therefore the apoptotic effect of AKBA, observed in granulocytic HL60 cells [146], may be explained by increased levels of free cellular AA.

BAs were reported as specific, nonredox-type inhibitors of 5-LO [294]. It is obvious that also additional factors are involved in suppression of cellular 5-LO activity since there is a difference of the IC_{50} values in intact cells (2-5 μM) and homogenates (15-50 μM) [357, 359]. Rapid and prolonged levels of Ca^{2+} and ROS cause suicide inactivation of 5-LO activity [103]. For this reason, preincubation of intact cells with (A)KBA may result in reduced 5-LO activity after suicide inactivation induced by Ca^{2+} and/or ROS. Paradoxically we observed that (A)KBA stimulated LT formation in PMNL when coincubated with AA [paper VI]. Also other ingredients of *B. serrata* extracts such as 3-oxo-tirucallic acid enhanced 5-LO product formation in ionophore-challenged PMNL [26]. As reported earlier, 5-LO activity depends on Ca^{2+} and activation of MAPK [352, paper I, II]. Surprisingly, AKBA-induced 5-LO activity was neither sensitive to inhibition of MAPK via SB203580 or U0126 nor to depletion of Ca^{2+} [paper VI].

This implicates that so far unknown 5-LO stimulatory factors or pathways may be activated by 11-keto-BAs.

Remarkably, the effects of (A)KBAs are similar to other chemotactic factors. Stimulation of neutrophils with PAF or fMLP caused a rapid and transient activation of MAPK and increased $[Ca^{2+}]_i$ [95, 232, 235]. Also characteristics of the fMLP signal such as time course, partial dependence on Ca^{2+} and PKC plus the involvement of PI 3-K, are conform with those induced by 11-keto-BAs [237]. Since G-proteins link the fMLP-receptor interaction to functional cellular responses [182], the BA-mediated signal could also be mediated by a GPCR and the 11-keto group might be essential for binding to this conceivable receptor. This is supported also by the fact that PTx suppressed AKBA induced Ca^{2+} mobilisation and MAPK activation. However, such a receptor was not identified so far.

E. Inhibitors of actin polymerization, effects on LT biosynthesis

Neutrophils provide the body´s first line of defense against bacterial infection, and are stimulated to move towards these sites by chemoattractans. Chemotaxis requires actin polymerisation followed by the formation of new filaments. Several reports were described that inhibition of actin polymerization potentiates the release of agonist-induced formation of AA metabolites [128, 254, 300], but so far the mechanisms involved were not elucidated. In this project we could show that inhibitiors of actin polymerization led to an elevation of $[Ca^{2+}]_i$ associated with an increase of free AA and 5-LO translocation. These events can increase LT formation [paper VII].

As outlined before, for LT biosynthesis several conditions have to be fulfilled including sufficient supply of AA by $cPLA_2$, activation of 5-LO and association of the enzyme with the NE [352]. Agonists, which induce LT formation, are also able to increase $[Ca^{2+}]_i$. $cPLA_2$ requires about 350-400 nM Ca^{2+} for activation whereas 5-LO shows a linear dependence on Ca^{2+} and saturates at this concentration [309]. Treating PMNL with the Ca^{2+} ionophore caused the release of about 800 nM $[Ca^{2+}]_i$ resulting in a prominent formation of LTs. However TG, which caused moderate increase of $[Ca^{2+}]_i$ (230 nM), led only to low amounts of 5-LO products [paper VII], implying that the extent of 5-LO products correlates with the concentration of $[Ca^{2+}]_i$. We observed that LB alone, which disrupts the microfilament organisation, caused an elevation of cellular Ca^{2+} levels [paper VII, 347]. Since TG plus LB gave additive effects regarding $[Ca^{2+}]_i$ (460 nM), it is conceivable that the TG-induced LT formation was upregulated by coaddition of LB [paper VII]. Although addition of LB on top of ionophore treatment resulted in additional Ca^{2+} mobilisation, there was neither an increase in liberation of AA nor of 5-LO activity. This is

not surprising since ionophore alone generated Ca^{2+} levels, which are high enough for maximal $cPLA_2$ activation.

Regulation of $cPLA_2$ and 5-LO via phosphorylation of serine residues is another aspect which should be considered [paper I. paper II, 113, 355], particularly when the Ca^{2+} level is low [270, 353]. Addition of LB caused rapid ERK2 and p38 MAPK activation but was much less effective compared to fMLP or ionophore [paper VII]. Since LB induced a prominent Ca^{2+} influx, we conclude that phosphorylation of $cPLA_2$ and/or 5-LO plays a minor role in the upregulating effect of LB. Supplementation of exogenous AA as substrate did not lead to an increased LT formation by LB [paper VII], confirming the hypothesis that LB elevates 5-LO product formation mainly by affecting $cPLA_2$. Even though LB promoted 5-LO translocation, activation of $cPLA_2$ seems to be the key step for substantial LT formation.

A variety of chemotactic agents, such as fMLP, PAF and C5a, not only activate the AA pathway but also induce actin polymerization via binding to GPCR [379]. It is of interest that $cPLA_2$ as well as 5-LO bind to certain proteins which are linked to the cytoskeleton. Vimentin, a component of cell intermediate filaments, interacts with the C2-domain of $cPLA_2$ [233] and also 5-LO was shown to bind to cytoskeletal proteins including α-actinin, actin and CLP [192, 265, 267]. The effects of actin polymerization on liberation of AA and LT formation, respectively, are discussed in a controversial manner. Several reports described that depolymerization of F-actin by CD or LB has an attenuating effect in stimulus-induced release of $[Ca^{2+}]_i$ and of AA release, in various cell types [67, 283, 292]. However, in accordance with our results, cytochalasin increased the $[Ca^{2+}]_i$ in human PMNL after stimulation with fMLP [243]. Similarly, inhibition of cytoskeletal rearrangement by botulinum C2 toxin amplifies ligand-evoked lipid mediator generation in human neutrophils [119]. Moreover, disruption of F-actin in human basophils also promotes events such as histamine release, LTC_4 release, and the intracellular Ca^{2+} signal [347]. In conclusion, the effect of actin polymerization on the AA pathway apparently depends on the cell type and the nature of the stimulus, but appears to be based on the Ca^{2+} level of the cell.

Our results implicate that the effects of LB are associated with activation of PLC isoenzymes and the Src family kinases. The PLC inhibitor U73122 [24] significantly suppressed the release of intracellular stored Ca^{2+}, evoked by LB [paper VII], whereas the structurally related negative control U73433 failed. It was reported that Src family kinases are capable of activating PLCγ1 and -2 isoforms in response to different chemoattractans [170, 260, 268]. We could demonstrate that the specific Src kinase inhibitors PP2 [135, 301] and SU6656 [23, 31] selectively blocked the effects of LB on Ca^{2+} mobilisation, AA release, 5-LO translocation, and 5-LO product formation. In contrast, the inactive compound PP3, used as a negative control for PP2, showed

no effect [paper VII]. Fgr, Hck, and Lyn are tyrosine protein kinases expressed in neutrophils [170]. Mice, deficient in Src family kinases Hck and Fgr, revealed defective adhesion-dependent neutrophil functions [201]. Additionally, there is evidence that disassembly of F-actin leads to an activation of Src kinases. For example, actin depolymerization, induced by LB, causes activation of phosphotyrosine kinases [93]. Moreover, CD induced increases in the phosphorylation levels of c-Src [302].

In conclusion, cytoskeletal dynamics and activation of Src kinases have an influence in AA metabolism. Our studies support the hypothesis that alteration of $[Ca^{2+}]_i$ via Src kinase signalling and accordingly liberation of AA via $cPLA_2$ is followed by the metabolism of AA by 5-LO, resulting in formation of proinflammatory LTs. The F-actin cytoskeleton is a fundamental component of all eukaryotic cells. It provides force and stability and plays an integral role in a diverse array of cellular processes including migration and adhesion of PMNL to sites of inflammation. Chemoattractant-induced actin polymerization may be regarded as a regulatory process, which controls Ca^{2+}-dependent functions. Metabolism of AA is one example.

VII. Summary

5-LO is the key enzyme in the biosynthesis of LTs and is predominantly expressed in myeloid cells, but was also found in dendritic cells. LTs are bioactive lipid mediators and play a fundamental role in a variety of inflammatory diseases such as asthma, psoriasis, arthritis or allergic rhinitis. Moreover, they are also involved in cancer cell proliferation, bone remodelling associated with osteoarthritis, and atherosclerosis. Based on these facts it is of considerable interest to develop drugs which target 5-LO. However, today no 5-LO inhibitor is available on the market. The only 5-LO inhibitor zileuton (Zyflo®, Abbott), which was on the US market, has been withdrawn from the market in June 2003. In order to identify an inhibitor of 5-LO for clinical application, such a compound should not only exhibit powerful efficacy in *in vitro* assays but also in *ex vivo* and *in vivo* models. For this reason the development of potential 5-LO inhibitors requires the knowledge about the detailed activation pathway of the 5-LO enzyme.

In resting cells 5-LO resides in the cytosol or in the soluble compartment of the nucleus. Upon stimulation, 5-LO translocates to the nuclear membrane and colocalizes with $cPLA_2$, FLAP and LTC_4S. At the nuclear membrane AA is released by $cPLA_2$ and presented by FLAP to 5-LO for LT formation.

The nonphysiological stimulus Ca^{2+} ionophore causes a rapid and prominent increase in intracellular Ca^{2+} which results in an intensive activation of 5-LO and consequently in substantial LT formation. Natural ligands such as fMLP, PAF, C5a or zymosan lead to a moderate Ca^{2+} mobilisation and thus, 5-LO activity is rather low. However, priming of leukocytes with cytokines, phorbol esters, LPS, or growth factors and subsequent treatment with natural ligands results in a more prominent increase in 5-LO product formation. In general, this elevated level of LTs is considered to be due to an increased liberation of AA by $cPLA_2$ resulting in higher substrate availability.

Ca^{2+}, ATP, PC and LOOH are factors, which are capable of modulating the *in vitro* activity of 5-LO. Much less is known about the factors that dominate the regulation of 5-LO in intact cells. Recent data revealed that p38-dependent MK-2/3 phosphorylates 5-LO and plays a significant role in the Ca^{2+}-independent cellular activation of 5-LO. Osmotic stress, heat shock, or chemical stress induces 5-LO product formation in parallel with p38 MAPK activation in PMNL. Additionally, inhibitor studies gave evidence for the assumption that also the MEK1/2 pathway is involved in the *in vivo* activation of 5-LO.

Indeed, using in-gel kinase- and *in vitro* kinase-assays, we could show, that 5-LO is a substrate for ERK1/2 [paper I]. Addition of polyunsaturated fatty acids such as AA or oleic acid

potentiated the rate of ERK2-phosphorylated 5-LO [paper I] but also of MK-2-phosphorylated 5-LO [paper II]. These effects seem to be specific for MK-2 and ERK2, since promotion of protein phosphorylation was restricted to 5-LO as substrate. Unfortunately, we failed to convincingly assess *in vivo* phosphorylation of 5-LO immunoprecipitated from stimulated $^{32}P_i$-labelled PMNL, MM6 or HL-60 cells. The experimental setup or rapid dephosphorylation might be the reason [paper I]. Studies with specific kinase inhibitors and mutated 5-LO enzyme prove that both, phosphorylation by MK-2 at Ser271 and by ERK1/2 at Ser663, contribute to the cellular activation of 5-LO in response to natural stimuli that do not prominently alter the intracellular Ca^{2+}-level [paper I, II]. In contrast, using Ca^{2+} ionophores, both, non-phosphorylatable and phosphorylatable 5-LO showed comparable enzyme activity. Together, phosphorylation of 5-LO by p38 MAPK-dependent MK-2/3 and ERKs is an alternative activation pathway of 5-LO. Moreover, these findings provide evidence that the utilization of assays, in which physiological stimuli and priming agents are operative, are more adequate for mimicking the physiological conditions of 5-LO activation in leukocytes.

Nonredox-type 5-LO inhibitors were originally designed to compete with AA at the catalytic site of 5-LO. In fact, ZM230487 and L-739.010 potently suppress LT biosynthesis in several *in vitro* and *ex vivo* test systems. However, they failed in clinical trials of chronic inflammatory diseases. In this study we could demonstrate that inhibition of 5-LO product formation by nonredox-type 5-LO inhibitors in human PMNL depends on the activation pathway of 5-LO [paper III]. Thus, compared with 5-LO product formation induced by Ca^{2+} ionophore, cell stress-induced 5-LO activity involving 5-LO kinase pathways required about 10- to 100-fold higher concentrations of ZM230487 and L-739.010 for comparable 5-LO inhibition. No such differences were observed for the iron ligand inhibitor BWA4C [paper III]. Further experiments revealed that Ca^{2+} is not required for sufficient 5-LO inhibition and that simultaneous stimulation with Ca^{2+} ionophore and cell stress did not restore the potent 5-LO inhibition. Moreover, studies with transiently transfected HeLa cells showed that the non-phosphorylatable 5-LO enzyme (5-LO Ser271Ala/Ser663Ala) is significantly more sensitive towards nonredox-type inhibitors than phosphorylatable wt 5-LO, when cells are stimulated under conditions, where 5-LO kinases are active [paper III]. In conclusion, our data indicate, that activation of 5-LO via phosphorylation strongly decreases the potency of nonredox-type 5-LO inhibitors as compared to conditions where 5-LO is stimulated by Ca^{2+}. However, incorporation of phosphate alone is not sufficient to protect 5-LO against these inhibitors, but requires cellular integrity. Based on these data, the application of new test systems should be taken into account for the development of novel 5-LO inhibitors which consider the activation pathway of 5-LO.

In the course of this study we examined the molecular profile of the novel nonredox-type 5-LO inhibitor CJ-13,610 in various *in vitro* test systems. In intact human PMNL, challenged with Ca^{2+} ionophore, CJ-13,610 potently suppressed 5-LO product formation with an IC_{50} = 0.07 µM. Supplementation of exogenous AA impaired the efficacy of CJ-13,610, implying a competitive mode of action [paper IV]. Previous data revealed that LOOH compete with the classical nonredox-type 5-LO inhibitors ZM230487 and L-739.010 at a putative regulatory site, and for this reason, the compounds were much less effeective at elevated peroxide levels or in broken cell systems. In analogy to ZM230487 and L-739.010, also CJ-13,610 failed to inhibit 5-LO in cell-free systems under nonreducing conditions up to 30 µM. However, inclusion of GPx activity restored the efficacy of CJ-13,610 (IC_{50}=0.3 µM) [paper IV]. In contrast to former members of this type of inhibitors, the potency of CJ-13,610 does not depend on the cell stimulus or the activation pathway of 5-LO. Thus, 5-LO product formation in PMNL induced by phosphorylation events was equally suppressed by CJ-13,610 as compared to Ca^{2+}-mediated 5-LO activation. In transfected HeLa cells, CJ-13,610 only slightly discriminated between phosphorylatable wt 5-LO and a mutant 5-LO that lacks phosphorylation sites [paper IV]. In summary, CJ-13,610 may posses considerable potential as a potent orally active nonredox-type 5-LO inhibitor that lacks certain disadvantages of former members of this class of 5-LO inhibitors. However, reduced efficacy should be taken into account in the treatment of diseases where elevated peroxide levels occur in the cell.

Extracts of *Boswellia serrata* gum resins have been traditionally used as a folk medicine to cure inflammatory and arthritic diseases. Furthermore, it was found that BAs, the active principles of these extracts, potently suppress the formation of proinflammatory LTs in intact cells by inhibiting 5-LO. However, significantly higher concentrations of BAs were required for equal inhibition of 5-LO in broken cells systems. Therefore, additional factors seem to be responsible for the potent inhibition of LT formation in intact cells.

11-keto-BAs activate p38 MAPK, ERK1/2 and stimulate Ca^{2+} mobilisation in human PMNL. Moreover, 11-keto-BAs induce the formation of ROS, release of AA, and subsequently LT biosynthesis [paper V, VI]. Inhibitor studies revealed that ROS formation is Ca^{2+}-dependent and is mediated by NADPH oxidase involving PI 3-K and MAPK activation [paper V, VI]. Also the release of AA depends on Ca^{2+} and ERK1/2 [paper VI]. Studies with different haematopoietic cells revealed that the activation of signal cascades by BAs depends on the cell type. The activation pattern of leukocytes by 11-keto-BAs is quite similar to chemoattractans such as fMLP or PAF, however, a specific receptor for BAs, mediating these signals, has not been identified so far. Apparently keto-BAs at relatively high concentrations cause functional

leukocyte responses, which are pro-inflammatory. The 5-LO activity-reducing effects in intact cells of BAs could be explained by pronounced elevated Ca^{2+} and ROS levels which may cause a suicide inactivation of 5-LO.

Finally, we investigated the effects of inhibitors of actin polymerization on 5-LO product formation upon stimulation with Ca^{2+} mobilizing agents [paper VII]. We found that substances that block actin polymerization such as latrunculin B (LB) and cytochalasin D (CD) enhance the release of AA as well as nuclear translocation of 5-LO and LT formation in isolated human PMNL challenged with TG. These effects were not observed when Ca^{2+} ionophore was used as stimulus. Investigation of the stimulatory mechanisms revealed that LB elicits Ca^{2+} mobilisation and potentiates agonist-induced elevation of intracellular Ca^{2+}, regardless of the nature of the agonist. LB caused a rapid but only moderate activation of p38 MAPK and ERKs. Selective Src family kinase inhibitors such as PP2 and SU6656 blocked LB- or CD-mediated Ca^{2+} mobilisation and suppressed the upregulatory effects on AA release and 5-LO product synthesis [paper VII]. Therefore, inhibitors of actin polymerization cause activation of Src family kinases that mediate enhancement of intracellular Ca^{2+} levels, thereby facilitating the release of AA and LT formation in response to certain agonists.

In conclusion, for the suitable development of novel agents directed against a certain target, knowledge about the cellular biology, in particular the regulation of the activity, is necessary. As exemplified in this study, phosphorylation of 5-LO is an aspect which influences the regulation of 5-LO activity and has a fundamental impact on enzyme inhibition. Further studies about the mode of action of inhibitors as well as the role of the cytoskeleton in regulation of 5-LO activity are essential for the development of new potential 5-LO inhibitor drugs.

VIII. Zusammenfassung

Leukotriene (LT) sind bioaktive Lipidmediatoren, die in einer Vielzahl von Entzündungskrankheiten wie z.B. Asthma, Psoriasis, Arthritis oder allergische Rhinitis involviert sind. Des weiteren spielen LT in der Pathogenese von Erkrankungen wie Krebs, Osteoarthritis oder Atherosklerose eine Rolle. Die 5-Lipoxygenase (5-LO) ist das Enzym, das für die Bildung von LTA_4 aus Arachidonsäure (AA) verantwortlich ist. Aufgrund der physiologischen Eigenschaften der LT, ist die Entwicklung von potentiellen Arzneistoffen, welche die 5-LO als Zielstruktur besitzen, von erheblichem Interesse. Bis heute jedoch ist kein 5-LO Hemmer auf dem europäischen Arzneimittelmarkt erhältlich. Der Wirkstoff Zileuton (Zyflo®, Abbott) wurde im Juni 2003 in den USA vom Markt genommen. Für eine erfolgreiche Behandlung der bereits erwähnten Krankheiten muss ein designierter Wirkstoff nicht nur in *in vitro* Untersuchungen sondern auch in *ex vivo* und *in vivo* Modellen eine ausgeprägte Wirkung zeigen. Aus diesem Grund erfordert die erfolgreiche Entwicklung eines potentiellen 5-LO Inhibitors ein genaues Wissen über die Aktivierungsvorgänge des Enzyms in der Zelle.

In Zellen befindet sich die 5-LO im Zytosol oder im löslichen Kompartement des Zellkerns. Nach Stimulierung wandert das Enzym zur Kernmembran und kolokalisiert mit zytosolischen Phospholipase A_2 ($cPLA_2$), dem 5-LO-aktivierenden-Protein (FLAP) und der LTC_4-Synthase. An der Kernmembran wird die AA mittels der $cPLA_2$ freigesetzt, durch das Helferprotein FLAP 5-LO präsentiert und anschließend zu LT metabolisiert.

Der unphysiologische Stimulus Ca^{2+}-Ionophor verursacht einen schnellen und deutlichen Anstieg des intrazellulären Ca^{2+}. Dies führt zur Aktivierung der 5-LO und folglich zur Bildung von LT. Natürliche Stimuli, wie N-Formyl-methionyl-leucyl-phenylalanin (fMLP), Plättchen aktivierender Faktor (PAF), C5a oder Zymosan verursachen eher einen moderaten Ca^{2+} Einstrom und somit eine geringere 5-LO-Aktivierung. Jedoch ergibt die Vorbehandlung von Zellen mit Zytokinen, Phorbolestern, Lipopolysacchariden oder Wachstumsfaktoren und anschließender Stimulation mit natürlichen Liganden einen prominenten Anstieg der 5-LO Aktivität. Dies ist vorwiegend auf die erhöhte Freisetzung von AA zurückzuführen. Ca^{2+}, ATP, Phosphatidylcholin und Lipidhydroperoxide sind Faktoren, welche die *in vitro* Aktivität der 5-LO beeinflussen. Neueste Daten zeigen, dass die p38-abhängige MK-2/3 5-LO phosphorylieren kann und dies eine entscheidende Rolle bei der Ca^{2+}-unabhängigen zellulären 5-LO-Aktivierung spielt. Osmotischer Stress, Hitzeschock oder chemischer Stress verursachen 5-LO-Produktbildung, die mit der Aktivierung von p38 MAPK einhergeht. Inhibitorstudien weisen darauf hin, dass der MEK1/2-Signalweg ebenfalls *in vivo* an der 5-LO Aktivierung beteiligt ist.

Tatsächlich konnten wir mittels „In-gel kinase“ und „*In vitro* kinase“ Untersuchungen zeigen, dass die 5-LO ein Substrat für die Extracellular signal-regulated kinase (ERK) und MK-2/3 darstellt. Der Zusatz von mehrfach ungesättigten Fettsäuren (UFA), wie AA oder Ölsäure, verstärkte den Phosphorylierungsgrad der 5-LO sowohl durch ERK1/2 [Paper I, II] als auch durch MK-2/3 [Paper II]. Diese verstärkende Wirkung von UFAs auf die Phosphorylierung der 5-LO ist spezifisch für ERK1/2 und MK-2/3, und wurde für andere Kinasen nicht beobachtet. Eine *in vivo*-Phosphorylierung der 5-LO, nach 5-LO Immunopräzipitation aus stimulierten PMNL, MM6 oder HL-60 Zellen, konnte jedoch nicht gezeigt werden. Die experimentellen Bedingungen oder eine zu schnelle Dephosphorylierung könnten hierfür die Ursachen sein [Paper I]. Ser271 (MK-2/3) und Ser663 (ERK1/2) wurden als Phosphorylierungsstellen der 5-LO identifiziert [Paper I, II]. Die genannten Kinasen sind demnach auch für die 5-LO Aktivierung durch natürliche Stimuli verantwortlich, die den zellulären Ca^{2+}-Spiegel kaum beeinflussen. Durch Austausch beider Serinreste (271/663) der 5-LO durch Alanin konnte gezeigt werden, dass die katalytische Aktivität des Enzyms nach Ca^{2+}-Stimulation nicht beeinflusst wird. Bei der Aktivierung durch Phosphorylierung zeigte die Doppelmutante jedoch eindeutig weniger Aktivität als das Wildtyp-Enzym. Wir können daraus schließen, dass Phosphorylierung der 5-LO durch ERK1/2 und/oder MK-2/3 einen alternativen Aktivierungsmechanismus neben Ca^{2+} darstellt.

Ursprünglich wurden Nonredox-5-LO-Inhibitoren als kompetetive Wirkstoffe entwickelt, die mit AA um die Bindung an die katalytische Domäne der 5-LO konkurrieren. Vertreter dieser Inhibitoren, wie ZM230487 und L-739,010, zeigen eine potente Hemmung der LT-Biosynthese in verschiedenen Testsystemen. Sie scheiterten jedoch in klinischen Studien. In dieser Arbeit konnten wir zeigen, dass die Wirksamkeit dieser Inhibitoren vom Aktivierungsweg der 5-LO abhängig ist [Paper III]. Verglichen mit 5-LO Aktivität, die durch den unphysiologischen Stimulus Ca^{2+}-Ionophor induziert wird, erfordert die Hemmung Zellstress-induzierter Aktivität eine 10- bis 100-fach höhere Konzentrationen der Nonredox-5-LO-Inhibitoren. Für den Eisenliganden BWA4C konnte dies nicht gezeigt werden [Paper III]. Studien mit transfizierten HeLA-Zellen unterstreichen diese Ergebnisse. Die nicht-phosphorylierbare 5-LO Mutante (Ser271Ala/Ser663Ala) war wesentlich sensitiver als der Wildtyp, wenn das Enzym durch 5-LO Kinasen aktiviert wurde [Paper III]. Somit zeigen diese Ergebnisse, dass, im Gegensatz zu Ca^{2+}, die 5-LO Aktivierung mittels Phosphorylierung die Wirksamkeit der Nonredox-Inhibitoren deutlich verringert. Der Einbau von Phosphatresten in das Enzym ist jedoch nicht allein verantwortlich für diesen Wirkungsverlust. Somit sollten bei der Entwicklung neuer 5-LO

Inhibitoren u.a. Testbedingungen gewählt werden, in denen die 5-LO Aktivität durch Phosphorylierung induziert wird.

Desweiteren wurde das pharmakologische Profil des neuen 5-LO Inhibitors CJ-13,610 mittels verschiedener *in vitro*-Testsysteme charakterisiert. In intakten PMNL, die durch Ca^{2+}-Ionophor stimuliert wurden, hemmte die Substanz die 5-LO Produktbildung mit einem IC_{50}-Wert von 70 nM. Wurde jedoch AA exogen hinzugefügt, verminderte sich die Wirkung und die erforderliche Konzentration des Inhibitors stieg an. Dies deutet auf eine kompetitive Wirkweise hin [Paper IV]. Die klassischen Nonredox-Inhibitoren ZM230487 und L-739,010 konkurrieren mit Fettsäurehydroperoxiden an einer möglichen regulatorischen Domäne des 5-LO Enzyms. Aus diesem Grund versagen diese Substanzen unter Bedingungen, in denen erhöhte Peroxidspiegel in der Zelle vorliegen. In Analogie zu ZM230487 und L-739,010 bleibt ebenfalls eine Hemmung der 5-LO Aktivität durch CJ-13,610 bei erhöhten Peroxidspiegeln ebenfalls aus [Paper IV]. Die Senkung des Peroxidspiegels durch Erneuerung der Glutathionperoxidaseaktivität stellt die Wirkung von CJ-13,610 in zellfreien Systemen (IC_{50}=0,3µM) wieder her. Im Gegensatz zu den bekannten Nonredox-Inhibitoren hängt die Wirkung von CJ-13,610 nicht vom Aktivierungsweg der 5-LO ab. Die LT-Biosynthese wird gleich stark unterdrückt, unabhängig davon, ob das Enzym durch Ca^{2+} oder durch Phosphorylierung aktiviert wird. Auch in transfizierten HeLa-Zellen zeigt sich kein Unterschied in der Hemmwirkung zwischen wt 5-LO oder der mutierten 5-LO, bei der die Phosphorylierungsstellen fehlen [Paper IV]. Aus diesem Grund besitzt CJ-13,610 ein beachtliches Potential als wirksamer 5-LO Inhibitor, dem einige der bekannten Nachteile der älteren Substanzen fehlen. Eine verminderte Wirkung ist jedoch unter Bedingungen zu erwarten, bei denen ein erhöhter Peroxidspiegel vorliegt.

Extrakte aus dem Harz des Weihrauchs *(Boswellia serrata)* werden in der traditionellen Volksmedizin bei der Behandlung von Entzündungskrankheiten eingesetzt. In polymorphkernigen Leukozyten (PMNL) hemmen Boswelliasäuren (BA), die aktiven Inhaltsstoffe des Weihrauchharzes, die Biosynthese der LT. In Zellhomogenaten jedoch sind für die gleiche Wirkung wesentlich höhere Konzentrationen erforderlich, was auf eine zusätzliche Wirkweise der BAs schließen lässt.

11-keto-BAs induzieren die Aktivierung von p38 MAPK, ERK1/2 und stimulieren die Ca^{2+}-Mobilisierung in PMNL. Zusätzlich verursachen BAs die Bildung von reaktiven Sauerstoffspezies (ROS), die Freisetzung von AA und die damit verbundene Bildung von LT [Paper V, VI]. Untersuchungen mit spezifischen Inhibitoren zeigten, dass diese induzierte ROS-Bildung Ca^{2+}-abhängig ist. Zudem wird die ROS-Bildung durch die NADPH-Oxidase

vermittelt, wobei eine Aktivierung von PI 3-K und MAPK mit einbezogen ist [Paper V, VI]. Die BA-induzierte AA-Freisetzung wird ebenfalls über den Ca^{2+}-Spiegel und die Aktivierung von ERK1/2 reguliert [Paper VI]. Untersuchungen mit verschiedenen hämatopoetischen Zellen haben ergeben, dass die Aktivierung von Signalkaskaden sehr stark vom Zelltyp abhängig ist. Interessanterweise zeigt die Aktivierung von Leukozyten durch 11-keto-BAs ein ähnliches Bild wie bei der Aktivierung durch fMLP oder PAF. Allerdings konnte bis jetzt kein spezifischer Rezeptor, der diese Signale vermittelt, identifiziert werden. Dagegen kann die reduzierende Wirkung von BAs bezüglich der 5-LO Aktivität durch erhöhte Ca^{2+}-Spiegel bzw. ROS-Spiegel erklärt werden, die dann zu einer Inaktivierung der 5-LO führen.

Schließlich wurde im Rahmen dieser Arbeit die Wirkung von Inhibitoren der Aktinpolymerisation auf die 5-LO Produktbildung untersucht. Inhibitoren wie Cytochalasin D (CD) oder Latrunculin B (LB), verstärken sowohl die AA-Freisetzung, die Translokation der 5-LO als auch die LT Bildung in isolierten PMNL, die mit Thapsigargin (TG) stimuliert wurden. Diese Wirkung konnte nicht beobachtet werden, wenn die Zellen mit Ca^{2+}-Ionophor behandelt wurden [Paper VII]. Nähere Untersuchungen ergaben, dass LB selbst einen Anstieg des $[Ca^{2+}]_i$ verursacht und einen, durch Agonisten verursachten, Ca^{2+}-Einstrom verstärkt. LB führt zu einer raschen, aber eher moderaten Aktivierung von p38 MAPK und ERKs. Selektive Inhibitoren von Kinasen der Src-Familie, wie PP2 und SU6656, blockieren den LB- oder CD-vermittelten Ca^{2+}-Anstieg und unterdrücken die hochregulierende Wirkung auf die AA-Freisetzung und LT-Bildung [Paper VII]. Somit führen Inhibitoren der Aktinpolymerisation zu einer Aktivierung von Src-Kinasen. Diese wiederum verstärken den Stimulus-induzierten Anstieg des intrazellulären Ca^{2+} und dadurch die Freisetzung von AA und die Bildung von LT.

Grundsätzlich ist es also von fundamentaler Bedeutung bei der Entwicklung von neuen Arzneistoffen die zellulären Zusammenhänge, insbesondere die Regulierung der Aktivität von Enzymen, zu kennen. Wie in dieser Arbeit gezeigt, hat die Phosphorylierung der 5-LO einen starken Einfluss auf die Regulation der 5-LO Aktivität und eine elementare Wirkung auf die Hemmung des Enzyms durch verschiedene Wirkstoffe.

IX. References

1. Abramovitz, M., Wong, E., Cox, M. E., Richardson, C. D., Li, C., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *Eur. J. Biochem.* 215, 105-111

2. Ahamed, J., and Ali, H. (2002) Distinct roles of receptor phosphorylation, G protein usage and MAP kinase activation on platelet-activating-factor-induced leukotriene C4 generation and chemokine production. *J. Biol. Chem.* 277, 22685-22691

3. Aharony, D., Redkar, B. D., Hubbs, S. J., and Stein, R. L. (1987) Kinetic studies on the inactivation of 5-lipoxygenase by 5(S)-hydroperoxyeicosatetraenoic acid. *Prostaglandins* 33, 85-100

4. Aharony, D., and Stein, R. L. (1986) Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase. *J. Biol. Chem.* 261, 11512-11519

5. Aiello, R. J., Bourassa, P. A., Lindsey, S., Weng, W., Freeman, A., and Showell, H. J. (2002) Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice.[see comment]. *Arterioscler. Thromb. Vasc. Biol.* 22, 443-449

6. Alanko, J., Sievi, E., Ahteenmaki, T., I., M., Vapaatalo, H., and Paratainen, J. (1998) Catechol estrogens as inhibitors of leukotriene synthesis. *Biochem. Pharmacol.* 55, 101-104

7. Albert, D., Bürkert, E., Steinhilber, D., and Werz, O. (2003) Induction of 5-lipoxygenase activation in polymorphonuclear leukocytes by 1-oleoyl-2-acetylglycerol. *Biochim. Biophys. Acta* 1631, 85-93.

8. Albert, D., Zündorf, I., Dingermann, T., Müller, W. E., Steinhilber, D., and Werz, O. (2002) Hyperforin is a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase. *Biochem. Pharmacol.* 64, 1767-1775

9. Allen, S., Dashwood, M., Morrison, K., and Yacoub, M. (1998) Differential leukotriene constrictor responses in human atherosclerotic coronary arteries. *Circulation* 97, 2406-2413

10. Allen, S. P., Chester, A. H., Piper, P. J., Sampson, A. P., Akl, E. S., and Yacoub, M. H. (1992) Effects of leukotrienes C4 and D4 on human isolated saphenous veins. *Br. J. Clin. Pharmacol.* 34, 409-414

11. Alonso, F., Henson, P. M., and Leslie, C. C. (1986) A cytosolic phospholipase in human neutrophils that hydrolyzes arachidonoyl-containing phosphatidylcholine. *Biochim. Biophys. Acta* 878, 273-280

12. Anderson, K. M., Seed, T., Jajeh, A., Dudeja, P., Byun, T., Meng, J., Ou, D., Bonomi, P., and Harris, J. E. (1996) An in vivo inhibitor of 5-lipoxygenase, MK886, at micromolar concentration induces apoptosis in U937 and CML cells. *Anticancer Res.* 16, 2589-2599

13. Arm, J. P. (2004) Leukotriene generation and clinical implications. *Allergy Asthma Proc.* 25, 37-42

14. Arnold, C., Albert, D., Fischer, L., Rådmark, O., Steinhilber, D., and Werz, O. (2004) 1-Oleoyl-2-acetlyglycerol mimics stimulatory effects of calcium on 5-lipoxygenase activity via a putative (phospho)lipid-binding site within the N-terminal C2-like domain. *Manuscript*

15. Avis, I., Hong, S. H., Martinez, A., Moody, T., Choi, Y. H., Trepel, J., Das, R., Jett, M., and Mulshine, J. L. (2001) Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. *FASEB J.* 15, 2007-2009.

16. Back, M. (2002) Functional characteristics of cysteinyl-leukotriene receptor subtypes. *Life Sci.* 71, 611-622

17. Back, M., Qiu, H., Haeggstrom, J. Z., and Sakata, K. (2004) Leukotriene B4 is an indirectly acting vasoconstrictor in guinea pig aorta via an inducible type of BLT receptor. *Am. J. Physiol.* 287, 419-424. Epub 2004 Mar 2011.

18. Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) Inhibition of calcium-independent phospholipase A2 prevents arachidonic acid incorporation and phospholipid remodeling in P388D1 macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 92, 8527-8531

19. Bates, M. E., Green, V. L., and Bertics, P. J. (2000) ERK1 and ERK2 activation by chemotactic factors in human eosinophils is interleukin 5-dependent and contributes to leukotriene C(4) biosynthesis. *J. Biol. Chem.* 275, 10968-10975.

20. Bell, R. L., Lanni, C., Malo, P. E., Brooks, D. W., Stewart, A. O., Hansen, R., Rubin, P., and Carter, G. W. (1993) Preclinical and clinical activity of zileuton and A-78773. *Ann. N. Y. Acad. Sci.* 696, 205-215

21. Bindu, P. H., Sastry, G. M., and Sastry, G. N. (2004) Characterization of calcium and magnesium binding domains of human 5-lipoxygenase. *Biochem. Biophys. Res. Commun.* 320, 461-467.

22. Bingham, C. O., 3rd, and Austen, K. F. (1999) Phospholipase A2 enzymes in eicosanoid generation. *Proc. Assoc. Am. Physicians* 111, 516-524

23. Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S. A. (2000) SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol. Cell. Biol.* 20, 9018-9027

24. Bleasdale, J. E., Bundy, G. L., Bunting, S., Fitzpatrick, F. A., Huff, R. M., Sun, F. F., and Pike, J. E. (1989) Inhibition of phospholipase C dependent processes by U-73,122. *Adv. Prostaglandin. Thromboxane. Leukot. Res.* 19, 590-593

25. Boden, S. E., Bertsche, T., Ammon, H. P., and Safayhi, H. (2000) MEK-1/2 inhibition prevents 5-lipoxygenase translocation in N- formylpeptide-challenged human neutrophils. *Int. J. Biochem. Cell Biol.* 32, 1069-1074.

26. Boden, S. E., Schweizer, S., Bertsche, T., Dufer, M., Drews, G., and Safayhi, H. (2001) Stimulation of leukotriene synthesis in intact polymorphonuclear cells by the 5-lipoxygenase inhibitor 3-oxo-tirucallic acid. *Mol. Pharmacol.* 60, 267-273.

27. Boeglin, W. E., Kim, R. B., and Brash, A. R. (1998) A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. *Proc. Natl. Acad. Sci. U. S. A.* 95, 6744-6749

28. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390, 622-625.

29. Borgeat, P., Hamberg, M., and Samuelsson, B. (1976) Transformation of arachidonic acid and homo-g-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. *J. Biol. Chem.* 251, 7816-7820

30. Borgeat, P., and Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. U. S. A.* 76, 2148-2152

31. Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. (2001) Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 98, 7319-7324

32. Brash, A. R. (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *J. Biol. Chem.* 274, 23679-23682.

33. Brash, A. R., Boeglin, W. E., and Chang, M. S. (1997) Discovery of a second 15S-lipoxygenase in humans. *Proc. Natl. Acad. Sci. U. S. A.* 94, 6148-6152

34. Brink, C., Dahlen, S. E., Drazen, J., Evans, J. F., Hay, D. W., Nicosia, S., Serhan, C. N., Shimizu, T., and Yokomizo, T. (2003) International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. *Pharmacol. Rev.* 55, 195-227

35. Brock, T. G., Anderson, J. A., Fries, F. P., Peters-Golden, M., and Sporn, P. H. (1999) Decreased leukotriene C4 synthesis accompanies adherence-dependent nuclear import of 5-lipoxygenase in human blood eosinophils. *J. Immunol.* 162, 1669-1676.

36. Brock, T. G., and Healy, A. M. (2000) Nuclear import of arachidonate 5-lipoxygenase. *Arch. Immunol. Ther. Exp. (Warsz).* 48, 481-486.

37. Brock, T. G., McNish, R. W., Bailie, M. B., and Peters-Golden, M. (1997) Rapid import of cytosolic 5-lipoxygenase into the nucleus of neutrophils after in vivo recruitment and in vitro adherence. *J. Biol. Chem.* 272, 8276-8280

38. Brooks, C. D., Stewart, A. O., Basha, A., Bhatia, P., Ratajczyk, J. D., Martin, J. G., Craig, R. A., Kolasa, T., Bouska, J. B., and Lanni, C. (1995) (R)-(+)-N-[3-[5-[(4-fluorophenyl)methyl]-2-thienyl]-1-methyl- 2-propynyl]-N-hydroxyurea (ABT-761), a second-generation 5-lipoxygenase inhibitor. *J. Med. Chem.* 38, 4768-4775

39. Brungs, M., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1994) On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGFß. *Biochem. Biophys. Res. Commun.* 205, 1572-1580

40. Brungs, M., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by

transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U. S. A.* 92, 107-111

41. Buhl, A. M., Avdi, N., Worthen, G. S., and Johnson, G. L. (1994) Mapping of the C5a receptor signal transduction network in human neutrophils. *Proc. Natl. Acad. Sci. U. S. A.* 91, 9190-9194

42. Bürkert, E., Arnold, C., Hammarberg, T., Rådmark, O., Steinhilber, D., and Werz, O. (2003) The C2-like beta-barrel domain mediates the Ca2+-dependent resistance of 5-lipoxygenase activity against inhibition by glutathione peroxidase-1. *J. Biol. Chem.* 278, 42846-42853

43. Bürkert, E., Rådmark, O., Samuelsson, B., Steinhilber, D., and Werz, O. (2001) Hypertonicity suppresses ionophore-induced product formation and translocation of 5-lipoxygenase in human leukocytes. *J. Leukoc. Biol.* 71, 477486

44. Bürkert, E., Rådmark, O., Steinhilber, D., and Werz, O. (2002) Monocyte-derived soluble protein confers 5-lipoxygenase activity Ca2+-dependent. *Biochem. Biophys. Res. Commun.* 295, 985-991

45. Bürkert, E., Szellas, D., Rådmark, O., Steinhilber, D., and Werz, O. (2003) Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. *J. Leukoc. Biol.* 73, 191-200.

46. Byrum, R. S., Goulet, J. L., Griffiths, R. J., and Koller, B. H. (1997) Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses. *J. Exp. Med.* 185, 1065-1075

47. Byrum, R. S., Goulet, J. L., Snouwaert, J. N., Griffiths, R. J., and Koller, B. H. (1999) Determination of the contribution of cysteinyl leukotrienes and leukotriene B4 in acute inflammatory responses using 5-lipoxygenase- and leukotriene A4 hydrolase-deficient mice. *J. Immunol.* 163, 6810-6819

48. Capodici, C., Pillinger, M. H., Han, G., Philips, M. R., and Weissmann, G. (1998) Integrin-dependent homotypic adhesion of neutrophils. Arachidonic acid activates Raf-1/Mek/Erk via a 5-lipoxygenase- dependent pathway. *J. Clin. Invest.* 102, 165-175

49. Carter, G. W., Young, P. R., Albert, D. H., Bouska, J., Dyer, R., Bell, R. L., Summers, J. B., and Brooks, D. W. (1991) 5-lipoxygenase inhibitory activity of zileuton. *J. Pharmacol. Exp. Ther.* 256, 929-937

50. Chang, L. C., and Wang, J. (2001) Signal transduction pathways for activation of extracellular signal-regulated kinase by arachidonic acid in rat neutrophils. *J. Leukoc. Biol.* 69, 659-665

51. Chanock, S. J., el Benna, J., Smith, R. M., and Babior, B. M. (1994) The respiratory burst oxidase. *J. Biol. Chem.* 269, 24519-24522

52. Charleson, S., Evans, J. F., Leger, S., Perrier, H., Prasit, P., Wang, Z., and Vickers, P. J. (1994) Structural requirements for the binding of fatty acids to 5-lipoxygenase-activating protein. *Eur. J. Pharmacol.* 267, 275-280

53. Chen, X. S., and Funk, C. D. (2001) The N-terminal "ß-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. *J. Biol. Chem.* 276, 811-818

54. Chen, X. S., Sheller, J. R., Johnson, E. N., and Funk, C. D. (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature* 372, 179-182.

55. Chen, X. S., Zhang, Y. Y., and Funk, C. D. (1998) Determinants of 5-lipoxygenase nuclear localization using green fluorescent protein/5-lipoxygenase fusion proteins. *J. Biol. Chem.* 273, 31237-31244

56. Claesson, H. E., and Dahlen, S. E. (1999) Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.* 245, 205-227.

57. Clancy, R. M., Dahinden, C. A., and Hugli, T. E. (1983) Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by N-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. *Proc. Natl. Acad. Sci. U. S. A.* 80, 7200-7204

58. Claria, J., and Serhan, C. N. (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. U. S. A.* 92, 9475-9479

59. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a calcium dependent translocation domain with homology to PKC and GAP. *Cell* 65, 1043-1051

60. Coffey, M. J., Phare, S. M., and Peters-Golden, M. (2002) Interaction between nitric oxide, reactive oxygen intermediates, and peroxynitrite in the regulation of 5-lipoxygenase metabolism. *Biochim. Biophys. Acta* 1584, 81-90

61. Coffey, M. J., Phare, S. M., and Peters-Golden, M. (2000) Prolonged exposure to lipopolysaccharide inhibits macrophage 5- lipoxygenase metabolism via induction of nitric oxide synthesis. *J. Immunol.* 165, 3592-3598.

62. Comporti, M. (1985) Lipid peroxidation and cellular damage in toxic liver injury. *Lab. Invest.* 53, 599-623

63. Corey, E. J., and Landsbury, J. P. T. (1983) Stereochemical course of 5-Lipoxygenation by rat basophil leukemic cell /RBL-1) and potato enzymes. *J. Am. Chem. Soc.* 105, 4093-4094

64. Covin, R. B., Brock, T. G., Bailie, M. B., and Peters-Golden, M. (1998) Altered expression and localization of 5-lipoxygenase accompany macrophage differentiation in the lung. *Am. J. Physiol.* 275, L303-310.

65. Crawley, G. C., Dowell, R. I., Edwards, P. N., Foster, S. J., McMillan, R. M., Walker, E. R., Waterson, D., Bird, T. G., Bruneau, P., and Giroaeau, J. M. (1992) Methoxytetrahydropyrans. A new series of selective and orally potent 5-lipoxygenase inhibitors. *J. Med. Chem.* 35, 2600-2609

66. Cronstein, B. N. (1994) Adenosine, an endogenous anti-inflammatory agent. *J. Appl. Physiol.* 76, 5-13.

67. Cybulsky, A. V., Takano, T., Papillon, J., Khadir, A., Bijian, K., and Le Berre, L. (2004) The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2. *Am. J. Physiol.* 286, 466-476

68. Dahinden, C. A., Zingg, J., Maly, F. E., and de Weck, A. L. (1988) Leukotriene production in human neutrophils primed by recombinant human granulocyte/macrophage colony-stimulating factor and stimulated with the complement component C5A and FMLP as second signals. *J. Exp. Med.* 167, 1281-1295

69. Dahlen, B., Kumlin, M., Ihre, E., Zetterstrom, O., and Dahlen, S. E. (1997) Inhibition of allergen-induced airway obstruction and leukotriene generation in atopic asthmatic subjects by the leukotriene biosynthesis inhibitor BAYx 1005. *Thorax* 52, 342-347

70. Dahlen, B., Kumlin, M., Margolskee, D. J., Larsson, C., Blomqvist, H., Williams, V. C., Zetterstrom, O., and Dahlen, S. E. (1993) The leukotriene-receptor antagonist MK-0679 blocks airway obstruction induced by inhaled lysine-aspirin in aspirin-sensitive asthmatics. *Eur. Respir. J.* 6, 1018-1026

71. Dahlen, S. E., Bjork, J., Hedqvist, P., Arfors, K. E., Hammarstrom, S., Lindgren, J. A., and Samuelsson, B. (1981) Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. U. S. A.* 78, 3887-3891

72. Dahlen, S. E., Hedqvist, P., Hammarstrom, S., and Samuelsson, B. (1980) Leukotrienes are potent constrictors of human bronchi. *Nature* 288, 484-486

73. Datta, K., Biswal, S. S., and Kehrer, J. P. (1999) The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, induces apoptosis independently of FLAP. *Biochem. J.* 340, 371-375

74. Datta, K., Biswal, S. S., Xu, J., Towndrow, K. M., Feng, X., and Kehrer, J. P. (1998) A relationship between 5-lipoxygenase-activating protein and bcl-xL expression in murine pro-B lymphocytic FL5.12 cells. *J. Biol. Chem.* 273, 28163-28169

75. de Carvalho, M. G., McCormack, A. L., Olson, E., Ghomashchi, F., Gelb, M. H., Yates, J. R., 3rd, and Leslie, C. C. (1996) Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A2 expressed in insect cells and present in human monocytes. *J. Biol. Chem.* 271, 6987-6997

76. De Caterina, R., Mazzone, A., Giannessi, D., Sicari, R., Pelosi, W., Lazzerini, G., Azzara, A., Forder, R., Carey, F., and Caruso, D. (1988) Leukotriene B4 production in human atherosclerotic plaques. *Biomed. Biochim. Acta* 47, 182-185

77. Dessen, A. (2000) Structure and mechanism of human cytosolic phospholipase A2. *Biochim. Biophys. Acta* 1488, 40-47

78. Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., and Wahli, W. (1996) The PPARalpha-leukotriene B4 pathway to inflammation control.[see comment]. *Nature* 384, 39-43

79. Dewas, C., Fay, M., Gougerot-Pocidalo, M. A., and El-Benna, J. (2000) The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in

formyl-methionyl-leucyl-phenylalanine-induced p47phox phosphorylation in human neutrophils. *J. Immunol.* 165, 5238-5244

80. Diez, E., Louis-Flamberg, P., Hall, R. H., and Mayer, R. J. (1992) Substrate specificities and properties of human phospholipases A2 in a mixed vesicle model. *J. Biol. Chem.* 267, 18342-18348

81. Ding, J., Vlahos, C. J., Liu, R., Brown, R. F., and Badwey, J. A. (1995) Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J. Biol. Chem.* 270, 11684-11691

82. Ding, X. Z., Iversen, P., Cluck, M. W., Knezetic, J. A., and Adrian, T. E. (1999) Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.* 261, 218-223.

83. Dixon, R. A. F., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., Evans, J. F., Gillard, J. W., and Miller, D. K. (1990) Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 343, 282-284

84. Doerfler, M. E., Danner, R. L., Shelhamer, J. H., and Parrillo, J. E. (1989) Bacterial lipopolysaccharides prime human neutrophils for enhanced production of leukotriene B4. *J. Clin. Invest.* 83, 970-977

85. Drazen, J. M., Austen, K. F., Lewis, R. A., Clark, D. A., Goto, G., Marfat, A., and Corey, E. J. (1980) Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 77, 4354-4358

86. Drazen, J. M., and Israel, E. (1995) Treatment of chronic stable asthma with drugs active on the 5-lipoxygenase pathway. *Int. Arch. Allergy Immunol.* 107, 319-320

87. Drazen, J. M., O'Brien, J., Sparrow, D., Weiss, S. T., Martins, M. A., Israel, E., and Fanta, C. H. (1992) Recovery of leukotriene E4 from the urine of patients with airway obstruction. *Am. Rev. Respir. Dis.* 146, 104-108

88. Drazen, J. M., Yandava, C. N., Dube, L., Szczerback, N., Hippensteel, R., Pillari, A., Israel, E., Schork, N., Silverman, E. S., Katz, D. A., and Drajesk, J. (1999) Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat. Genet.* 22, 168-170

89. Dwyer, J. H., Allayee, H., Dwyer, K. M., Fan, J., Wu, H., Mar, R., Lusis, A. J., and Mehrabian, M. (2004) Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.* 350, 29-37

90. Evans, J. F. (2003) The cysteinyl leukotriene receptors. *Prostaglandins Leukot. Essent. Fatty Acids* 69, 117-122

91. Evans, J. F., Nathaniel, D. J., Zamboni, R. J., and Ford-Hutchinson, A. W. (1985) Leukotriene A3. A poor substrate but a potent inhibitor of rat and human neutrophil leukotriene A4 hydrolase. *J. Biol. Chem.* 260, 10966-10970

92. Falgueyret, J. P., Denis, D., MacDonald, D., Hutchinson, J. H., and Riendeau, D. (1995) Characterization of the arachidonate and ATP binding sites of human 5-lipoxygenase

using photoaffinity labeling and enzyme immobilization. *Biochemistry (Mosc).* 34, 13603-13611

93. Fan, L., Di Ciano-Oliveira, C., Weed, S. A., Craig, A. W., Greer, P. A., Rotstein, O. D., and Kapus, A. (2004) Actin depolymerization-induced tyrosine phosphorylation of cortactin: the role of Fer kinase.[see comment]. *Biochem. J.* 380, 581-591

94. Fan, X. M., Tu, S. P., Lam, S. K., Wang, W. P., Wu, J., Wong, W. M., Yuen, M. F., Lin, M. C., Kung, H. F., and Wong, B. C. (2004) Five-lipoxygenase-activating protein inhibitor MK-886 induces apoptosis in gastric cancer through upregulation of p27kip1 and bax. *J. Gastroenterol. Hepatol.* 19, 31-37

95. Ferby, I. M., Waga, I., Sakanaka, C., Kume, K., and Shimizu, T. (1994) Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J. Biol. Chem.* 269, 30485-30488

96. Ferry, D. R., Deakin, M., Baddeley, J., Daryanani, S., Bramhall, S., Anderson, D. A., Wakelam, M. J., Doran, J., Pemberton, G., Young, A. M., Buckels, J., and Kerr, D. J. (2000) A phase II study of the 5-lipoxygenase inhibitor, CV6504, in advanced pancreatic cancer: correlation of clinical data with pharmacokinetic and pharmacodynamic endpoints. *Ann. Oncol.* 11, 1165-1170

97. Fiore, S., Maddox, J. F., Perez, H. D., and Serhan, C. N. (1994) Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J. Exp. Med.* 180, 253-260

98. Fiore, S., Romano, M., Reardon, E. M., and Serhan, C. N. (1993) Induction of functional lipoxin A4 receptors in HL-60 cells. *Blood* 81, 3395-3403

99. Fiorucci, S., Meli, R., Bucci, M., and Cirino, G. (2001) Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? *Biochem. Pharmacol.* 62, 1433-1438

100. Flamand, L., Borgeat, P., Lalonde, R., and Gosselin, J. (2004) Release of anti-HIV mediators after administration of leukotriene B4 to humans. *J. Infect. Dis.* 189, 2001-2009. Epub 2004 Apr 2030.

101. Flamand, N., Surette, M. E., Picard, S., Bourgoin, S., and Borgeat, P. (2002) Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Mol. Pharmacol.* 62, 250-256.

102. Flohe, L., Gunzler, W., Jung, G., Schaich, E., and Schneider, F. (1971) [Glutathione peroxidase. II. Substrate specificity and inhibitory effects of substrate analogues]. *Hoppe. Seylers Z. Physiol. Chem.* 352, 159-169.

103. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1994) 5-Lipoxygenase. *Annu. Rev. Biochem.* 63, 383-417.

104. Fruchtmann, R., Mohrs, K. H., Hatzelmann, A., Raddatz, S., Fugmann, B., Junge, B., Horstmann, H., and Müller-Peddinghaus, R. (1993) In vitro pharmacology of BAY X1005, a new inhibitor of leukotriene synthesis. *Agents Actions* 38, 188-195

105. Fruteau de Laclos, B., and Borgeat, P. (1988) Conditions for the formation of the oxo derivatives of arachidonic acid from platelet 12-lipoxygenase and soybean 15-lipoxygenase. *Biochim. Biophys. Acta* 958, 424-433

106. Funk, C. D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871-1875.

107. Funk, C. D. (1999) Targeted disruption of 5-lipoxygenase. *Novel inhibitors of leukotrienes, G. Folco, B.samualsson, R.C. Murphy (ED) Birkhäuser Verlag Basel/switzerland*, 101-111

108. Funk, C. D., and Chen, X. S. (2000) 5-Lipoxygenase and leukotrienes. Transgenic mouse and nuclear targeting studies. *Am. J. Respir. Crit. Care Med.* 161, 120-124.

109. Funk, C. D., Chen, X. S., Sheller, J. R., Tu, Y. P., and Irvin, C. G. (1998) The role of 5-lipoxygenase products in a mouse model of allergic airway inflammation. *Lung biology in health and disease: Eicosanoids, aspirin and asthma* 114, 187-200

110. Funk, C. D., Hoshiko, S., Matsumoto, T., Rådmark, O., and Samuelsson, B. (1989) Characterization of the human 5-lipoxygenase gene. *Proc. Natl. Acad. Sci. U. S. A.* 86, 2587-2591

111. Garcia, C., Boyce, B. F., Gilles, J., Dallas, M., Qiao, M., Mundy, G. R., and Bonewald, L. F. (1996) Leukotriene B4 stimulates osteoclastic bone resorption both in vitro and in vivo. *J. Bone Miner. Res.* 11, 1619-1627

112. Ghosh, J., and Myers, C. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 95

113. Gijon, M. A., and Leslie, C. C. (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J. Leukoc. Biol.* 65, 330-336

114. Gillard, J., Ford-Hutchinson, A. W., Chan, C., Charleson, S., Denis, D., Foster, A., Fortin, R., Leger, S., McFarlane, C. S., and Morton, H. (1989) L-663,536 (MK-886) (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2 - dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. *Can. J. Physiol. Pharmacol.* 67, 456-464

115. Glaser, T., Winter, S., Groscurth, P., Safayhi, H., Sailer, E. R., Ammon, H. P., Schabet, M., and Weller, M. (1999) Boswellic acids and malignant glioma: induction of apoptosis but no modulation of drug sensitivity. *Br. J. Cancer* 80, 756-765

116. Goetzl, E. J., and Sun, F. F. (1979) Generation of unique mono-hydroxy-eicosatetraenoic acids from arachidonic acid by human neutrophils. *J. Exp. Med.* 150, 406-411

117. Goulet, J. l., Snouwaert, J. N., Latour, A. M., Coffman, T. M., and Koller, B. H. (1994) Altered inflammatory responses in leukotriene-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 91, 12852-12856

118. Griffiths, R. J., Smith, M. A., Roach, M. L., Stock, J. L., Stam, E. J., Milici, A. J., Scampoli, D. N., Eskra, J. D., Byrum, R. S., Koller, B. H., and McNeish, J. D. (1997) Collagen-induced arthritis is reduced in 5-lipoxygenase-activating protein-deficient mice. *J. Exp. Med.* 185, 1123-1129

119. Grimminger, F., Sibelius, U., Aktories, K., Suttorp, N., and Seeger, W. (1991) Inhibition of cytoskeletal rearrangement by botulinum C2 toxin amplifies ligand-evoked lipid mediator generation in human neutrophils. *Mol. Pharmacol.* 40, 563-571

120. Grinstein, S., and Furuya, W. (1992) Chemoattractant-induced tyrosine phosphorylation and activation of microtubule-associated protein kinase in human neutrophils. *J. Biol. Chem.* 267, 18122-18125

121. Gupta, S., Srivastava, M., Ahmad, N., Sakamoto, K., Bostwick, D. G., and Mukhtar, H. (2001) Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer* 91, 737-743.

122. Gut, J. D., Goldman, D. W., Jamieson, G. C., and Trudell, J. R. (1987) Convertion of leukotriene A4 to leukotriene B4: catalysis by human liver microsomes under anaerobic conditions. *Arch. Biochem. Biophys.* 259, 497-509

123. Haeggstrom, J. Z. (2000) Structure, Function and Regulation of Leukotriene A4 Hydrolase. *Am. J. Respir. Crit. Care Med.* 161, S25-S31

124. Haeggstrom, J. Z., Wetterholm, A., Shapiro, R., Vallee, B. L., and Samuelsson, B. (1990) Leukotriene A4 hydrolase: a zinc metalloenzyme. *Biochem. Biophys. Res. Commun.* 172, 965-970

125. Haeggstrom, J. Z., Wetterholm, A., Vallee, B. L., and Samuelsson, B. (1990) Leukotriene A4 hydrolase: an epoxide hydrolase with peptidase activity. *Biochem. Biophys. Res. Commun.* 173, 431-437

126. Hafstrom, I., Palmblad, J., Malmsten, C. L., Rådmark, O., and Samuelsson, B. (1981) Leukotriene B4--a stereospecific stimulator for release of lysosomal enzymes from neutrophils. *FEBS Lett.* 130, 146-148

127. Hajjar, D. P., and Pomerantz, K. B. (1992) Signal transduction in atherosclerosis: integration of cytokines and the eicosanoid network. *FASEB J.* 6, 2933-2941

128. Ham, E. A., Soderman, D. D., Zanetti, M. E., Dougherty, H. W., McCauley, E., and Kuehl, F. A., Jr. (1983) Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *Proc. Natl. Acad. Sci. U. S. A.* 80, 4349-4353

129. Hamberg, M., and Samuelsson, B. (1974) Prostaglandine endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U. S. A.* 71, 3400-3404

130. Hammarberg, T., Kuprin, S., Rådmark, O., and Holmgren, A. (2001) EPR investigation of the active site of recombinant human 5- lipoxygenase: inhibition by selenide. *Biochemistry (Mosc).* 40, 6371-6378.

131. Hammarberg, T., Provost, P., Persson, B., and Rådmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J. Biol. Chem.* 275, 38787-38793

132. Hammarberg, T., and Rådmark, O. (1999) 5-lipoxygenase binds calcium. *Biochemistry (Mosc).* 38, 4441-4447

133. Hammarberg, T., Zhang, Y. Y., Lind, B., Rådmark, O., and Samuelsson, B. (1995) Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. *Eur. J. Biochem.* 230, 401-407.

134. Hanaka, H., Shimizu, T., and Izumi, T. (2002) Nuclear-localization-signal-dependent and nuclear-export-signal-dependent mechanisms determine the localization of 5-lipoxygenase. *Biochem. J.* 361, 505-514.

135. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem.* 271, 695-701

136. Haribabu, B., Verghese, M. W., Steeber, D. A., Sellars, D. D., Bock, C. B., and Snyderman, R. (2000) Targeted disruption of the leukotriene B(4) receptor in mice reveals its role in inflammation and platelet-activating factor-induced anaphylaxis.[see comment]. *J. Exp. Med.* 192, 433-438

137. Hazan-Halevy, I., Seger, R., and Levy, R. (2000) The Requirement of Both Extracellular Signal-Regulated Kinase and p38 Mitogen-activated Protein Kinase for Stimulation of Cytosolic Phospholiase A2 Activity by either FCgammaRIIA or FCgammaRIIIB in Human Neutrophils. *J. Cell Biol.* 275, 12416-12423

138. Healy, A. M., Peters-Golden, M., Yao, J. P., and Brock, T. G. (1999) Identification of a bipartite nuclear localization sequence necessary for nuclear import of 5-lipoxygenase. *J. Biol. Chem.* 274, 29812-29818

139. Hedqvist, P., Dahlen, S. E., Gustafsson, L., Hammarstrom, S., and Samuelsson, B. (1980) Biological profile of leukotrienes C4 and D4. *Acta Physiol. Scand.* 110, 331-333

140. Heise, C. E., O'Dowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D. S., Stocco, R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Jr., Zeng, Z., Liu, Q., Ma, L., Clements, M. K., Coulombe, N., Liu, Y., Austin, C. P., George, S. R., O'Neill, G. P., Metters, K. M., Lynch, K. R., and Evans, J. F. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. *J. Biol. Chem.* 275, 30531-30536

141. Helgadottir, A., Manolescu, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorsteinsdottir, U., Samani, N. J., Gudmundsson, G., Grant, S. F., Thorgeirsson, G., Sveinbjornsdottir, S., Valdimarsson, E. M., Matthiasson, S. E., Johannsson, H., Gudmundsdottir, O., Gurney, M. E., Sainz, J., Thorhallsdottir, M., Andresdottir, M., Frigge, M. L., Topol, E. J., Kong, A., Gudnason, V., Hakonarson, H., Gulcher, J. R., and Stefansson, K. (2004) The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.* 36, 233-239

142. Hemak, J., Gale, D., and Brock, T. G. (2002) Stuctural characterization of the catalytic domain of the human 5-lipoxygenase enzyme. *J. Mol. Model.* 8, 102-112

143. Henderson, W. R., Jr. (1994) The role of leukotrienes in inflammation. *Ann. Intern. Med.* 121, 684-697

144. Herlaar, E., and Brown, Z. (1999) p38 MAPK signalling cascades in inflammatory disease. *Mol. Med. Today* 5, 439-447

145. Hii, C. S., Ferrante, A., Edwards, Y. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1995) Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C-dependent mechanism. *J. Biol. Chem.* 270, 4201-4204

146. Hoernlein, R. F., Orlikowsky, T., Zehrer, C., Niethammer, D., Sailer, E. R., Simmet, T., Dannecker, G. E., and Ammon, H. P. (1999) Acetyl-11-keto-beta-boswellic acid induces apoptosis in HL-60 and CCRF-CEM cells and inhibits topoisomerase I. *J. Pharmacol. Exp. Ther.* 288, 613-619

147. Hoshiko, S., Rådmarck, O., and Samuelsson, B. (1990) Characterization of the human 5-lipoxygenase gene promoter. *Proc. Natl. Acad. Sci. U. S. A.* 87, 9073-9077

148. Hosoi, T., Koguchi, Y., Sugikawa, E., Chikada, A., Ogawa, K., Tsuda, N., Suto, N., Tsunoda, S., Taniguchi, T., and Ohnuki, T. (2002) Identification of a novel human eicosanoid receptor coupled to G(i/o). *J. Biol. Chem.* 277, 31459-31465

149. Huang, H. S., Chen, C. J., Lu, H. S., and Chang, W. C. (1998) Identification of a lipoxygenase inhibitor in A431 cells as a phospholipid hydroperoxide glutathione peroxidase. *FEBS Lett.* 424, 22-26

150. Huang, W. W., Garcia-Zepeda, E. A., Sauty, A., Oettgen, H. C., Rothenberg, M. E., and Luster, A. D. (1998) Molecular and biological characterization of the murine leukotriene B4 receptor expressed on eosinophils. *J. Exp. Med.* 188, 1063-1074

151. Hurley, J. H., and Misra, S. (2000) Signaling and subcellular targeting by membrane-binding domains. *Annu. Rev. Biophys. Biomol. Struct.*, 49-79.

152. Imai, H., Narashima, K., Arai, M., Sakamoto, H., Chiba, N., and Nakagawa, Y. (1998) Suppression of leukotriene formation in RBL-2H3 cells that overexpressed phospholipid hydroperoxide glutathione peroxidase. *J. Biol. Chem.* 273, 1990-1997

153. In, K. H., Asano, K., Beier, D., Grobholz, J., Finn, P. W., Silverman, E. K., Silverman, E. S., Collins, T., Fischer, A. R., Keith, T. P., Serino, K., Kim, S. W., De Sanctis, G. T., Yandava, C., Pillari, A., Rubin, P., Kemp, J., Israel, E., Busse, W., Ledford, D., Murray, J. J., Segal, A., Tinkleman, D., and Drazen, J. M. (1997) Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. *J. Clin. Invest.* 99, 1130-1137

154. Irvin, C. G., Tu, Y. P., Sheller, J. R., and Funk, C. D. (1997) 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. *Am. J. Physiol.* 272, 1053-1058.

155. Israel, E., Dermarkarian, R., Rosenberg, M., Sperling, R., Taylor, G., Rubin, P., and Drazen, J. M. (1990) The effects of a 5-lipoxygenase inhibitor on asthma induced by cold, dry air.[see comment]. *N. Engl. J. Med.* 323, 1740-1744

156. Israel, E., Rubin, P., Kemp, J. P., Grossman, J., Pierson, W., Siegel, S. C., Tinkelman, D., Murray, J. J., Busse, W., and Segal, A. T. (1993) The effect of inhibition of 5-lipoxygenase by zileuton in mild-to-moderate asthma. *Ann. Intern. Med.* 119, 1059-1066

157. Izumi, T., Hoshiko, S., Rådmark, O., and Samuelsson, B. (1990) Cloning of the cDNA for human 12-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A.* 87, 7477-7481

158. Jakobsson, P. J., Mancini, J. A., and Ford-Hutchinson, A. W. (1996) Identification and characterization of a novel human microsomal glutathione S-transferase with leukotriene C4 synthase activity and significant sequence identity to 5-lipoxygenase-activating protein and leukotriene C4 synthase. *J. Biol. Chem.* 271, 22203-22210

159. Jakobsson, P. J., Mancini, J. A., Riendeau, D., and Ford-Hutchinson, A. W. (1997) Identification and characterization of a novel microsomal enzyme with glutathione-dependent transferase and peroxidase activities. *J. Biol. Chem.* 272, 22934-22939

160. Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., and Persson, B. (1999) Common structural features of MAPEG -- a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci.* 8, 689-692

161. Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., and Persson, B. (2000) Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily. *Am. J. Respir. Crit. Care Med.* 161, S20-24.

162. Janssen-Timmen, U., Vickers, P. J., Wittig, U., Lehmann, W. D., Stark, H. J., Fusenig, N. E., Rosenbach, T., Rådmark, O., Samuelsson, B., and Habenicht, A. J. R. (1995) Expression of 5-lipoxygenase in differentiating human skin keratinocytes. *Proc. Natl. Acad. Sci. U. S. A.* 92, 6966-6970

163. Johnson, G. J., and Lapadat, R. (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK and p38 Protein Kinases. *Science* 298, 1911-1912

164. Jones, C. E., Holden, S., Tenaillon, L., Bhatia, U., Seuwen, K., Tranter, P., Turner, J., Kettle, R., Bouhelal, R., Charlton, S., Nirmala, N. R., Jarai, G., and Finan, P. (2003) Expression and characterization of a 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid receptor highly expressed on human eosinophils and neutrophils. *Mol. Pharmacol.* 63, 471-477

165. Jones, S. M., Luo, M., Healy, A. M., Peters-Golden, M., and Brock, T. G. (2002) Structural and functional criteria reveal a new nuclear import sequence on the 5-lipoxygenase protein. *J. Biol. Chem.* 277, 38550-38556.

166. Jones, S. M., Luo, M., Peters-Golden, M., and Brock, T. G. (2003) Identification of two novel nuclear import sequences on the 5-lipoxygenase protein. *J. Biol. Chem.* 278, 10257-10263

167. Kargman, S., Vickers, P. J., and Evans, J. F. (1992) A23187-induced translocation of 5-lipoxygenase in osteosarcoma cells. *J. Cell Biol.* 119, 1701-1709

168. Kennedy, B. P., Diehl, R. E., Boie, Y., Adam, M., and Dixon, R. A. (1991) Gene characterization and promoter analysis of the human 5-lipoxygenase-activating protein (FLAP). *J. Biol. Chem.* 266, 8511-8516

169. Klan, N., Seuter, S., Schnur, N., Jung, M., and Steinhilber, D. (2003) Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. *Biol. Chem.* 384, 777-785

170. Korade-Mirnics, Z., and Corey, S. J. (2000) Src kinase-mediated signaling in leukocytes. *J. Leukoc. Biol.* 68, 603-613

171. Kramer, R. M., Checani, G. C., Deykin, A., Pritzker, C. R., and Deykin, D. (1986) Solubilization and properties of Ca2+-dependent human platelet phospholipase A2. *Biochim. Biophys. Acta* 878, 394-403

172. Krump, E., Lemay, G., and Borgeat, P. (1996) Adenosine A2 receptor-induced inhibition of leukotriene B4 synthesis in whole blood ex vivo. *Br. J. Pharmacol.* 117, 1639-1644

173. Krump, E., Picard, S., Mancini, J., and Borgeat, P. (1997) Suppression of leukotriene B4 biosynthesis by endogenous adenosine in ligand-activated human neutrophils. *J. Exp. Med.* 186, 1401-1406.

174. Krump, E., Sanghera, J. S., Pelech, S. L., Furuya, W., and Grinstein, S. (1997) Chemotactic peptide N-formyl-met-leu-phe activation of p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase-2 in human neutrophils. *J. Biol. Chem.* 272, 937-944.

175. Kühn, H. (2000) Structural basis for the positional specificity of lipoxygenases. *Prostaglandins Other Lipid Mediat.* 62, 255-270

176. Kühn, H., and Thiele, B. J. (1999) The diversity of the lipoxygenase family. Many sequence data but little information on biological significance. *FEBS Lett.* 449, 7-11

177. Kühn, H., Walther, M., and Kuban, R. J. (2002) Mammalian arachidonate 15-lipoxygenases structure, function, and biological implications. *Prostaglandins Other Lipid Mediat.* 68-69, 263-290

178. Kulkarni, S., Das, S., Funk, C. D., Murray, D., and Cho, W. (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. *J. Biol. Chem.* 277, 13167-13174.

179. Kusner, E. J., Buckner, C. K., Dea, D. M., DeHaas, C. J., Marks, R. L., and Krell, R. D. (1994) The 5-lipoxygenase inhibitors ZD2138 and ZM230487 are potent and selective inhibitors of several antigen-induced guinea-pig pulmonary responses. *Eur. J. Pharmacol.* 257, 285-292

180. Kyriakis, J. M., and Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioassays* 18, 567-577

181. Labat, C., Ortiz, J. L., Norel, X., Gorenne, I., Verley, J., Abram, T. S., Cuthbert, N. J., Tudhope, S. R., Norman, P., and Gardiner, P. (1992) A second cysteinyl leukotriene receptor in human lung. *J. Pharmacol. Exp. Ther.* 263, 800-805

182. Lad, P. M., Olson, C. V., Grewal, I. S., and Scott, S. J. (1985) A pertussis toxin-sensitive GTP-binding protein in the human neutrophil regulates multiple receptors, calcium mobilization, and lectin-induced capping. *Proc. Natl. Acad. Sci. U. S. A.* 82, 8643-8647

183. Lam, B. K., and Austen, K. F. (2002) Leukotriene C4 synthase: a pivotal enzyme in cellular biosynthesis of the cysteinyl leukotrienes. *Prostaglandins Other Lipid Mediat.* 68-69, 511-520

184. Lam, B. K., Penrose, J. F., Freeman, G. J., and Austen, K. F. (1994) Expression cloning of a cDNA for human leukotriene C4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A4. *Proc. Natl. Acad. Sci. U. S. A.* 91, 7663-7667

185. Lam, S., Chan, H., LeRiche, J. C., Chan-Yeung, M., and Salari, H. (1988) Release of leukotrienes in patients with bronchial asthma. *J. Allergy Clin. Immunol.* 81, 711-717

186. Laufer, S. (2003) Role of eicosanoids in structural degradation in osteoarthritis. *Curr. Opin. Rheumatol.* 15, 623-627

187. Laufer, S., Tries, S., Augustin, J., and Dannhardt, G. (1994) Pharmacological profile of a new pyrrolizine derivative inhibiting the enzymes cyclo-oxygenase and 5-lipoxygenase. *Arzneimittelforschung.* 44, 629-636

188. Lee, J. C., Kumar, S., Griswold, D. E., Underwood, D. C., Votta, B. J., and Adams, J. L. (2000) Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology* 47, 185-201.

189. Lee, T. H., Horton, C. E., Kyan-Aung, U., Haskard, D., Crea, A. E., and Spur, B. W. (1989) Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Clin. Sci.* 77, 195-203

190. Leff, J. A., Busse, W. W., Pearlman, D., Bronsky, E. A., Kemp, J., Hendeles, L., Dockhorn, R., Kundu, S., Zhang, J., Seidenberg, B. C., and Reiss, T. F. (1998) Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction.[see comment]. *N. Engl. J. Med.* 339, 147-152

191. Lepley, R. A., and Fitzpatrick, F. A. (1998) 5-Lipoxygenase compartmentalization in granulocytic cells is modulated by an internal bipartite nuclear localizing sequence and nuclear factor kappa B complex formation. *Arch. Biochem. Biophys.* 356, 71-76

192. Lepley, R. A., and Fitzpatrick, F. A. (1994) 5-lipoxygenase contains a functional Src homology 3- binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins. *J. Biol. Chem.* 269, 24163-24168

193. Lepley, R. A., and Fitzpatrick, F. A. (1996) Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells. *Arch. Biochem. Biophys.* 331, 141-144

194. Lepley, R. A., Muskardin, D. T., and Fitzpatrick, F. A. (1996) Tyrosine kinase activity modulates catalysis and translocation of cellular 5-lipoxygenase. *J. Biol. Chem.* 271, 6179-6184

195. Leslie, C. C. (1991) Kinetic properties of a high molecular mass arachidonoyl-hydrolyzing phospholipase A2 that exhibits lysophospholipase activity. *J. Biol. Chem.* 266, 11366-11371

196. Lewis, T. A., Bayless, L., Eckman, J. B., Ellis, J. L., Grewal, G., Libertine, L., Marie Nicolas, J., Scannell, R. T., Wels, B. F., Wenberg, K., and Wypij, D. M. (2004) 5-lipoxygenase inhibitors with histamine H(1) receptor antagonist activity. *Bioorg. Med. Chem. Lett.* 14, 2265-2268.

197. Liebmann, C. (2001) Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. *Cell. Signal.* 13, 777-785

198. Lin, L. L., Lin, A. Y., and DeWitt, D. L. (1992) Interleukin-1 alpha induces the accumulation of cytosolic phospholipase A2 and the release of prostaglandin E2 in human fibroblasts. *J. Biol. Chem.* 267, 23451-23454

199. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* 72, 269-278

200. Liu, J. J., Nilsson, A., Oredsson, S., Badmaev, V., Zhao, W. Z., and Duan, R. D. (2002) Boswellic acids trigger apoptosis via a pathway dependent on caspase-8 activation but independent on Fas/Fas ligand interaction in colon cancer HT-29 cells. *Carcinogenesis* 23, 2087-2093

201. Lowell, C. A., Fumagalli, L., and Berton, G. (1996) Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. *J. Cell Biol.* 133, 895-910

202. Luo, M., Jones, S. M., Peters-Golden, M., and Brock, T. G. (2003) Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B4 synthetic capacity. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12165-12170

203. Luo, M., Jones, S. M., Phare, S. M., Coffey, M. J., Peters-Golden, M., and Brock, T. G. (2004) Protein kinase A inhibits leukotriene synthesis by phosphorylation of 5-lipoxygenase on serine 523. *J. Biol. Chem.* 279, 41512-41520

204. Lynch, K. R., O'Neill, G. P., Liu, Q., Im, D. S., Sawyer, N., Metters, K. M., Coulombe, N., Abramovitz, M., Figueroa, D. J., Zeng, Z., Connolly, B. M., Bai, C., Austin, C. P., Chateauneuf, A., Stocco, R., Greig, G. M., Kargman, S., Hooks, S. B., Hosfield, E., Williams, D. L., Jr., Ford-Hutchinson, A. W., Caskey, C. T., and Evans, J. F. (1999) Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 399, 789-793

205. Maccarrone, M., Corasaniti, M. T., Guerrieri, P., Nistico, G., and Agro, A. F. (1996) Nitric oxide-donor compounds inhibit lipoxygenase activity. *Biochem. Biophys. Res. Commun.* 219, 128-133

206. Maddox, J. F., Hachicha, M., Takano, T., Petasis, N. A., Fokin, V. V., and Serhan, C. N. (1997) Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. *J. Biol. Chem.* 272, 6972-6978

207. Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J. C., and Hellmiss, R. (1989) Molecular cloning and amino acid sequence of rat kidney aminopeptidase M: a member of a super family of zinc-metallohydrolases. *Biochem. Biophys. Res. Commun.* 161, 236-241

208. Mancini, J. A., Abramovitz, M., Cox, M. E., Wong, E., Charleson, S., Perrier, H., Wang, Z., Prasit, P., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein is an arachidonate binding protein. *FEBS Lett.* 318, 277-281

209. Mancini, J. A., and Evans, J. F. (1995) Cloning and characterization of the human leukotriene A4 hydrolase gene. *Eur. J. Biochem.* 231, 65-71

210. Mancini, J. A., Prasit, P., Coppolino, M. G., Charleson, P., Leger, S., Evans, J. F., Gillard, J. W., and Vickers, P. J. (1992) 5-Lipoxygenase-activating protein is the target of a novel hybrid of two classes of leukotriene biosynthesis inhibitors. *Mol. Pharmacol.* 41, 267-272

211. Mancini, J. A., Waterman, H., and Riendeau, D. (1998) Cellular oxygenation of 12-hydroxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid by 5-lipoxygenase is stimulated by 5-lipoxygenase-activating protein. *J. Biol. Chem.* 273, 32842-32847

212. Mandal, A. K., Skoch, J., Bacskai, B. J., Hyman, B. T., Christmas, P., Miller, D., Yamin, T. T., Xu, S., Wisniewski, D., Evans, J. F., and Soberman, R. J. (2004) The membrane organization of leukotriene synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 101, 6587-6592. Epub 2004 Apr 6514.

213. Mano, T., Okumura, Y., Sakakibara, M., Okumura, T., Tamura, T., Miyamoto, K., and Stevens, R. W. (2004) 4-[5-Fluoro-3-[4-(2-methyl-1H-imidazol-1-yl)benzyloxy]phenyl]-3,4,5,6- tetrahydro-2H-pyran-4-carboxamide, an orally active inhibitor of 5-lipoxygenase with improved pharmacokinetic and toxicology characteristics. *J. Med. Chem.* 47, 720-725

214. Mano, T., Stevens, R. W., Ando, K., Nakao, K., Okumura, Y., Sakakibara, M., Okumura, T., Tamura, T., and Miyamoto, K. (2003) Novel imidazole compounds as a new series of potent, orally active inhibitors of 5-lipoxygenase. *Bioorg. Med. Chem.* 11, 3879-3887.

215. Marom, Z., Shelhamer, J. H., Bach, M. K., Morton, D. R., and Kaliner, M. (1982) Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am. Rev. Respir. Dis.* 126, 449-451

216. Matsumoto, T., Funk, C. D., Rådmark, O., Hoog, J. O., Jornvall, H., and Samuelsson, B. (1988) Molecular cloning and amino acid sequence of human 5-lipoxygenase.[erratum appears in Proc Natl Acad Sci U S A 1988 May;85(10):3406]. *Proc. Natl. Acad. Sci. U. S. A.* 85, 26-30

217. McMahon, B., and Godson, C. (2004) Lipoxins: endogenous regulators of inflammation. *Am. J. Physiol.* 286, 189-201

218. McMahon, B., Stenson, C., McPhillips, F., Fanning, A., Brady, H. R., and Godson, C. (2000) Lipoxin A4 antagonizes the mitogenic effects of leukotriene D4 in human renal mesangial cells. Differential activation of MAP kinases through distinct receptors. *J. Biol. Chem.* 275, 27566-27575

219. McMillan, R. M., Spruce, K. E., Crawley, G. C., Walker, E. R., and Foster, S. J. (1992) Pre-clinical pharmacology of ICI D2138, a potent orally-active non-redox inhibitor of 5-lipoxygenase. *Br. J. Pharmacol.* 107, 1042-1047

220. McMillan, R. M., and Walker, E. R. (1992) Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.* 13, 323-330

221. Medina, J. F., Wetterholm, A., Rådmark, O., Shapiro, R., Haeggstrom, J. Z., Vallee, B. L., and Samuelsson, B. (1991) Leukotriene A4 hydrolase: determination of the three zinc-binding ligands by site-directed mutagenesis and zinc analysis. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7620-7624

222. Mehrabian, M., and Allayee, H. (2003) 5-lipoxygenase and atherosclerosis. *Curr. Opin. Lipidol.* 14, 447-457.

223. Mehrabian, M., Allayee, H., Wong, J., Shih, W., Wand, X., Shaposhnik, Z., Funk, C. D., and Jusis, A. (2002) Identification of 5-Lipoxygenase as a Major Gene Contributing to Atherosclerosis Susceptibility in Mice. *Circ. Res.* 91, 120-126

224. Michelassi, F., Landa, L., Hill, R. D., Lowenstein, E., Watkins, W. D., Petkau, A. J., and Zapol, W. M. (1982) Leukotriene D4: a potent coronary artery vasoconstrictor associated with impaired ventricular contraction. *Science* 217, 841-843

225. Miller, D. K., Gillard, J. W., Vickers, P. J., Sadowski, S., Léveillé, C., Mancini, J. A., Charleson, P., Dixon, R. A. F., Ford-Hutchinson, A. W., Fortin, R., Gauthier, J. Y., Rodkey, J., Rosen, R., Rouzer, C. A., Sigal, I. S., Strader, C. D., and Evans, J. F. (1990) Identification and isolation of a membrane protein necessary for leukotriene production. *Nature* 343, 278-281

226. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.* 9, 168-173

227. Morgenstern, R., Guthenberg, C., and Depierre, J. W. (1982) Microsomal glutathione S-transferase. Purification, initial characterization and demonstration that it is not identical to the cytosolic glutathione S-transferases A, B and C. *Eur. J. Biochem.* 128, 243-248

228. Mrsny, R. J., Gewirtz, A. T., Siccardi, D., Savidge, T., Hurley, B. P., Madara, J. L., and McCormick, B. A. (2004) Identification of hepoxilin A3 in inflammatory events: a required role in neutrophil migration across intestinal epithelia. *Proc. Natl. Acad. Sci. U. S. A.* 101, 7421-7426. Epub 2004 May 7423.

229. Mueller, M. J., Blomster, M., Oppermann, U. C., Jornvall, H., Samuelsson, B., and Haeggstrom, J. Z. (1996) Leukotriene A4 hydrolase: protection from mechanism-based inactivation by mutation of tyrosine-378. *Proc. Natl. Acad. Sci. U. S. A.* 93, 5931-5935

230. Muralidhar, B., Carpenter, K. L., Muller, K., Skepper, J. N., and Arends, M. J. (2004) Potency of arachidonic acid in polyunsaturated fatty acid-induced death of human monocyte-macrophages: implications for atherosclerosis. *Prostaglandins Leukot. Essent. Fatty Acids* 71, 251-262.

231. Naccache, P. H., McColl, S. R., Caon, A. C., and Borgeat, P. (1989) Arachidonic acid-induced mobilization of calcium in human neutrophils: evidence for a multicomponent mechanism of action. *Br. J. Pharmacol.* 97, 461-468

232. Naccache, P. H., Volpi, M., Showell, H. J., Becker, E. L., and Sha'afi, R. I. (1979) Chemotactic factor-induced release of membrane calcium in rabbit neutrophils. *Science* 203, 461-463

233. Nakatani, Y., Tanioka, T., Sunaga, S., Murakami, M., and Kudo, I. (2000) Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A(2)alpha. *J. Biol. Chem.* 275, 1161-1168

234. Nasser, S. M., Bell, G. S., Hawksworth, R. J., Spruce, K. E., MacMillan, R., Williams, A. J., Lee, T. H., and Arm, J. P. (1994) Effect of the 5-lipoxygenase inhibitor ZD2138 on allergen-induced early and late asthmatic responses. *Thorax* 49, 743-748

235. Ng, D. S., and Wong, K. (1989) Effect of platelet-activating factor (PAF) on cytosolic free calcium in human peripheral blood mononuclear leukocytes. *Res. Commun. Chem. Pathol. Pharmacol.* 64, 351-354

236. Nicholson, D. W., Ali, A., Vaillancourt, J. P., Calaycay, J. R., Mumford, R. A., Zamboni, R. J., and Ford-Hutchinson, A. W. (1993) Purification to homogeneity and the N-terminal sequence of human leukotriene C4 synthase: a homodimeric glutathione S-transferase composed of 18-kDa subunits. *Proc. Natl. Acad. Sci. U. S. A.* 90, 2015-2019

237. Nick, J. A., Avdi, N. J., Young, S. K., Knall, C., Gerwins, P., Johnson, G. L., and Worthen, G. S. (1997) Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J. Clin. Invest.* 99, 975-986

238. Nicosia, S., Capra, V., and Rovati, G. E. (2001) Leukotrienes as mediators of asthma. *Pulm. Pharmacol. Ther.* 14, 3-19

239. Nigam, S., Patabhiraman, S., Ciccoli, R., Ishdorj, G., Schwarz, K., Petrucev, B., Kuhn, H., and Haeggstrom, J. Z. (2004) The Rat Leukocyte-type 12-Lipoxygenase Exhibits an Intrinsic Hepoxilin A3 Synthase Activity. *J. Biol. Chem.* 279, 29023-29030. Epub 22004 May 29023.

240. Noguchi, M., Miyano, M., Kuhara, S., Matsumoto, T., and Noma, M. (1994) Interfacial kinetic reaction of human 5-lipoxygenase. *Eur. J. Biochem.* 222, 285-292.

241. Noguchi, M., Miyano, M., and Matsumoto, T. (1996) Physicochemical characterization of ATP binding to human 5-lipoxygenase. *Lipids* 31, 367-371

242. Noguchi, M., Miyano, M., Matsumoto, T., and Noma, M. (1994) Human 5-lipoxygenase associates with phosphatidylcholine liposomes and modulates LTA(4) synthetase activity. *Biochim. Biophys. Acta* 1215, 300-306

243. Nowak, D., Bialasiewicz, P., Antczak, A., Krol, M., and Piasecka, G. (1995) Effect of cytochalasin B on intracellular free calcium concentration in human polymorphonuclear leukocytes after repeated stimulation with n-formyl-methionyl-leucyl-phenylalanine. *Int. J. Clin. Lab. Res.* 25, 116-120

244. Ochi, K., Yoshimoto, T., Yamamoto, S., Taniguchi, K., and Miyamoto, T. (1983) Arachidonate 5-lipoxygenase of guinea pig peritoneal polymorphonuclear leukocytes. Activation by adenosine 5'-triphosphate. *J. Biol. Chem.* 258, 5754-5758

245. O'Flaherty, J. T., Kuroki, M., Nixon, A. B., Wijkander, J., Yee, E., Lee, S. L., Smitherman, P. K., Wykle, R. L., and Daniel, L. W. (1996) 5-Oxo-eicosanoids and hematopoietic cytokines cooperate in stimulating neutrophil function and the mitogen-activated protein kinase pathway. *J. Biol. Chem.* 271, 17821-17828

246. O'Flaherty, J. T., and Nishihira, J. (1987) 5-Hydroxyeicosatetraenoate promotes Ca2+ and protein kinase C mobilization in neutrophils. *Biochem. Biophys. Res. Commun.* 148, 575-581

247. O'Flaherty, J. T., and Rossi, A. G. (1993) 5-hydroxyicosatetraenoate stimulates neutrophils by a stereospecific, G protein-linked mechanism. *J. Biol. Chem.* 268, 14708-14714

248. Öhd, J., Wikström, K., and Sjölander, A. (2000) Leukotrienes induce cell-survival signaling in intestinal epithelial cells. *Gastroenterology* 119, 1007-1018

249. Ohuchi, K., Sugawara, T., Watanabe, M., Hirasawa, N., Tsurufuji, S., Fujiki, H., Sugimura, T., and Christensen, S. B. (1987) Stimulation of arachidonic acid metabolism in rat peritoneal macrophages by thapsigargin, a non-(12-O-tetradecanoylphorbol-13-acetate) (TPA)-type tumor promoter. *J. Cancer Res. Clin. Oncol.* 113, 319-324

250. Örning, L., Gierse, J., Duffin, K., Bild, G., Krivi, G., and Fitzpatrick, F. A. (1992) Mechanism-based inactivation of leukotriene A4 hydrolase/aminopeptidase by leukotriene A4. Mass spectrometric and kinetic characterization. *J. Biol. Chem.* 267, 22733-227339

251. Örning, L., Kaijser, L., and Hammarstrom, S. (1985) In vivo metabolism of leukotriene C4 in man: urinary excretion of leukotriene E4. *Biochem. Biophys. Res. Commun.* 130, 214-220

252. Örning, L., Krivi, G., and Fitzpatrick, F. A. (1991) Leukotriene A4 hydrolase. Inhibition by bestatin and intrinsic aminopeptidase activity establish its functional resemblance to metallohydrolase enzymes. *J. Biol. Chem.* 266, 1375-1378

253. Owman, C., Nilsson, C., and Lolait, S. J. (1996) Cloning of cDNA encoding a putative chemoattractant receptor. *Genomics* 37, 187-194

254. Palmer, R. M., and Salmon, J. A. (1983) Release of leukotriene B4 from human neutrophils and its relationship to degranulation induced by N-formyl-methionyl-leucyl-phenylalanine, serum-treated zymosan and the ionophore A23187. *Immunology* 50, 65-73

255. Papayianni, A., Serhan, C. N., and Brady, H. R. (1996) Lipoxin A4 and B4 inhibit leukotriene-stimulated interactions of human neutrophils and endothelial cells. *J. Immunol.* 156, 2264-2272

256. Park, Y. S., Lee, J. H., Bondar, J., Harwalkar, J. A., Safayhi, H., and Golubic, M. (2002) Cytotoxic action of acetyl-11-keto-beta-boswellic acid (AKBA) on meningioma cells. *Planta Med.* 68, 397-401

257. Penrose, J. F., Spector, J., Baldasaro, M., Xu, K., Boyce, J., Arm, J. P., Austen, K. F., and Lam, B. K. (1996) Molecular cloning of the gene for human leukotriene C4 synthase. Organization, nucleotide sequence, and chromosomal localization to 5q35. *J. Biol. Chem.* 271, 11356-11361

258. Penzo, D., Petronilli, V., Angelin, A., Cusan, C., Colonna, R., Scorrano, L., Pagano, F., Prato, M., Di Lisa, F., and Bernardi, P. (2004) Arachidonic acid released by phospholipase A(2) activation triggers Ca(2+)-dependent apoptosis through the mitochondrial pathway. *J. Biol. Chem.* 279, 25219-25225

259. Percival, M. D., Denis, D., Riendeau, D., and Gresser, M. J. (1992) Investigation of the mechanism of non-turnover-dependent inactivation of purified human 5-lipoxygenase. *Eur. J. Biochem.* 210, 109-117

260. Perskvist, N., Zheng, L., and Stendahl, O. (2000) Activation of human neutrophils by Mycobacterium tuberculosis H37Ra involves phospholipase C gamma 2, Shc adapter protein, and p38 mitogen-activated protein kinase. *J. Immunol.* 164, 959-965

261. Peters-Golden, M., and Brock, T. G. (2003) 5-lipoxygenase and FLAP. *Prostaglandins Leukot. Essent. Fatty Acids* 69, 99-109

262. Peters-Golden, M., and Brock, T. G. (2001) Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett.* 487, 323-326

263. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) A model for p53-induced apoptosis.[see comment]. *Nature* 389, 300-305

264. Powell, W. S., Chung, D., and Gravel, S. (1995) 5-Oxo-6,8,11,14-eicosatetraenoic acid is a potent stimulator of human eosinophil migration. *J. Immunol.* 154, 4123-4132

265. Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B., and Rådmark, O. (2001) 5-Lipoxygenase interacts with Coactosin-Like Protein. *J. Biol. Chem.* 276, 16520-16527

266. Provost, P., Doucet, J., Stock, A., Gerisch, G., Samuelsson, B., and O., R. (2001) Coactosin-like Protein, a Human F-actin-binding protein: Critical role of Lysin-75. *Biochem. J.* 359, 255-263

267. Provost, P., Samuelsson, B., and Rådmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1881-1885.

268. Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G. M. (1995) G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase.Shc adapter protein signaling complexes.[erratum appears in J Biol Chem 1995 Oct 13;270(41):24622]. *J. Biol. Chem.* 270, 19969-19973

269. Puustinen, T., Scheffer, M. M., and Samuelsson, B. (1988) Regulation of the human leukocyte 5-lipoxygenase: stimulation by micromolar calcium levels and phosphatidylcholine vesicles. *Biochim. Biophys. Acta* 960, 261-267

270. Qiu, Z. H., Gijon, M. A., deCarvalho, M. S., Spencer, D. M., and Leslie, C. C. (1998) The role of calcium and phosphorylation of cytosolic phospholipase A(2) in regulating arachidonic acid release in macrophages. *J. Biol. Chem.* 273, 8203-8211

271. Rådmark, O. (2003) 5-lipoxygenase-derived leukotrienes: mediators also of atherosclerotic inflammation.[comment]. *Arterioscler. Thromb. Vasc. Biol.* 23, 1140-1142

272. Rådmark, O. (2002) Arachidonate 5-lipoxygenase. *Prostaglandins Other Lipid Mediat.* 68-69, 211-234.

273. Rådmark, O., Shimizu, T., Jornvall, H., and Samuelsson, B. (1984) Leukotriene A4 hydrolase in human leukocytes. Purification and properties. *J. Biol. Chem.* 259, 12339-12345.

274. Reddy, K. V., Hammarberg, T., and Rådmark, O. (2000) Mg2+ activates 5-lipoxygenase in vitro: dependency on concentrations of phosphatidylcholine and arachidonic acid. *Biochemistry (Mosc).* 39, 1840-1848

275. Reynolds, C. H. (1988) 5-Lipoxygenase and leukotriene synthesis: effects of calcium ions and of inhibitors. *Prostaglandins* 36, 59-68

276. Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., and Dennis, E. A. (1993) Metal ion and salt effects on the phospholipase A2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A2. *Biochim. Biophys. Acta* 1167, 272-280

277. Riddick, C. A., Ring, W. L., Baker, J. R., Hodulik, C. R., and Bigby, T. D. (1997) Dexamethasone increases expression of 5-lipoxygenase and its activating protein in human monocytes and THP-1 cells. *Eur. J. Biochem.* 246, 112-118

278. Riendeau, D., Denis, D., Choo, L. Y., and Nathaniel, D. J. (1989) Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation. *Biochem. J.* 263, 565-572.

279. Riendeau, D., Falgueyret, J. P., Guay, J., Ueda, N., and Yamamoto, S. (1991) Pseudoperoxidase activity of 5-lipoxygenase stimulated by potent benzofuranol and N-hydroxyurea inhibitors of the lipoxygenase reaction. *Biochem. J.* 274, 287-292

280. Riendeau, D., Falgueyret, J. P., Meisner, D., Sherman, M. M., Laliberte, F., and Street, I. P. (1993) Interfacial catalysis and production of a high ratio of leukotriene A4 to 5-HPETE by 5-lipoxygenase in a coupled assay with phospholipase A2. *J. Lipid Mediat.* 6, 23-30

281. Romano, M., Catalano, A., Nutini, M., D'Urbano, E., Crescenzi, C., Claria, J., Libner, R., Davi, G., and Procopio, A. (2001) 5-lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. *FASEB J.* 15, 2326-2336.

282. Romano, M., and Claria, J. (2003) Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. *FASEB J.* 17, 1986-1995

283. Rosado, J. A., Jenner, S., and Sage, S. O. (2000) A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J. Biol. Chem.* 275, 7527-7533

284. Ross, R. (1999) Atherosclerosis--an inflammatory disease.[see comment]. *N. Engl. J. Med.* 340, 115-126

285. Roux, P. P., and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68, 320-344

286. Rouzer, C. A., and Kargman, S. (1988) Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* 263, 10980-10988

287. Rouzer, C. A., Rands, E., Kargman, S., Jones, R. E., Register, R. B., and Dixon, R. A. (1988) Characterization of cloned human leukocyte 5-lipoxygenase expressed in mammalian cells. *J. Biol. Chem.* 263, 10135-10140

288. Rouzer, C. A., and Samuelsson, B. (1986) The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS Lett.* 204, 293-296.

289. Rouzer, C. A., and Samuelsson, B. (1985) On the nature of the 5-lipoxygenase reaction in human leukocytes: Enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. U. S. A.* 82, 6040-6044

290. Rouzer, C. A., and Samuelsson, B. (1987) Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A.* 84, 7393-7397

291. Rouzer, C. A., Shimizu, T., and Samuelsson, B. (1985) On the nature of the 5-lipoxygenase reaction in human leukocytes: characterization of a membrane-associated stimulatory factor. *Proc. Natl. Acad. Sci. U. S. A.* 82, 7505-7509.

292. Sabala, P., Targos, B., Caravelli, A., Czajkowski, R., Lim, D., Gragnaniello, G., Santella, L., and Baranska, J. (2002) Role of the actin cytoskeleton in store-mediated calcium entry in glioma C6 cells. *Biochem. Biophys. Res. Commun.* 296, 484-491

293. Safayhi, H., Boden, S. E., Schweizer, S., and Ammon, H. P. (2000) Concentration-dependent potentiating and inhibitory effects of Boswellia extracts on 5-lipoxygenase product formation in stimulated PMNL. *Planta Med.* 66, 110-113

294. Safayhi, H., Mack, T., Sabieraj, J., Anazodo, M. I., Subramanian, L. R., and Ammon, H. P. (1992) Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. *J. Pharmacol. Exp. Ther.* 261, 1143-1146

295. Safayhi, H., Rall, B., Sailer, E. R., and Ammon, H. P. (1997) Inhibition by boswellic acids of human leukocyte elastase. *J. Pharmacol. Exp. Ther.* 281, 460-463

296. Safayhi, H., and Sailer, E. R. (1997) Anti-inflammatory actions of pentacyclic triterpenes. *Planta Med.* 63, 487-493

297. Safayhi, H., Sailer, E. R., and Ammon, H. P. (1995) Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-beta-boswellic acid. *Mol. Pharmacol.* 47, 1212-1216

298. Sailer, E. R., Schweizer, S., Boden, S. E., Ammon, H. P. T., and Safayhi, H. (1998) Characterization of an acetyl-11-keto-ß-boswellic acid and arachidonate-binding regulatory site of 5-lipoxygenase using photoaffinity labeling. *Eur. J. Biochem.* 256, 364-368

299. Sala, A., Bolla, M., Zarini, S., Müller-Peddinghaus, R., and Folco, G. (1996) Release of leukotriene A4 versus leukotriene B4 from human polymorphonuclear leukocytes. *J. Biol. Chem.* 271, 17944-17948

300. Salari, H., Braquet, P., Naccache, P., and Borgeat, P. (1985) Characterization of effect of N-formyl-methionyl-leucyl-phenylalanine on leukotriene synthesis in human polymorphonuclear leukocytes. *Inflammation* 9, 127-138

301. Salazar, E. P., and Rozengurt, E. (1999) Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. *J. Biol. Chem.* 274, 28371-28378

302. Samarakoon, R., and Higgins, P. J. (2003) Pp60c-src mediates ERK activation/nuclear localization and PAI-1 gene expression in response to cellular deformation. *J. Cell. Physiol.* 195, 411-420

303. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 220, 568-575

304. Samuelsson, B., Borgeat, P., Hammarstrom, S., and Murphy, R. C. (1979) Introduction of a nomenclature: leukotrienes. *Prostaglandins* 17, 785-787

305. Samuelsson, B., Dahlén, S. E., Lindgren, J. Å., Rouzer, C. A., and Serhan, C. N. (1987) Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* 237, 1171-1176

306. Sapirstein, A., and Bonventre, J. V. (2000) Specific physiological roles of cytosolic phospholipase A2 as defined by gene knockouts. *Biochim. Biophys. Acta* 1488, 139-148

307. Sarau, H. M., Ames, R. S., Chambers, J., Ellis, C., Elshourbagy, N., Foley, J. J., Schmidt, D. B., Muccitelli, R. M., Jenkins, O., Murdock, P. R., Herrity, N. C., Halsey, W., Sathe, G., Muir, A. I., Nuthulaganti, P., Dytko, G. M., Buckley, P. T., Wilson, S., Bergsma, D. J., and Hay, D. W. (1999) Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Mol. Pharmacol.* 56, 657-663

308. Sayers, I., Barton, S., Rorke, S., Sawyer, J., Peng, Q., Beghe, B., Ye, S., Keith, T., Clough, J. B., Holloway, J. W., Sampson, A. P., and Holgate, S. T. (2003) Promoter polymorphism in the 5-lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) genes and asthma susceptibility in a Caucasian population. *Clin. Exp. Allergy* 33, 1103-1110

309. Schatz-Munding, M., Hatzelmann, A., and Ullrich, V. (1991) The involvement of extracellular calcium in the formation of 5-lipoxygenase metabolites by human polymorphonuclear leukocytes. *Eur. J. Biochem.* 197, 487-493

310. Schatz-Munding, M., and Ullrich, V. (1992) Priming of human polymorphonuclear leukocytes with granulocyte-macrophage colony-stimulating factor involves protein kinase C rather than enhanced calcium mobilisation. *Eur. J. Biochem.* 204, 705-712

311. Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L. L. (1995) Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *J. Biol. Chem.* 270, 30749-30754

312. Serhan, C. N., Hamberg, M., Samuelsson, B., Morris, J., and Wishka, D. G. (1986) On the stereochemistry and biosynthesis of lipoxin B. *Proc. Natl. Acad. Sci. U. S. A.* 83, 1983-1987

313. Serhan, C. N., Nicolaou, K. C., Webber, S. E., Veale, C. A., Dahlen, S. E., Puustinen, T. J., and Samuelsson, B. (1986) Lipoxin A. Stereochemistry and biosynthesis. *J. Biol. Chem.* 261, 16340-16345

314. Shao, Y., Ho, C. T., Chin, C. K., Badmaev, V., Ma, W., and Huang, M. T. (1998) Inhibitory activity of boswellic acids from Boswellia serrata against human leukemia HL-60 cells in culture. *Planta Med.* 64, 328-331

315. Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., and Sportsman, J. R. (1991) Molecular cloning and expression of human Ca(2+)-sensitive cytosolic phospholipase A2. *J. Biol. Chem.* 266, 14850-14853

316. Shimizu, T., Izumi, T., Seyama, Y., Tadokoro, K., Rådmark, O., and Samuelsson, B. (1986) Characterization of leukotriene A4 synthase from murine mast cells: evidence for its identity to arachidonate 5-lipoxygenase. *Proc. Natl. Acad. Sci. U. S. A.* 83, 4175-4179

317. Sigal, E., Craik, C. S., Highland, E., Grunberger, D., Costello, L. L., Dixon, R. A., and Nadel, J. A. (1988) Molecular cloning and primary structure of human 15-lipoxygenase. *Biochem. Biophys. Res. Commun.* 157, 457-464

318. Silverman, E. S., and Drazen, J. M. (1999) The biology of 5-lipoxygenase: function, structure, and regulatory mechanisms. *Proc. Assoc. Am. Physicians* 111, 525-536

319. Six, D. A., and Dennis, E. A. (2000) The expanding suprfamiliy of phospholipase A2 enzymes: classification and characterization. *Biochim. Biophys. Acta* 1488, 1-19

320. Skorey, K. I., and Gresser, M. J. (1998) Calcium is not required for 5-lipoxygenase activity at high phosphatidyl choline vesicle concentrations. *Biochemistry (Mosc).* 37, 8027-8034

321. Smith, W. G., Shaffer, A. F., Currie, J. L., Thompson, J. M., Kim, S., Rao, T., and Isakson, P. C. (1995) Characterization of 5-lipoxygenase inhibitors in biochemical and functional in vivo assays. *J. Pharmacol. Exp. Ther.* 275, 1332-1338

322. Soberman, R. J., and Christmas, P. (2003) The organization and consequences of eicosanoid signaling. *J. Clin. Invest.* 111, 1107-1113

323. Spanbroek, R., Grabner, R., Lotzer, K., Hildner, M., Urbach, A., Ruhling, K., Moos, M. P., Kaiser, B., Cohnert, T. U., Wahlers, T., Zieske, A., Plenz, G., Robenek, H., Salbach, P., Kühn, H., Rådmark, O., Samuelsson, B., and Habenicht, A. J. (2003) Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 100, 1238-1243.

324. Spanbroek, R., Stark, H. J., Janssen-Timmen, U., Kraft, S., Hildner, M., Andl, T., Bosch, F. X., Fusenig, N. E., Bieber, T., Rådmark, O., Samuelsson, B., and Habenicht, A. J. (1998) 5-Lipoxygenase expression in Langerhans cells of normal human epidermis. *Proc. Natl. Acad. Sci. U. S. A.* 95, 663-668

325. Steinhilber, D. (1999) 5-Lipoxygenase: a target for antiinflammatory drugs revisited. *Curr. Med. Chem.* 6, 71-85

326. Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, I., Danielsson, C., Kahlen, J.-P., Nayeri, S., Schräder, M., and Carlberg, C. (1995) The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. *J. Biol. Chem.* 270, 7037-7040

327. Steinhilber, D., Hoshiko, S., Grunewald, J., Rådmark, O., and Samuelsson, B. (1993) Serum factors regulate 5-lipoxygenase activity in maturating HL60 cells. *Biochim. Biophys. Acta* 1178, 1-8

328. Straif, D., Werz, O., Kellner, R., Bahr, U., and Steinhilber, D. (2000) Glutathione peroxidase-1 but not 4 is involved in the regulation of cellular 5-lipoxygenase activity in monocytic cells. *Biochem. J.* 349, 455-461

329. Sun, D., McDonnel, M., Chen, X. S., Lakkis, M. M., Li, H., Isaacs, S. N., H., E. S., Patel, P. I., and Funk, C. D. (1998) Human 12(R)-lipoxygenase and the mouse ortholog. Molecular cloning, expression and gene chromosomal assignment. *J. Biol. Chem.* 273, 33540-33547

330. Surette, M. E., Krump, E., Picard, S., and Borgeat, P. (1999) Activation of leukotriene synthesis in human neutrophils by exogenous arachidonic acid: inhibition by adenosine A(2a) receptor agonists and crucial role of autocrine activation by leukotriene B(4). *Mol. Pharmacol.* 56, 1055-1062

331. Sutherland, M., Schewe, T., and Nigam, S. (2000) Biological actions of the free acid of hepoxilin A3 on human neutrophils. *Biochem. Pharmacol.* 59, 435-440

332. Tager, A. M., Dufour, J. H., Goodarzi, K., Bercury, S. D., von Andrian, U. H., and Luster, A. D. (2000) BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis.[see comment]. *J. Exp. Med.* 192, 439-446

333. Tager, A. M., and Luster, A. D. (2003) BLT1 and BLT2: the leukotriene B(4) receptors. *Prostaglandins Leukot. Essent. Fatty Acids* 69, 123-134

334. Takano, T., Fiore, S., Maddox, J. F., Brady, H. R., Petasis, N. A., and Serhan, C. N. (1997) Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. *J. Exp. Med.* 185, 1693-1704

335. Tateson, J. E., Randall, R. W., Reynolds, C. H., Jackson, W. P., Bhattacherjee, P., Salmon, J. A., and Garland, L. G. (1988) Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment in vitro and ex vivo. *Br. J. Pharmacol.* 94, 528-539

336. Theorell, H., Holman, R. T., and Akeson, A. (1947) Crystalline Lipoxydase. *Acta Chem. Scand.* 1, 571-576

337. Thunnissen, M. M., Nordlund, P., and Haeggstrom, J. Z. (2001) Crystal structure of human leukotriene A(4) hydrolase, a bifunctional enzyme in inflammation. *Nat. Struct. Biol.* 8, 131-135

338. Tijsterman, M., and Plasterk, R. H. (2004) Dicers at RISC; the mechanism of RNAi.[comment]. *Cell* 117, 1-3

339. Turner, C. R., Smith, W. B., Andresen, C. J., Eggler, J. F., and Watson, J. W. (1996) The effect of 5-lipoxygenase inhibition on Ascaris antigen (Ag)-induced responses in atopic monkeys. *Inflamm. Res.* 45, 42-49

340. Uhl, J., Klan, N., Rose, M., Entian, K. D., Werz, O., and Steinhilber, D. (2002) The 5-Lipoxygenase Promoter is Regulated by DNA Methylation. *J. Leukoc. Biol.* 277, 4374-4379

341. Underwood, D. C., Osborn, R. R., Newsholme, S. J., Torphy, T. J., and Hay, D. W. (1996) Persistent airway eosinophilia after leukotriene (LT) D4 administration in the guinea pig: modulation by the LTD4 receptor antagonist, pranlukast, or an interleukin-5 monoclonal antibody. *Am. J. Respir. Crit. Care Med.* 154, 850-857

342. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Role of cytosolic phospholipase A(2) in allergic response and parturition. *Nature* 390, 618-622

343. Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K. D., Roveri, A., Schomburg, D., and Flohe, L. (1995) Diversity of Glutathione Peroxidases. *Methods Enzymol.* 252, 38-53

344. Ursini, F., Maiorino, M., and Roveri, A. (1997) Phospholipid hydroperoxide glutathione peroxidase (PHGPx): more than an antioxidant enzyme? *Biomed. Environ. Sci.* 10, 327-332.

345. Vallee, B. L., and Auld, D. S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry (Mosc).* 29, 5647-5659.

346. Vick, B. A., and Zimmerman, D. C. (1987) In Biochemistry of plants: A comprehensive treatise. *Academic Press* 9, 53-90

347. Vilarino, N., and MacGlashan, D. W., Jr. (2004) Actin cytoskeleton-dependent down-regulation of early IgE-mediated signaling in human basophils. *J. Leukoc. Biol.* 75, 928-937

348. Vonakis, B. M., and Vanderhoek, J. Y. (1990) A calcium-independent 5-lipoxygenase system in mast/basophil PT-18 cells. *Biochim. Biophys. Acta* 1045, 142-146

349. Weinblatt, M. E., Kremer, J. M., Coblyn, J. S., Helfgott, S., Maier, A. L., Petrillo, G., Henson, B., Rubin, P., and Sperling, R. (1992) Zileuton, a 5-lipoxygenase inhibitor in rheumatoid arthritis. *J. Rheumatol.* 19, 1537-1541

350. Weitzel, F., and Wendel, A. (1993) Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J. Biol. Chem.* 268, 6288-6292.

351. Werz, O. (2002) 5-Lipoxygenase: Cellular Biology and Molecular Pharmacology. *Current Drug Targets-Inflammation & Allergy* 1, 23-44

352. Werz, O. (2004) 5-Lipxygenase: Regulation and Pharmacology. *Medicinal Chemistry Reviews*

353. Werz, O., Bürkert, E., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood* 99, 1044-1052

354. Werz, O., Klemm, J., Rådmark, O., and Samuelsson, B. (2001) p38 MAP kinase mediates stress-induced leukotriene synthesis in a human B-lymphocyte cell line. *J. Leukoc. Biol.* 70, 830-838

355. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2000) 5-Lipoxygenase is phosphorylated by p38 kinase dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5261-5266

356. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2001) Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood* 97, 2487-2495.

357. Werz, O., Schneider, N., Brungs, M., Sailer, E. R., Safayhi, H., Ammon, H. P. T., and Steinhilber, D. (1997) A test system for leukotriene synthesis inhibitors based on the in-vitro differentiation of the human leukemic cell lines HL-60 and Mono Mac 6. *Naunyn. Schmiedebergs Arch. Pharmacol.* 356, 441-445

358. Werz, O., and Steinhilber, D. (1996) Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells - The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. *Eur. J. Biochem.* 242, 90-97

359. Werz, O., Szellas, D., Henseler, M., and Steinhilber, D. (1998) Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.* 54, 445-451.

360. Werz, O., Szellas, D., Steinhilber, D., and Rådmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPKAP kinase-2. *J. Biol. Chem.* 277, 14793-14800

361. Wetterholm, A., and Haeggstrom, J. Z. (1992) Leukotriene A4 hydrolase: an anion activated peptidase. *Biochim. Biophys. Acta* 1123, 275-281

362. Wetterholm, A., Macchia, L., and Haeggstrom, J. Z. (1994) Zinc and other divalent cations inhibit purified leukotriene A4 hydrolase and leukotriene B4 biosynthesis in human polymorphonuclear leukocytes. *Arch. Biochem. Biophys.* 311, 263-271

363. White, J. R., Naccache, P. H., Molski, T. F., Borgeat, P., and Sha'afi, R. I. (1983) Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factor. *Biochem. Biophys. Res. Commun.* 113, 44-50

364. Winking, M., Sarikaya, S., Rahmanian, A., Jodicke, A., and Boker, D. K. (2000) Boswellic acids inhibit glioma growth: a new treatment option? *J. Neurooncol.* 46, 97-103

365. Wong, A., Cook, M. N., Foley, J. J., Sarau, H. M., Marshall, P., and Hwang, S. M. (1991) Influx of Extracellular Calcium Is Required for the Membrane Translocation of 5-Lipoxygenase and Leukotriene Synthesis. *Biochemistry (Mosc).* 30, 9346-9354

366. Wong, A., Cook, M. N., Hwang, S. M., Sarau, H. M., Foley, J. J., and Crooke, S. T. (1992) Stimulation of leukotriene production and membrane translocation of 5-

lipoxygenase by cross-linking of the IgE receptors in RBL-2H3 cells. *Biochemistry (Mosc).* 31, 4046-4053

367. Woods, J. W., Evans, J. F., Ethier, D., Scott, S., Vickers, P. J., Hearn, L., Heibein, J. A., Charleson, S., and Singer, II (1993) 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *J. Exp. Med.* 178, 1935-1946

368. Wu, X., Biswal, S. S., and Kehrer, J. P. (2003) Roles of 5-lipoxygenase activating protein in cell proliferation and apoptosis. *Cell Biol. Toxicol.* 19, 135-143

369. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* 387, 620-624

370. Yokomizo, T., Izumi, T., and Shimizu, T. (2001) Leukotriene B4: Metabolism and Signal Transduction. *Arch. Biochem. Biophys.* 385, 231-241

371. Yokomizo, T., Kato, K., Hagiya, H., Izumi, T., and Shimizu, T. (2001) Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. *J. Biol. Chem.* 276, 12454-12459

372. Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000) A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders.[see comment]. *J. Exp. Med.* 192, 421-432

373. Yoshimoto, T., Soberman, R. J., Spur, B., and Austen, K. F. (1988) Properties of highly purified leukotriene C4 synthase of guinea pig lung. *J. Clin. Invest.* 81, 866-871

374. Yoshimoto, T., and Takahashi, Y. (2002) Arachidonate 12-lipoxygenases. *Prostaglandins Other Lipid Mediat.* 68-69, 245-262

375. Yuan, J. H., Birkmeier, J., Yang, D. C., Hribar, J. D., Liu, N., Bible, R., Hajdu, E., Rock, M., and Schoenhard, G. (1996) Isolation and identification of metabolites of leukotriene A4 hydrolase inhibitor SC-57461 in rats. *Drug Metab. Dispos.* 24, 1124-1133

376. Zhang, Y. Y., Hammarberg, T., Rådmark, O., Samuelsson, B., Ng, C. F., Funk, C. D., and Loscalzo, J. (2000) Analysis of a nucleotide-binding site of 5-lipoxygenase by affinity labelling: binding characteristics and amino acid sequences. *Biochem. J.* 351 Pt 3, 697-707.

377. Zhang, Y. Y., Lind, B., Rådmark, O., and Samuelsson, B. (1993) Iron content of human 5-lipoxygenase, effects of mutations regarding conserved histidine residues. *J. Biol. Chem.* 268, 2535-2541.

378. Zhao, L., Moos, M. P., Grabner, R., Pedrono, F., Fan, J., Kaiser, B., John, N., Schmidt, S., Spanbroek, R., Lotzer, K., Huang, L., Cui, J., Rader, D. J., Evans, J. F., Habenicht, A. J., and Funk, C. D. (2004) The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. *Nat. Med.* 10, 966-973. Epub 2004 Aug 2022.

379. Zhelev, D. V., Alteraifi, A. M., and Chodniewicz, D. (2004) Controlled pseudopod extension of human neutrophils stimulated with different chemoattractants. *Biophys. J.* 87, 688-695.

380. Zhu, X., Sano, H., Kim, K. P., Sano, A., Boetticher, E., Munoz, N. M., Cho, W., and Leff, A. R. (2001) Role of mitogen-activated protein kinase-mediated cytosolic phospholipase A2 activation in arachidonic acid metabolism in human eosinophils. *J. Immunol.* 167, 461-468.

X. Appendix (Paper I-VII)

Paper I: Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2002) Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.* 16, 1441-1443.

Paper II: Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2003) 5-Lipoxygenase activation by MAPKAPK-2 and ERKs. *Adv Exp Med Biol* 525, 129-132.

Paper III: Fischer, L., Szellas, D., Rådmark, O., Steinhilber, D., and Werz, O. (2003) Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB J.* 17, 949-951.

Paper IV: Fischer, L., Steinhilber, D., and Werz, O. (2004) Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610. *Br. J. Pharmacol.* 142, 861-868. Epub 2004 Jun 2014.

Paper V: Altmann, A., Fischer, L., Schubert-Zsilavecz, M., Steinhilber, D., and Werz, O. (2002) Boswellic acids activate p42 MAPK and p38 MAPK and stimulate Ca^{2+} mobilisation. *Biochem. Biophys. Res. Commun.* 290, 185-190.

Paper VI: Altmann, A., Pöckel, D., Fischer, L., Schubert-Zsilavecz, M., Steinhilber, D., and Werz, O. (2004) Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leukocytes. *Br. J. Pharmacol.* 141, 223-232. Epub 2003 Dec 2022.

Paper VII: Fischer, L., Bürkert, E., Steinhilber, D., and Werz, O. (2005) Inhibitors of actin polymerization upregulate arachidonic acid release and 5-lipoxygenase activation by upregulation of Ca^{2+} mobilisation in polymorphonuclear leukocytes involving Src family kinases. *Manuscript.*

I

The FASEB Journal express article 10.1096/fj.01-0909fje. Published online July 1, 2002.

Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes

Oliver Werz,* Eva Bürkert,* Lutz Fischer,* Dagmar Szellas,* David Dishart,[†] Bengt Samuelsson,[†] Olof Rådmark,[†] and Dieter Steinhilber*

*Institute of Pharmaceutical Chemistry, University of Frankfurt, D-60439 Frankfurt, Germany; [†]Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II Karolinska Institutet, S-171 77 Stockholm, Sweden.

Corresponding author: Oliver Werz, Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie Strasse 9, D-60439 Frankfurt, Germany. E-mail: o.werz@pharmchem.uni-frankfurt.de

ABSTRACT

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of proinflammatory leukotrienes. Here, we demonstrate that extracellular signal-regulated kinases (ERKs) can phosphorylate 5-LO *in vitro*. Efficient phosphorylation required the presence of unsaturated fatty acids and was abolished when Ser-663 was mutated to alanine. In intact HeLa cells stimulated with arachidonic acid (AA), impaired 5-LO product formation was evident in cells expressing the S663A-5-LO mutant compared with cells expressing wild-type 5-LO. For Mono Mac 6 cells, priming with phorbol myristate acetate (PMA) before stimulation with ionophore was required for ERK1/2 activation and efficient 5-LO phosphorylation, in parallel with substantial AA release and 5-LO product formation. Inhibition of PKC by GF109203x or MEK1/2 by U0126 (or PD98059) abolished the 5-LO up-regulation effects of PMA. In contrast, these inhibitors failed to suppress 5-LO product formation induced by stimuli such as AA plus ionophore, which apparently do not involve the ERK1/2 pathway. Based on inhibitor studies, ERKs are also involved in AA-stimulated 5-LO product formation in PMNL, whereas a role for ERKs is not apparent in 5-LO activation induced by ionophore or cell stress. Finally, the data suggest that ERKs and p38 MAPK-regulated MAPKAPKs can act in conjunction to stimulate 5-LO by phosphorylation.

Key words: arachidonic acid • leukotriene • p38 MAP kinase

Release of arachidonic acid (AA) and activation of 5-lipoxygenase (5-LO) in response to a variety of external stimuli initiates the biosynthesis of proinflammatory leukotrienes (LTs), a family of lipid mediators with pivotal roles in asthma and inflammatory disorders (1). Activated 5-LO catalyzes the formation of 5-hydroperoxyeicosatetraenoic acid from AA and the subsequent dehydration to LTA_4, an unstable epoxide, which is further converted to LTB_4, C_4, D_4 and E_4 (2).

Attention has been focused on the activation of cellular 5-LO, which involves enzyme translocation from a soluble locus to the nuclear envelope, where it colocalizes with cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase-activating protein (for review, see ref 3). Activation of 5-LO can be induced by various soluble and particulate stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), cytokines, immune complexes, and microbes and by ionophores or thapsigargin, which raise the intracellular Ca^{2+} levels (for review, see ref 4). Ca^{2+} activates 5-LO *in vitro* and *in vivo* (see ref 5 and references therein), and it was recently found that the putative N-terminal C2-like domain of 5-LO binds Ca^{2+} and is important for translocation to the nucleus and association with membranes (6–8).

Phosphorylation appears to activate 5-LO in various leukocytes depending on the stimulus (9–14). p38 mitogen-activated protein kinase (MAPK)-regulated MAPK-activated protein kinases (MAPKAPKs, MKs) have been identified as 5-LO kinases that phosphorylate 5-LO at Ser-271 in vitro, which is promoted by unsaturated fatty acids (UFAs) (10, 11). Of interest, stimulation of polymorphonuclear leukocytes (PMNL) by cell stress induces Ca^{2+}-independent 5-LO product formation involving p38 MAPK (13).

Extracellular signal-regulated kinases (ERK)1/2 are typically activated by growth-related stimuli through the Raf-1/MEK1/2 protein kinase cascade. Investigation of substrate specificity using synthetic peptides revealed that the Pro-Xaa-Ser/Thr-Pro motif represents the optimal primary sequence for ERK1/2 phosphorylation (15, 16). However, whereas many protein substrates such as $cPLA_2$, epidermal growth factor receptor, myelin basic protein (MBP), and ELK-1 conform to this motif (for review, see ref 17), the minimal consensus sequence for ERK1/2 is Ser/Thr-Pro. Thus, in addition to primary sequence requirements, recognition of substrates may depend on their structure.

Based on inhibitor studies, previous reports suggested an involvement of ERK1/2 in agonist-induced 5-LO product formation and translocation in granulocytes or differentiated HL60 cells (9, 18–23). Although 5-LO contains a putative ERK phosphorylation motif, ERKs could not be demonstrated to phosphorylate 5-LO in vitro (24). In this study, we show that ERKs phosphorylate 5-LO in vitro and that this phosphorylation was strongly stimulated by UFAs. We provide evidence for the involvement of ERK1/2 signaling and 5-LO phosphorylation in the activation of 5-LO in intact cells.

MATERIALS AND METHODS

Human transforming growth factor $beta_1$ ($TGF\beta_1$) was purified from outdated platelets as previously described (25). Calcitriol (1,25-dihydroxyvitamin D_3) was from Biomol (Plymouth Meeting, PA). Human recombinant 5-LO was expressed in *Escherichia coli* (26) and purified as described previously (5). Anti-5-LO antiserum (AK7, 1551) was affinity purified on a 5-LO column. Materials and sources: RPMI 1640, GIBCO BRL (Life Technologies, Rockville, MD); fetal calf serum and bovine insulin, Sigma (Deisenhofen, Germany); [γ-^{32}P]ATP (110 TBq/mmol), Amersham; activated (rat, recombinant) ERK2 isoform and 5(S)-HPETE, Biomol; activated GST-MK2, Upstate Biotechnology (Lake Placid, NY); arachidonic acid, arachidic acid, linoleic acid, linolenic acid, palmitic acid, oleic acid, Ca-ionophore A23187, fMLP, MBP and

PMA, Sigma; PD 98059 and U0126, Alexis (Grünberg, Germany); SB203580 and GF109203x, Calbiochem (Bad Soden, Germany); high-performance liquid chromatography (HPLC) solvents, Merck (Darmstadt, Germany).

Cells and transient transfections

MM6 cells were cultured and differentiated with TGFβ_1 and calcitriol as previously described (27). Cells were harvested by centrifugation (200*g*, 10 min, room temperature) and washed once in phosphate-buffered saline (PBS) pH 7.4. Human PMNL were freshly isolated from leukocyte concentrates obtained from healthy donors at St Markus Hospital as previously described (13). HeLa cells were maintained and transiently transfected using the Ca^{2+} phosphate method as previously described (11).

Site-directed mutagenesis, expression, and purification of 5-LO proteins

The codons for Ser-271 and Ser-663 in the plasmid pT3-5LO were mutated using the QuikChange kit from Stratagene (La Jolla, CA) as previously described (7). The mutated DNA was confirmed using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech), followed by analysis on a Applied Biosystem PRISM 377 sequencer (carried out by KISeq, Core Facilities at Karolinska Institutet). *E. coli* MV1190 was transformed with mutated or wild-type (wt) DNA; recombinant 5-LO proteins were expressed at 27°C and purified as previously described (7). The mutated plasmids pcDNA3.1-5LO-S663A and pcDNA3.1-5LO-S271A-S663A were prepared from pcDNA3.1-5LO (28) in the same manner.

Western blotting

For determination of phosphorylated ERK1/2, MM6 cells (3×10^6) or PMNL (5×10^6) were resuspended in 100 µl PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) and stimulated at 37°C. Incubations were stopped by addition of the same volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS [w/v]), 10% β-mercaptoethanol) and heated at 95°C for 6 min. Total cell lysates corresponding to 0.5×10^6 cells in 20 µl were mixed with 4 µl of glycerol/0.1% bromphenolblue (1:1, vol/vol) and analyzed by SDS-PAGE using a Mini Protean system (Bio-Rad, Munich, Germany) on a 10% gel. After electroblot to nitrocellulose membrane (Hybond C, Amersham), blocking with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl (TBS), membranes were washed and incubated with phospho-specific ERK1/2 (Thr202/Tyr204) antibodies (AB) (New England Biolabs, Frankfurt, Germany) overnight at 4°C. Immunoreactive proteins were visualized using alkaline phosphatase conjugated IgGs as previously described (26).

Immunoprecipitation

For preparation of immunoprecipitates (IPs), MM6 cells (1×10^7) were resuspended in 1 ml PGC buffer and stimulated. After 2 min at 37°C, cells were lysed by addition of 1 ml ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 % NP-40,

50 mM NaF, 2 mM Na_3VO_4, 25 mM ß-glycerophosphate, 1 mM sodium pyrophosphate, 10 mM 4-nitrophenyl phosphate, 1 mM PMSF, 10 µg/ml leupeptin, and 60 µg/ml soybean trypsin inhibitor). After sonification (1 × 5 sec), supernatants were obtained by centrifugation of the lysates (16,000*g*, 10 min, 4°C), precleared by incubation with 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated with 2 µl of phospho-ERK1/2 (Thr202/Tyr204) AB overnight at 4°C. The immune complexes were precipitated (3 h at 4°C) with 20 µl of A/G Plus-Agarose and washed twice with lysis buffer and twice with kinase buffer and immediately used for protein kinase assays.

Protein kinase assays

Phosphorylation of 5-LO and MBP in vitro was performed as described previously (10). In brief, purified recombinant 5-LO or MBP (40 pmol each) were preincubated in the absence or presence of fatty acids with the activated kinases (MK2, ERK2) or the ERK1/2-IPs from MM6 cells in presence of ATP (100 µM) and [γ-^{32}P]ATP (100 nCi/µl) for 30 min at 30°C. The reaction was terminated by addition of the same volume of SDS-b, and samples (20 µl) were separated by SDS-PAGE. Proteins were first visualized by Coomassie staining to ensure correct loading of protein. Phosphorylated proteins were analyzed using a Phosphorimager Fuji FLA 3000.

Determination of 5-lipoxygenase product formation

PMNL (5×10^6), HeLa (2×10^6), or MM6 cells (3×10^6) in 1 ml PGC buffer were preincubated for 30 min at 37°C in the presence or absence of the indicated inhibitors. Then, cells were stimulated with the indicated agents. After 10 min at 37°C, the reaction was stopped with 1 ml methanol and 30 µl 1 N HCl, and 500 µl PBS were added. Formed 5-LO products were extracted and analyzed by HPLC as previously described (29). 5-LO product formation is expressed as ng 5-LO products per 10^6 cells, which includes the all-trans isomers of LTB_4, LTB_4, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), and 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) for MM6 and HeLa cells. For PMNL, the 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid [5(*S*),12(*S*)-DiHETE] is also included in the calculation. 5-HETE and 5-HPETE coelute as one major peak; integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected, and oxidation products of LTB_4 were not determined.

Determination of [^{3}H]arachidonic acid release from phospholipids

MM6 cells were resuspended at 2×10^6/ml in RPMI 1640 medium containing 4.8 nM [^{3}H]AA (corresponding to 0.25 µCi/ml, specific activity 200 Ci/mmol) and incubated for 90 min at 37°C. Thereafter, cells were centrifuged and washed to remove unincorporated [^{3}H]AA. Labeled cells (5×10^6) were resuspended in 1 ml PGC buffer containing 2 mg/ml fatty acid free albumin. The samples were preincubated at 37°C with or without additives before the addition of ionophore A23187 (5 µM). After 10 min, the reaction was stopped with 1 ml methanol and 30 µl 1 N HCl, and 500 µl PBS were added and [^{3}H]AA was extracted and analyzed by HPLC as described previously (12).

RESULTS

5-LO is efficiently phosphorylated by ERK2 *in vitro* in the presence of unsaturated fatty acids, role of Ser-663

Because several reports have proposed a role for ERK1/2 in activation of the 5-LO pathway (9, 19–24), we addressed whether ERKs are actually capable of phosphorylating 5-LO. In vitro kinase assays were conducted using purified recombinant 5-LO as substrate and purified activated ERK2 as kinase; activated GST-MK2 was used as a positive control (see refs 10 and 11).

As shown in Figure 1A, 5-LO was dose-dependently phosphorylated by active ERK2 and MK2 in vitro. The 5-LO phosphorylation rates of ERK2 appeared somewhat lower than those obtained by MK2, and compared to MBP (which is an excellent in vitro substrate for ERKs [30]), 5-LO was less efficiently phosphorylated by ERK2. Thus, about 20- to 30-fold higher amounts of kinase were required for similar phosphate incorporation into 5-LO vs. MBP (40 pmol each).

Recently, we found that UFAs promote 5-LO phosphorylation by MK2, but not by the 5-LO kinases PKA or CaMKII (11). AA increased 5-LO phosphorylation by ERK2 in a dose-dependent fashion up to 25-fold (at 50 µM AA, Fig. 1B). Opposite effects of AA were observed when MBP was used as substrate. Thus, AA dose-dependently decreased ERK2 activity toward MBP, and at 50 µM AA, phosphorylation of MBP was almost completely abolished. In addition to AA, the UFAs oleic acid and linoleic acid increased ERK2 activity toward 5-LO. In contrast, the saturated arachidic acid and palmitic acid as well as 5-HETE, the hydroxylated metabolite of AA, failed to enhance 5-LO phosphorylation rates (Fig. 1C).

The minimal consensus sequence for ERK1/2 is Ser/Thr-Pro, and such a motif is present in 5-LO (YLSP at residues 661-664). In order to identify phosphorylation sites, Ser-663 in 5-LO was mutated to alanine (S663A-5-LO) and subjected to phosphorylation by ERK2. As shown in Figure 1D**,** in the absence of UFAs, wt-5-LO as well as the S663A-5-LO mutant were only marginally phosphorylated by ERK2. However, a striking difference between wt- and S663A-5-LO was apparent when the kinase reaction was performed in presence of AA. Whereas AA increased the phosphorylation of wt-5-LO, AA (or oleate, data not shown) led to only a marginal increase in the phosphorylation rate (about twofold) for the S663A mutant, indicating that enhanced phosphate incorporation due to UFAs requires the Ser-663 residue in 5-LO.

ERK activation correlates to 5-LO activation in MM6 cells

For MM6 cells, priming with PMA before stimulation with ionophore was found to be required for prominent AA-release and 5-LO product formation (12), and we attempted to correlate ERK activity to 5-LO activation. In accordance with the effect on 5-LO product synthesis, treatment of MM6 cells with ionophore alone failed to activate ERK1/2, but priming with PMA for 10 min with or without subsequent ionophore treatment resulted in a clear activation of ERK1/2, preferentially ERK2 (Fig. 2A, lower band). Inhibition of the upstream kinases protein kinase C

(PKC) by GF109203x ($IC_{50} \approx 0.1$ μM) or MEK1/2 by U0126 ($IC_{50} \approx 0.3$ μM) (or PD98059, $IC_{50} \approx 3$ μM, data not shown), dose-dependently reduced ERK1/2 activation by PMA.

Next, we determined the ability of endogenous ERK1/2, immunoprecipitated from MM6 cells, to phosphorylate 5-LO *in vitro*. MBP was used as control, and 50 μM AA was included when 5-LO phosphorylation was assayed. ERK1/2 derived from MM6 cells that had been primed with PMA before stimulation with ionophore markedly phosphorylated 5-LO (and MBP), and pretreatment of the cells with U0126 (3 μM) prevented the effect of PMA (Fig. 2B). Stimulation of MM6 cells with ionophore alone gave no significant change in immunoprecipitated ERK1/2 activity, compared with untreated control cells. When kinase activity toward 5-LO was determined in absence of exogenous AA, only faint bands of phosphorylated 5-LO were detected (data not shown).

ERKs are involved in PMA-up-regulated 5-LO product formation in MM6 cells

We determined the contribution of ERK1/2 in PMA-up-regulated AA release and 5-LO product formation in MM6 cells using specific inhibitors. Priming with PMA enhanced ionophore-induced [^{3}H] AA release up to sixfold, and inhibition of PKC by GF109203x (0.3 μM) or ERK1/2 activity by U0126 (1 μM) or PD98059 (10 μM) suppressed the liberation of AA (Fig. 3A).

In accordance with previous results (12), priming with PMA increased ionophore-stimulated 5-LO product formation in MM6 cells up to sevenfold in the absence of exogenous substrate, but also in the presence of exogenous AA, a significant ($P < 0.01$) increase (twofold) in 5-LO product formation was found (Fig. 3 B, C). No 5-LO products were detected when cells were stimulated with PMA alone (data not shown). The up-regulation effects of PMA were efficiently blocked by GF109203x ($IC_{50} \approx 0.2$ μM) as well as by U0126 ($IC_{50} \approx 0.3$ μM) or PD98059 ($IC_{50} \approx 5$ μM, data not shown) at concentrations that correlated with the suppression of PMA-induced ERK1/2 activation (compare Fig. 2A). However, neither GF109203x nor U0126 (or PD98059, not shown) blocked 5-LO product formation induced by ionophore plus AA in nonprimed cells, indicating that the inhibitors basically reduced PMA-up-regulated 5-LO activity. In broken cell preparations, the IC_{50} values for 5-LO catalytic activity were determined at >10 μM for GF109203x, >10 μM for U0126 and >100 μM for PD98059 (data not shown). Thus, direct effects of these kinase inhibitors on 5-LO catalytic activity, at the concentrations that abolished PMA-priming, are negligible.

ERKs are involved in AA-induced 5-LO product formation in PMNL

AA can activate ERKs in PMNL, predominantly ERK2, involving G protein-coupled receptors (31), and it was demonstrated that AA induces 5-LO product formation in PMNL (11, 32–34). In Ca^{2+}-free medium, a dose-dependent induction of 5-LO product formation by AA was observed that was accompanied with the phosphorylation of ERK2 (Fig. 4A). The 5-LO product pattern is shown in a HPLC chromatogram obtained from PMNL stimulated with 40 μM AA (Fig. 4B). U0126 and PD98059 were used to investigate the involvement of ERKs in 5-LO product formation. Exogenous AA was added to all incubations to exclude reduced 5-LO product

synthesis due to alterations in substrate supply, by possible inhibition of $cPLA_2$ (35). PMNL were treated with AA (60 μM) alone, with AA (10 μM) plus 2.5 μM ionophore (leading to prominent Ca^{2+} influx), or with AA (40 μM) plus 0.3 M NaCl (which activates 5-LO independent of Ca^{2+} involving p38 MAPK pathways (13). As shown in Fig. 4C (left panel), AA-induced 5-LO product formation was partially reduced by U0126 (0.3–3 μM) to about 50–60% at inhibitor concentrations that prevented ERK2 activation (Fig. 4C**,** right panel). In contrast, 5-LO product formation induced by AA plus ionophore or by AA plus NaCl (conditions that apparently activate 5-LO independent of ERK1/2) was hardly affected by U0126. Similar results were obtained with PD98059 (data not shown).

Furthermore, in PMNL (depleted from adenosine to augment 5-LO product synthesis from endogenous substrate (36)), 5-LO product synthesis induced by the physiological agonist fMLP (no exogenous AA) correlated to ERK1/2 activation and was completely reduced by 3 μM U0126 (Fig. 4D). When exogenous AA (5 μM) was included, only partial (~41%) reduction of fMLP-induced 5-LO product formation was detected (Fig. 4E), suggesting that alterations in endogenous AA supply contribute to impaired 5-LO product synthesis. Combination of U0126 with the p38 MAPK inhibitor SB203580 (10 μM), gave no further 5-LO inhibition (data not shown). Together, suppression of 5-LO product formation by ERK activation inhibitors depended on the stimulus and on the signal transduction pathway utilized to activate 5-LO.

Role of putative phosphorylation sites in 5-LO for product formation

To investigate the impact of 5-LO phosphorylation for enzyme activation in intact cells, we transiently transformed HeLa cells with plasmids encoding wt-5-LO or S663A-5-LO, and 5-LO product formation was determined. As shown in Fig. 5, HeLa cells expressing wt- or S663A-5-LO gave similar prominent product formation after stimulation with both ionophore (10 μM) and AA (0–60 μM), whereas 5-LO product formation for the S663A mutant was significantly lower compared with wt-5-LO, when cells were treated with AA (10–60 μM) alone. Thus, for S663A-5-LO, the amount of the formed products after incubation with AA (40 or 60 μM) was only 18 or 22% of the products obtained after incubation with AA plus ionophore the corresponding ratio for wt-5-LO was 41 or 43% (Fig. 5).

ERKs act in conjunction with MK2 to activate 5-LO

AA-induced 5-LO product formation in PMNL was also partially suppressed (by 40%) by the p38 MAPK inhibitor SB203580 (10 μM). Intriguingly, combining U0126 (3 μM) with SB203580 (10 μM) caused 5-LO suppression by 83% (Fig. 6A), suggesting that both ERKs and p38 MAPK pathways are involved in AA-induced 5-LO product formation. Again, formation of 5-LO products induced by Ca^{2+}-dependent pathways, using AA plus ionophore, was hardly suppressed by combinations of the kinase inhibitors.

Recently, we also found reduced 5-LO product formation in HeLa cells transformed with S271A-5-LO vs. wt-5-LO after stimulation with AA (11). To investigate whether both Ser-271 and Ser-663 could be of importance for AA-induced 5-LO activity in intact cells, we transfected HeLa cells with plasmids encoding S271A-S663A-5-LO and determined 5-LO product

generation. The expression levels of the S271A-S663A mutant were only 30–40% compared with the wt-5-LO (insert Fig. 6B), which correlate with the reduced formation of 5-LO products in broken cell preparations (data not shown). In relation to the protein expression levels, S271A-S663A-5-LO gave comparable 5-LO product formation after incubation with AA plus ionophore as found for wt-5-LO. However, for wt-5-LO the ratio of AA- to AA plus ionophore-induced 5-LO product synthesis at 10–60 μM AA was 24–43%, for S663A-5-LO 11–22%, and for the S271A-S663A-5-LO 5–14% (Fig. 6B). Thus, at low Ca^{2+} levels, phosphorylation at both Ser-663 and Ser-271 appears to be required for 5-LO product formation in intact cells.

DISCUSSION

Based on inhibitor studies, the ERK signaling pathway has been implicated in the activation of 5-LO, but the precise mechanisms involved have not been elucidated yet. For example, PD98059 blocked 5-LO activity and translocation in ionophore-stimulated HL-60 cells (9, 18) and U0126 suppressed LT synthesis in untreated or IL-5 primed eosinophils as well as in neutrophils challenged by fMLP (19, 20, 23). Also in PAF-stimulated RBL-2H3 cells, LTC_4 generation (but not CCL2 production) was blocked by U0126 (22). Note that $cPLA_2$, which provides AA as substrate for 5-LO, is regulated by ERKs (35, 37). Thus, it is not clear from some of these studies (19, 20, 22, 23) whether reduced 5-LO activity by ERK inhibitors is due to impaired AA availability. However, also in the presence of exogenous AA, PD98059 blocked 5-LO activity in HL-60 cells (9), suggesting a more direct role of ERK in regulating 5-LO.

Although in previous studies 5-LO phosphorylation by ERKs could not be detected (24), we found that ERK2 and ERK1/2 immunoprecipitated from MM6 cells phosphorylate human recombinant 5-LO in vitro. These opposite findings could be related to different assay conditons, for example, purity, integrity, and the amount of 5-LO used or kinase buffer composition. Also, compared with the excellent in vitro substrate MBP (30), 5-LO is a rather poor substrate for ERK2, and ERK2 activity toward 5-LO was not readily detectable in in-gel kinase assays (compare ref 10 and 11). However, UFAs (but not saturated fatty acids or 5-HETE) markedly enhanced 5-LO phosphorylation by ERK2, and endogenous ERKs derived from MM6 cells required AA for efficient phosphorylation of 5-LO. Previously, we found that UFAs also promote 5-LO phosphorylation at Ser-271 by MK2, but not by PKA or CaMKII (11), and phosphorylation of other MK2 substrates (e.g., heat shock protein 27) was not enhanced. Thus, the ability of UFAs to promote protein phosphorylation seems to be specific for 5-LO as substrate and is restricted to particular 5-LO kinases. The underlying mechanisms are unclear, presumably UFAs lead to exposure of the serine residues or favor substrate recognition and access of the kinases (compare ref 11). Because mutation of Ser-663 to alanine abolished substantial phosphorylation in presence of AA, it appears that Ser-663 is an ERK phosphorylation site in 5-LO.

It was shown that in response to ionophore, which activates ERKs and p38 MAPK pathways in leukocytes (38), 5-LO phosphorylation occurred in intact HL-60 cells (24), although only a very small amount of the total 5-LO protein was phosphorylated. We failed to convincingly assess in vivo phosphorylation after 5-LO immunoprecipitation from stimulated PMNL, MM6 or HL-60 cells preincubated with $^{32}P_i$. This might be attributable to inefficient ^{32}P-labelling of 5-LO due to

experimental settings or dephosphorylation during timely long experimental procedures. However, it is also conceivable that (in contrast to $cPLA_2$) only a small fraction of the cellular 5-LO is subject to phosphorylation. This small activated pool of 5-LO in turn may activate the bulk of enzyme via 5-LO derived lipid hydroperoxides that convert the active site iron from the ferrous to the ferric state, a process that is essential for initializing the 5-LO catalytic redox cycle (39, 40). Along these lines, it was implied also by others that on stimulation of neutrophils by AA, only a small amount of 5-LO is initially activated by Ca^{2+}-independent mechanisms before activation of the bulk of 5-LO (34). The stimulus-dependent difference in 5-LO product synthesis of the S663A 5-LO mutant compared to wt-5-LO in HeLa cells suggests that 5-LO phosphorylation by ERKs indeed plays a role for 5-LO activation in intact cells.

Recent studies have proposed that 5-LO in intact PMNL can be activated by at least two different pathways: either by elevation of intracellular Ca^{2+} (using ionophore as stimulus), or by a cell stress-induced, p38 MAPK-regulated pathway which is Ca^{2+}-independent (13). For $cPLA_2$, which is substrate for ERKs, p38 MAPK and p38 MAPK-regulated kinases (41, 42), stimuli leading to activation of MAPKs (PMA) and $cPLA_2$ phosphorylation caused AA release also at basal Ca^{2+} levels (43), whereas Ca^{2+}-mobilizing agents (ionophore) stimulated AA release when phosphorylation of $cPLA_2$ was blocked (35). Our studies confirm the hypothesis of Ca^{2+}- and/or phosphorylation-mediated 5-LO enzyme activation. Thus, 5-LO product formation in HeLa cells expressing wt- or S663A-5-LO was quantitatively the same when cells had been stimulated with AA plus ionophore, where Ca^{2+} is the predominant 5-LO activator and phosphorylation might be of minor importance. However, the S663A-5-LO mutant, lacking the ERK1/2 phosphorylation site, produced significantly lower amounts of 5-LO metabolites when an ERK-dependent stimulus such as AA was used. It was shown that AA causes pronounced ERK activation in various cell types (31, 44, 45) but leads to only moderate Ca^{2+} fluxes (13, 32, 33).

Notably, most of the natural ligands that stimulate LT synthesis in PMNL (e.g., fMLP, PAF, C5a, or AA) activate ERKs (31, 46–48) but lead to rather moderate elevations of intracellular Ca^{2+} compared with the nonphysiological stimulus ionophore (32, 33, 49, 50). In this respect, ERK activation inhibitors failed to suppress 5-LO product synthesis induced by ionophore or by ERK-independent phosphorylation pathways (via p38 MAPK using cell stress as stimulus), whereas AA-induced 5-LO product synthesis significantly correlated with ERK activity (Fig. 4). For 5-LO activation induced by fMLP (that causes Ca^{2+} mobilization in PMNL [50]), both Ca^{2+} and phosphorylation events may contribute (13), explaining less pronounced 5-LO inhibition by U0126 (Fig. 4E). Intriguingly, exogenous addition of 5-HPETE or 5-HETE to neutrophils stimulated ERKs, involving G protein-linked receptors, and conversion of AA by 5-LO was required for ERK activity (31, 51), implying autoregulatory loops for AA-induced 5-LO activation via ERKs.

Also in MM6 cells induction and up-regulation of 5-LO product formation significantly coincided with ERK activity, confirming a direct role of ERKs in 5-LO activation. Thus, ionophore alone failed to activate ERK1/2 in MM6 cells for unknown reasons, and 5-LO product synthesis from endogenous AA was low. Preincubation with PMA induced ERK activity and also 5-LO product synthesis, which was highly sensitive to specific ERK activation inhibitors at concentrations that were similar to those required to prevent ERK activation. This reduced 5-LO

product synthesis in PMA-primed cells might be due to impaired AA availability. However, PMA-up-regulated 5-LO product formation was abolished by GF109203x, U0126 and PD98059 also in presence of exogenous AA, implying a direct stimulatory effect of ERKs on 5-LO and possible alterations in the supply of endogenous AA should be negligible. Apparently in MM6 cells, both phosphorylation processes and elevated Ca^{2+} levels are of importance for substantial 5-LO product synthesis.

Finally, our data suggest that enzyme phosphorylation by ERK2 and MK2 at multiple sites can act in conjunction to activate 5-LO (Fig. 7). In PMNL, AA and fMLP also activate p38 MAPK leading to activation of the 5-LO kinase MK2 (11, 36, 45). Thus enzyme phosphorylation by ERK2 and MK2 at multiple sites might be necessary for AA-induced 5-LO activation, supported by the synergistic effects of U0126 and SB203580 (Fig. 6A). Moreover, for HeLa cells the ratio of AA to AA/ionophore-induced 5-LO product formation of S271A-S663A-5-LO, lacking the phosphorylation sites for MK2 and for ERKs, was significantly lower when compared with 5-LO mutants lacking only one phosphorylation site (S271A-5-LO or S663A-5-LO) (compare ref 11). Synergistic actions of MAPK pathways (depending on the stimulus) were also observed for the activation of $cPLA_2$ (35, 52).

In conclusion, our data show that ERKs, particularly in the presence of UFAs, are capable of phosphorylating 5-LO, which may contribute to 5-LO product synthesis. Importantly, ERKs can act in conjunction with p38 MAPK-regulated MKs to activate 5-LO in intact cells, where UFAs such as AA, might play a central role in the convergence of MAPK signaling cascades, leading to phosphorylation and activation of 5-LO. These findings might provide the molecular basis of 5-LO activation in leukocytes in response to particular agonists and priming agents and might provide new strategies for pharmacological intervention with LT biosynthesis during inflammatory diseases.

ACKNOWLEDGMENTS

We thank Hélène Ax:son Johnson, Astrid Neuß and Agneta Nordberg for expert technical assistance. This study was supported by grants from the Deutsche Pharmazeutische Gesellschaft, the Swedish Medical Research Council (03X-217), from the EU (QLG1-CT-2001-01521), and from the Verum Foundation.

REFERENCES

1. Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C. A., and Serhan, C. N. (1987) Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* 237, 1171–1176

2. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 220, 568–575

3. Peters-Golden, M., and Brock, T. G. (2000) Intracellular compartmentalization of leukotriene biosynthesis. *Am J Respir Crit Care Med* 161, S36–40.

4. Rådmark, O. (2000) The molecular biology and regulation of 5-lipoxygenase. *Am J Respir Crit Care Med* 161, S11–15

5. Hammarberg, T., and Rådmark, O. (1999) 5-lipoxygenase binds calcium. *Biochemistry* 38, 4441–4447

6. Kulkarni, S., Das, S., Funk, C. D., Murray, D., and Cho, W. (2002) A molecular basis of specific subcellular localization of the C2-like domain of 5-lipoxygenase. *J Biol Chem* 277, 13167–13174

7. Hammarberg, T., Provost, P., Persson, B., and Rådmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J Biol Chem* 275, 38787–38793

8. Chen, X. S., and Funk, C. D. (2001) The N-terminal "ß-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. *J Biol Chem* 276, 811-818

9. Lepley, R. A., and Fitzpatrick, F. A. (1996) Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells. *Arch Biochem Biophys* 331, 141–144

10. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2000) 5-Lipoxygenase is phosphorylated by p38 kinase dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. USA* 97, 5261–5266

11. Werz, O., Szellas, D., Steinhilber, D., and Rådmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPKAP kinase-2. *J Biol Chem* 277, 14793–14800

12. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2001) Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood* 97, 2487–2495

13. Werz, O., Buerkert, E., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood* 99, 1044–1052

14. Werz, O., Klemm, J., Rådmark, O., and Samuelsson, B. (2001) p38 MAP kinase mediates stress-induced leukotriene synthesis in a human B-lymphocyte cell line. *J Leuk Biol* 70, 830–838

15. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J Biol Chem* 266, 22159–22163

16. Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991) Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J Biol Chem* 266, 15180–15184

17. Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 268, 14553–14556

18. Lepley, R. A., and Fitzpatrick, F. A. (1998) 5-Lipoxygenase compartmentalization in granulocytic cells is modulated by an internal bipartite nuclear localizing sequence and nuclear factor kappa B complex formation. *Arch Biochem Biophys* 356, 71–76

19. Bates, M. E., Green, V. L., and Bertics, P. J. (2000) ERK1 and ERK2 activation by chemotactic factors in human eosinophils is interleukin 5-dependent and contributes to leukotriene C(4) biosynthesis. *J Biol Chem* 275, 10968–10975.

20. Boden, S. E., Bertsche, T., Ammon, H. P., and Safayhi, H (2000) MEK-1/2 inhibition prevents 5-lipoxygenase translocation in N- formylpeptide-challenged human neutrophils *Int J Biochem Cell Biol* 32, 1069–1074

21. Boden, S. E., Schweizer, S., Bertsche, T., Dufer, M., Drews, G., and Safayhi, H. (2001) Stimulation of leukotriene synthesis in intact polymorphonuclear cells by the 5-lipoxygenase inhibitor 3-oxo-tirucallic acid. *Mol Pharmacol* 60, 267–273

22. Ahamed, J., and Ali, H. (2002) Distinct roles of receptor phosphorylation, G protein usage and MAP kinase activation on platelet-activating-factor-induced leukotriene C4 generation and chemokine production. *J Biol Chem*, 277, 22685–22691

23. Zhu, X., Sano, H., Kim, K. P., Sano, A., Boetticher, E., Munoz, N. M., Cho, W., and Leff, A. R. (2001) Role of mitogen-activated protein kinase-mediated cytosolic phospholipase A2 activation in arachidonic acid metabolism in human eosinophils. *J Immunol* 167, 461–468.

24. Lepley, R. A., Muskardin, D. T., and Fitzpatrick, F. A. (1996) Tyrosine kinase activity modulates catalysis and translocation of cellular 5-lipoxygenase. *J Biol Chem* 271, 6179–6184

25. Werz, O., Brungs, M., and Steinhilber, D. (1996) Purification of transforming growth factor beta1 from human platelets. *Pharmazie* 51, 893–896

26. Hammarberg, T., Zhang, Y. Y., Lind, B., Rådmark, O., and Samuelsson, B. (1995) Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. *Eur J Biochem* 230, 401–407

27. Brungs, M., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D-3. *Proc Natl Acad Sci USA* 92, 107–111

28. Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B., and Rådmark, O. (2001) 5-Lipoxygenase interacts with coactosin-like protein. *J Biol Chem* 276, 16520–16527.

29. Werz, O., and Steinhilber, D. (1996) Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells--The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. *Eur J Biochem* 242, 90–97

30. Erickson, A. K., Payne, D. M., Martino, P. A., Rossomando, A. J., Shabanowitz, J., Weber, M. J., Hunt, D. F., and Sturgill, T. W. (1990) Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. *J Biol Chem* 265, 19728–19735.

31. Capodici, C., Pillinger, M. H., Han, G., Philips, M. R., and Weissmann, G. (1998) Integrin-dependent homotypic adhesion of neutrophils. Arachidonic acid activates Raf-1/Mek/Erk via a 5-lipoxygenase- dependent pathway. *J Clin Invest* 102, 165–175

32. Naccache, P. H., McColl, S. R., Caon, A. C., and Borgeat, P. (1989) Arachidonic acid-induced mobilization of calcium in human neutrophils: evidence for a multicomponent mechanism of action. *Br J Pharmacol* 97, 461–468

33. Schatzmunding, M., Hatzelmann, A., and Ullrich, V. (1991) The Involvement of Extracellular Calcium in the Formation of 5-Lipoxygenase Metabolites by Human Polymorphonuclear Leukocytes. *Eur J Biochem* 197, 487–493

34. Surette, M. E., Krump, E., Picard, S., and Borgeat, P. (1999) Activation of leukotriene synthesis in human neutrophils by exogenous arachidonic acid: inhibition by adenosine A(2a) receptor agonists and crucial role of autocrine activation by leukotriene B(4). *Mol Pharmacol* 56, 1055–1062

35. Gijon, M. A., Spencer, D. M., Siddiqi, A. R., Bonventre, J. V., and Leslie, C. C. (2000) Cytosolic phospholipase A2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A2 regulation. *J Biol Chem* 275, 20146–20156

36. Krump, E., Sanghera, J. S., Pelech, S. L., Furuya, W., and Grinstein, S. (1997) Chemotactic peptide N-formyl-met-leu-phe activation of p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase-2 in human neutrophils. *J Biol Chem* 272, 937–944

37. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* 72, 269–278

38. Elzi, D. J., Bjornsen, A. J., MacKenzie, T., Wyman, T. H., and Silliman, C. C. (2001) Ionomycin causes activation of p38 and p42/44 mitogen-activated protein kinases in human neutrophils. *Am J Physiol Cell Physiol* 281, C350–C360.

39. Rouzer, C. A., and Samuelsson, B. (1986) The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS Lett.* 204, 293-296

40. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1994) 5-Lipoxygenase. *Annu. Rev. Biochem.* 63, 383–417

41. Gijon, M. A., and Leslie, C. C. (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J Leukoc Biol* 65, 330–336

42. Hefner, Y., Borsch-Haubold, A. G., Murakami, M., Wilde, J. I., Pasquet, S., Schieltz, D., Ghomashchi, F., Yates, J. R., 3rd, Armstrong, C. G., Paterson, A., Cohen, P., Fukunaga, R., Hunter, T., Kudo, I., Watson, S. P., and Gelb, M. H. (2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *J Biol Chem* 275, 37542–37551

43. Qiu, Z. H., Gijon, M. A., deCarvalho, M. S., Spencer, D. M., and Leslie, C. C. (1998) The role of calcium and phosphorylation of cytosolic phospholipase A(2) in regulating arachidonic acid release in macrophages. *J Biol Chem* 273, 8203–8211

44. Hii, C. S., Ferrante, A., Edwards, Y. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulos, A., and Murray, A. W. (1995) Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C-dependent mechanism. *J Biol Chem* 270, 4201–4204

45. Hii, C. S., Huang, Z. H., Bilney, A., Costabile, M., Murray, A. W., Rathjen, D. A., Der, C. J., and Ferrante, A. (1998) Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukemic cells, and human neutrophils. Evidence for cell type-specific activation of mitogen-activated protein kinases. *J Biol Chem* 273, 19277–19282

46. Ferby, I. M., Waga, I., Sakanaka, C., Kume, K., and Shimizu, T. (1994) Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J Biol Chem* 269, 30485–30488

47. Buhl, A. M., Avdi, N., Worthen, G. S., and Johnson, G. L. (1994) Mapping of the C5a receptor signal transduction network in human neutrophils. *Proc Natl Acad Sci U S A* 91, 9190–9194

48. Grinstein, S., and Furuya, W. (1992) Chemoattractant-induced tyrosine phosphorylation and activation of microtubule-associated protein kinase in human neutrophils. *J Biol Chem* 267, 18122–18125

49. Naccache, P. H., Volpi, M., Showell, H. J., Becker, E. L., and Sha'afi, R. I. (1979) Chemotactic factor-induced release of membrane calcium in rabbit neutrophils. *Science* 203, 461–463

50. White, J. R., Naccache, P. H., Molski, T. F., Borgeat, P., and Sha'afi, R. I. (1983) Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factor. *Biochem Biophys Res Commun* 113, 44–50

51. Chang, L. C., and Wang, J. P. (2001) Signal transduction pathways for activation of extracellular signal- regulated kinase by arachidonic acid in rat neutrophils. *J Leukoc Biol* 69, 659–665

52. Hazan-Halevy, I., Seger, R., and Levy, R. (2000) The requirement of both extracellular regulated kinase and p38 mitogen- activated protein kinase for stimulation of cytosolic phospholipase A(2) activity by either FcgammaRIIA or FcgammaRIIIB in human neutrophils. A possible role for Pyk2 but not for the Grb2-Sos-Shc complex. *J Biol Chem* 275, 12416–12423

Received January 28, 2002; revised May 15, 2002.

Fig. 1

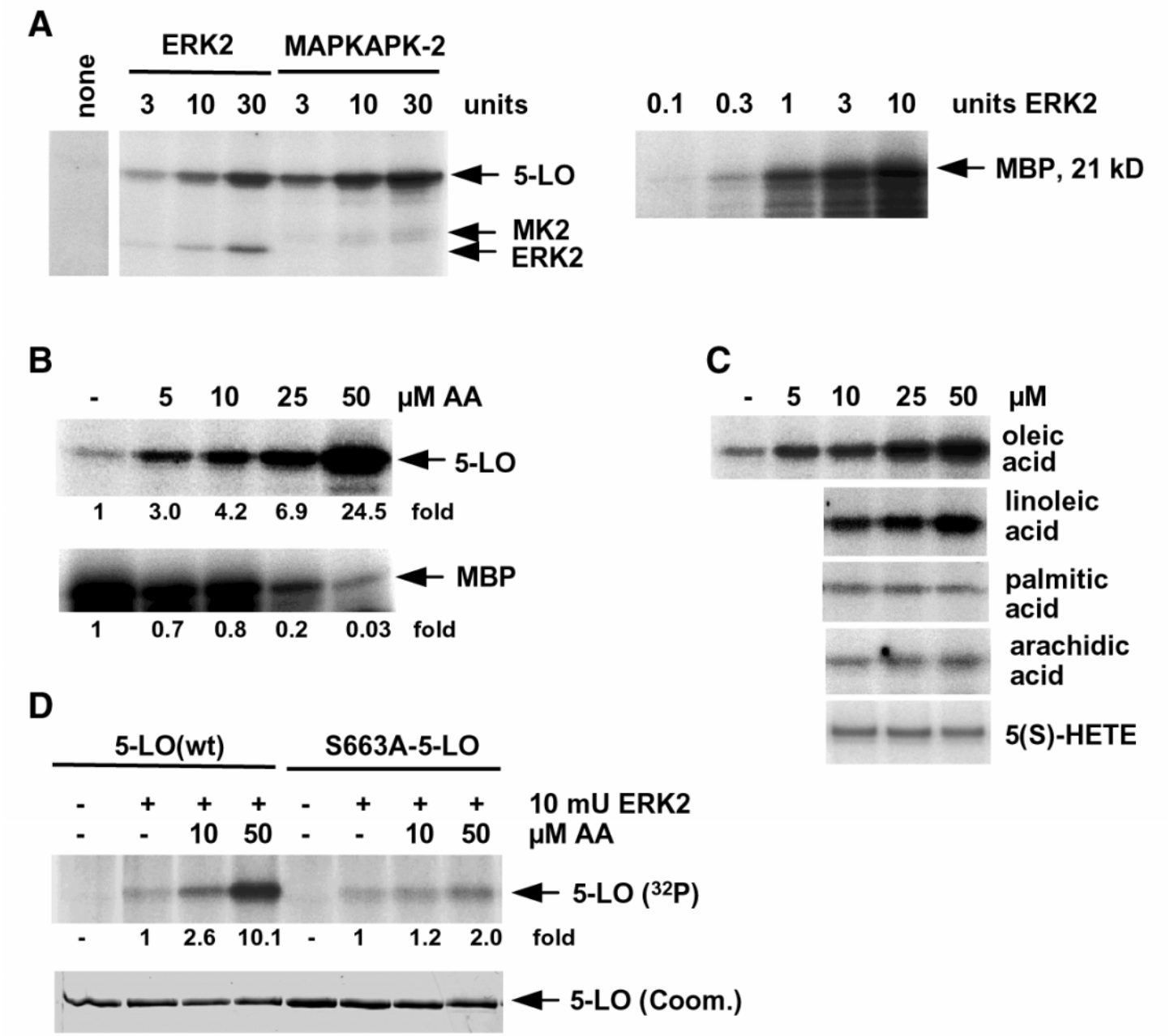


Figure 1. Extracellular signal-regulated kinase (ERK)2 phosphorylates 5-lipoxygenase (5-LO) in vitro: effects of unsaturated fatty acids. A) Phosphorylation of 5-LO (left panel) and myelin basic protein (right panel). Phosphorylation of the proteins (40 pmol each) was determined by in vitro kinase assay as described in Materials and Methods. Arrows indicate the positions of the corresponding proteins. One mU of each kinase (MK2 and ERK2) incorporates 1 pmol of phosphate into a standard substrate peptide. Because the standard substrates are different for the different kinases, the unit amounts are not strictly comparable. **B)** Arachidonic acid (AA) promotes 5-LO phosphorylation by ERK2. **C)** Effects of fatty acids on 5-LO phosphorylation by ERK2. **D)** Ser-663 is required for AA-induced increase in 5-LO phosphorylation by ERK2. The proteins (40 pmol each) were incubated with 10 mU active ERK2 in the absence or presence of the fatty acids, and phosphorylation was determined by in vitro kinase assays. Equal amounts of 5-LO and S663A-5-LO protein were determined by Coomassie staining. Results are representative of three separate experiments.

Fig. 2

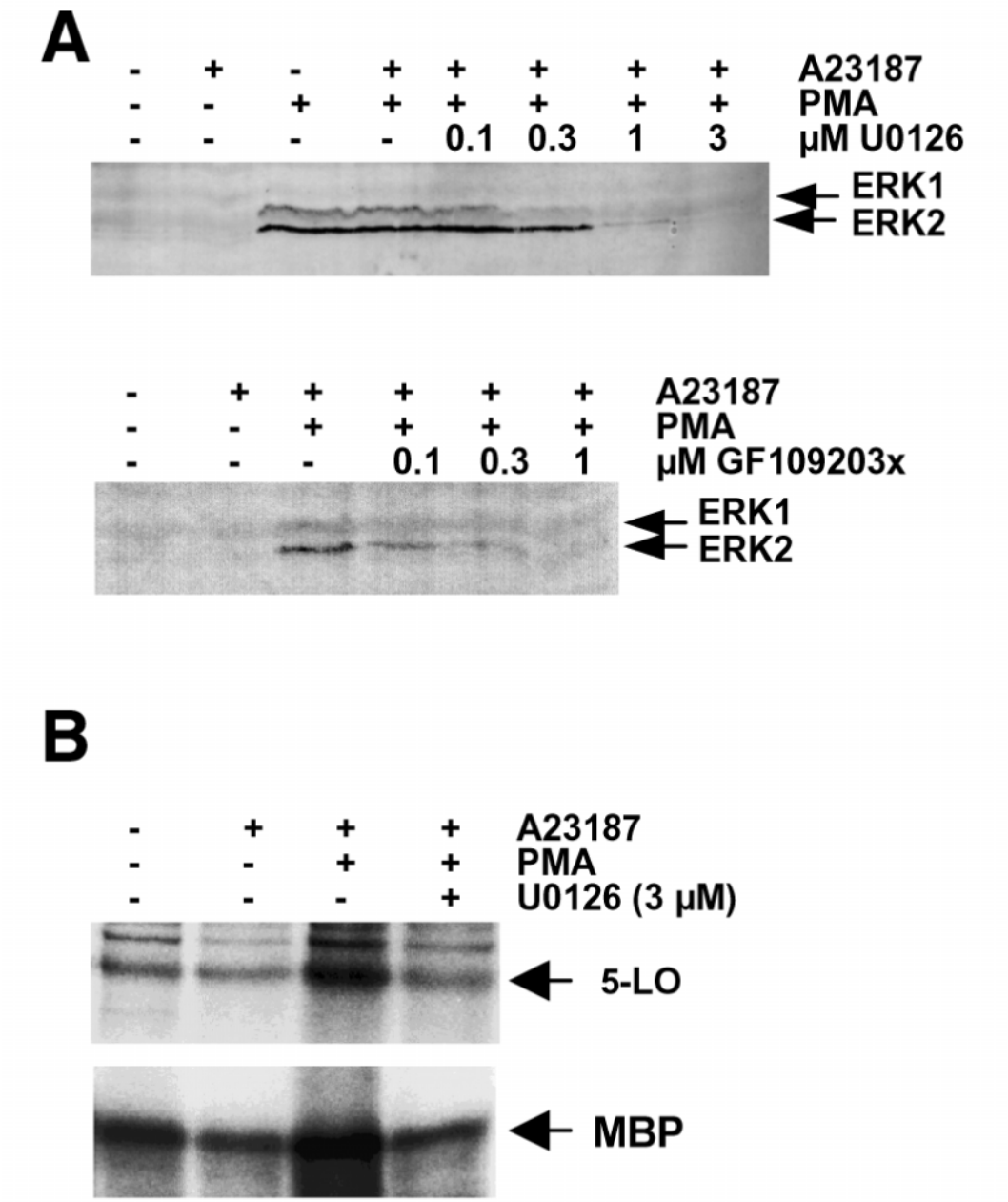


Figure 2. Activation of ERKs in MM6 cells. A) ERK1/2 activation by phorbol myristate acetate (PMA) involves protein kinase C and MEK1/2. MM6 cells (3×10^6) in 100 µl phosphate-buffered saline pH 7.4 containing 1 mg/ml glucose and 1 mM Ca^{2+} (PGC buffer) were preincubated with U0126 or GF109203x for 20 min at 37°C and then primed with or without 100 nM PMA for 10 min before addition of 5 µM ionophore as indicated. After 2.5 min, incubations were terminated by addition of the same volume of SDS-b, and ERK1/2 activation was determined by Western blot using specific antibodies (AB) that detect the dually phosphorylated forms of ERK1/2. **B)** Phosphorylation of 5-LO and myelin basic protein by ERKs immunoprecipitated from MM6 cells. MM6 cells (1×10^7 in 1 ml PGC buffer) were preincubated with 3 µM U0126 for 20 min at 37°C and then primed with or without 100 nM PMA for 10 min before addition of 5 µM ionophore as indicated. After 2 min, cells were lysed, and phosphorylated ERK1/2 were immunoprecipitated as described in Material and Methods. Phosphorylation of 5-LO (in the presence of 50 µM arachidonic acid [AA]) and MBP (no AA) by aliquots of phospho-ERK1/2-IPs was determined by in vitro kinase assay. Results are representative of three separate experiments.

Fig. 3

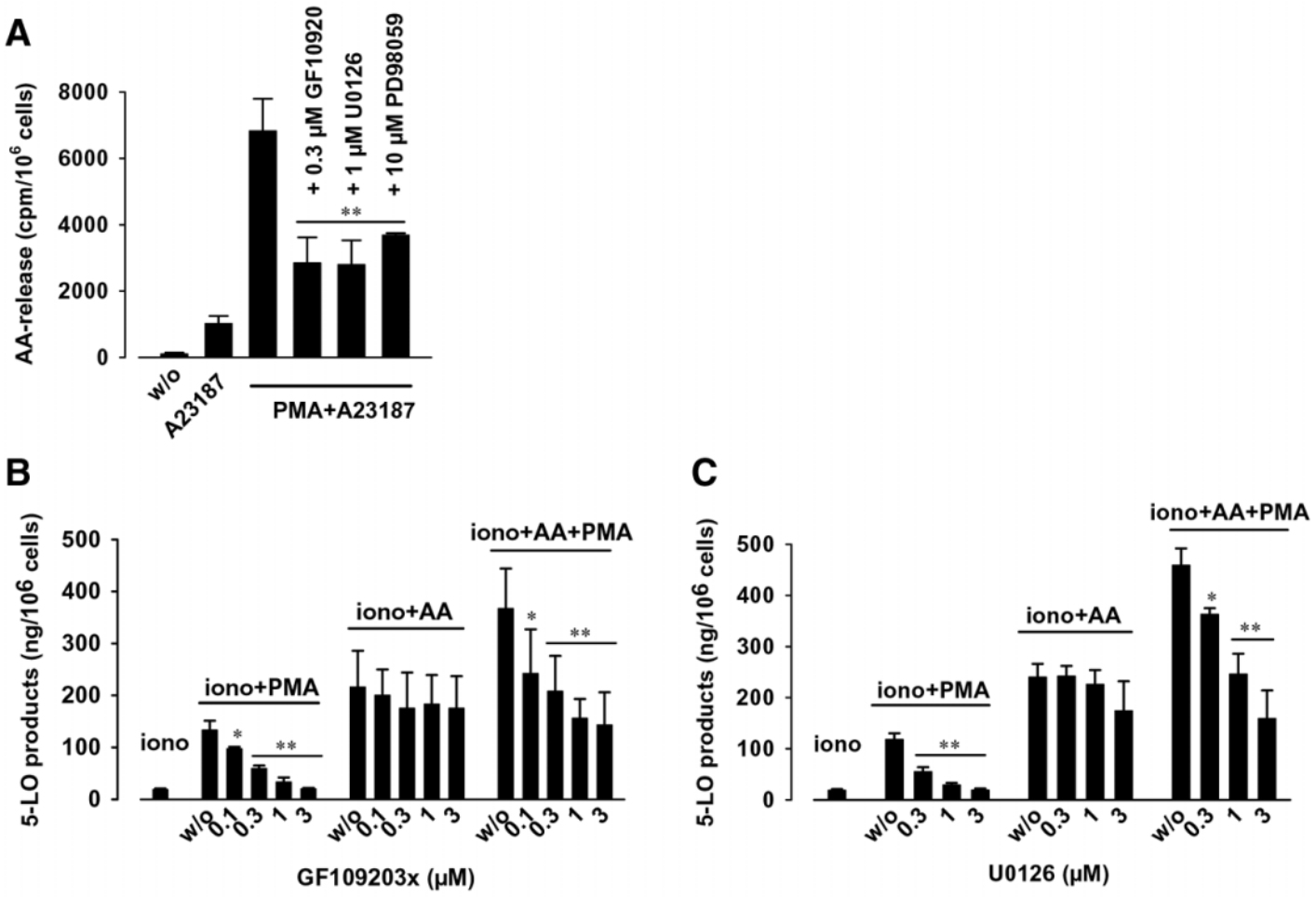


Figure 3. Involvement of ERKs in arachidonic acid release and 5-LO product formation in MM6 cells.
(A) Inhibition of PKC or MEK1/2 suppresses PMA-induced [^{3}H]AA release**.** MM6 cells (2×10^6/ml in RPMI 1640 medium) were prelabeled with 0.25 µCi/ml [^{3}H]AA for 90 min at 37°C. After unincorporated [^{3}H]AA was removed, cells (5×10^6 in 1 ml phosphate-buffered saline pH 7.4 containing 1 mg/ml glucose, 1 mM Ca^{2+}, and 2 mg/ml fatty acid free albumin) were preincubated with the indicated inhibitors for 20 min at 37°C, primed in absence or presence of PMA for 10 min at 37°C before addition of 5 µM ionophore. After 10 min, free [^{3}H]AA was determined by high-performance liquid chromatography (HPLC). Results are given as mean + SE, $n = 3$. **B**, **C)** Inhibition of PKC or MEK1/2 suppresses PMA-induced 5-LO product formation. MM6 cells (3×10^6) in 1 ml PGC buffer were preincubated with the inhibitors (GF109203x (**B**) or U0126 (**C**)) for 20 min at 37°C and then primed with or without 100 nM PMA for 10 min at 37°C before addition of 5 µM ionophore with or without 40 µM of exogenous AA as indicated. After another 10 min, 5-LO product formation was determined by HPLC. Results are given as mean + SE, $n = 4$–5. Student t test, *$P < 0.05$ and **$P < 0.01$.

Fig. 4

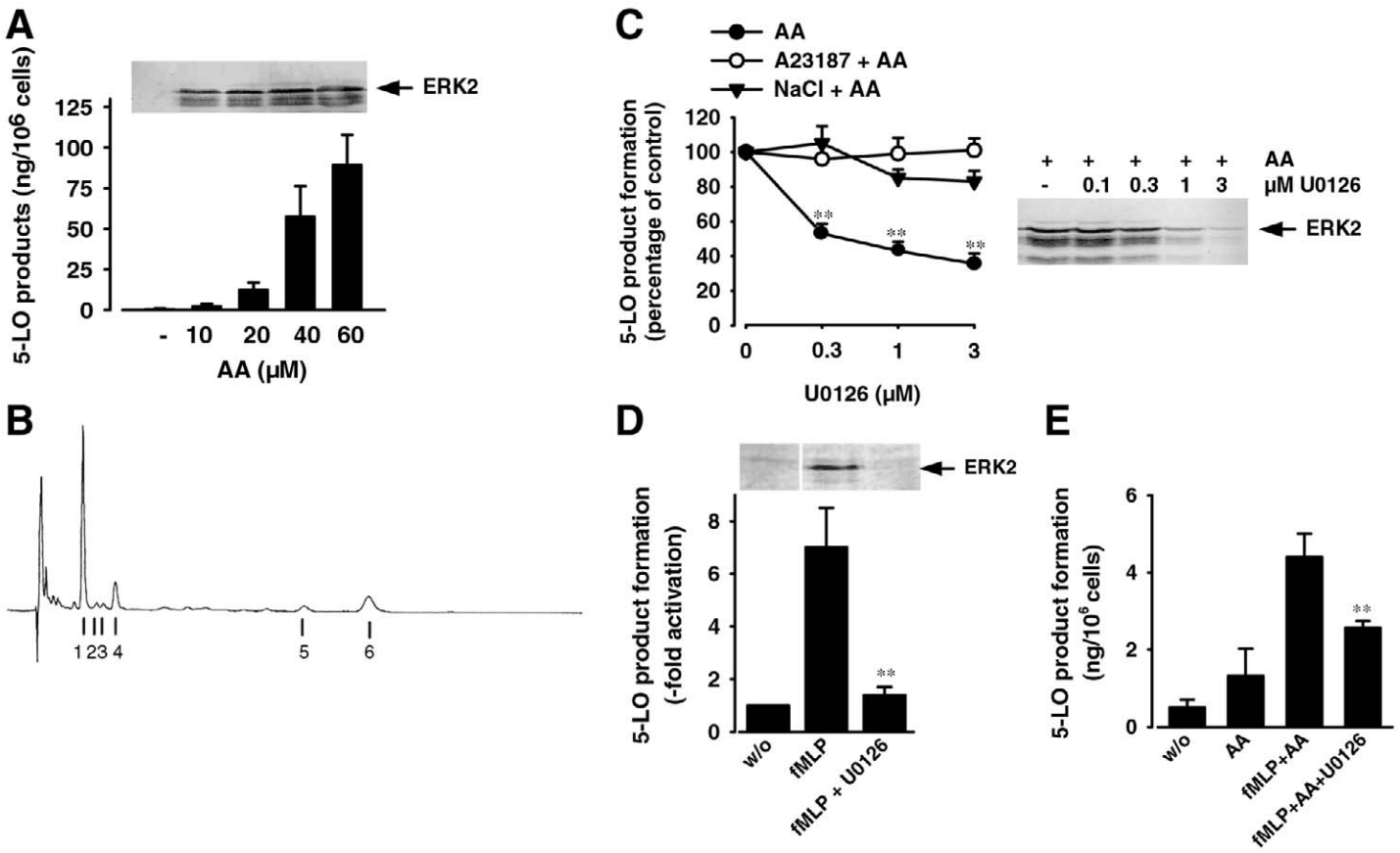


Figure 4. Involvement of ERKs in 5-LO product formation in polymorphonuclear leukocytes (PMNLs). **A)** AA-induced 5-LO product formation and activation of ERK2. To determine the activation of ERKs, 5×10^6 PMNL were resuspended in 100 µl phosphate-buffered saline (PBS) containing 1 mM EDTA and stimulated with the indicated concentration of AA at 37°C. After 2 min, incubations were terminated and phosphorylation of ERK2 was determined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by Western blot (WB). To determine 5-LO product formation, 5×10^6 PMNL were resuspended in 1 ml PBS containing 1 mM EDTA. 5-LO products were determined 10 min after stimulation by high-performance liquid chromatography (HPLC). Results are given as mean +SE, $n = 3–4$. **B)** Typical HPLC chromatogram of 5-LO products extracted from PMNL that were stimulated with 40 µM AA for 5 min at 37°C. 1: prostaglandin B_2; 2, 3: trans-isomers of LTB_4; and 4: LTB4; all recorded at 280 nm. 5: 12-HETE recorded at 235 nm; 6: 5-HETE recorded at 235 nm. **C)** Effects of U0126 on 5-LO product formation. For stimulation with 60 µM AA, 5×10^6 PMNL were resuspended in 1 ml PBS containing 1 mM EDTA. Alternatively, for stimulation with 2.5 µM ionophore plus 10 µM AA or with 300 mM NaCl plus 40 µM AA, cells were resuspended in phosphate-buffered saline, pH 7.4, containing 1 mg/ml glucose and 1 mM Ca^{2+} (PGC buffer). Before stimulation, cells were preincubated with U0126 for 30 min at 37°C. Ten minutes after addition of stimuli, 5-LO product formation was determined by HPLC. Results are given as mean +SE, $n = 3–4$. Student t test: $^{**}P < 0.01$. The control values (100%) in absence of inhibitors were 57.1 ± 7.7, 53.7 ± 9.8, and 100.7 ± 2.2 ng/10^6 cells stimulated with 60 µM AA, NaCl plus AA, and ionophore plus AA, respectively. To determine the activation of ERKs (right panel), PMNL (5×10^6 in 100 µl PBS containing 1 mM EDTA buffer) were preincubated with U0126 for 30 min at 37°C before addition of 60 µM AA. After 2 min, incubations were terminated and phosphorylated ERK2 was determined by WB. **D)** Activation of ERKs and 5-LO by N-formyl-methionyl-leucyl-phenylalanine (fMLP). PMNL (5×10^6/ ml PGC buffer) were preincubated with 3 µM U0126 for 25 min at 37°C. Then, adenosine deaminase (5 U/ml) was added 5 min before stimulation with 1 µM fMLP. Incubations were terminated after 2 min to determine phosphorylated ERK2 by WB. To determine 5-LO product formation, incubations were terminated after 10 min. Results are given as mean +SE, $n = 3$. Student t test: $^{**}P < 0.01$. **E)** Effects of U0126 on fMLP-induced 5-LO product formation in presence of exogenous AA. PMNL were treated as previously described, except that 5 µM AA was added with fMLP, and 5-LO product formation was determined. Results are given as mean +SE, $n = 4$. Student t test: $^{**}P < 0.01$.

Fig. 5

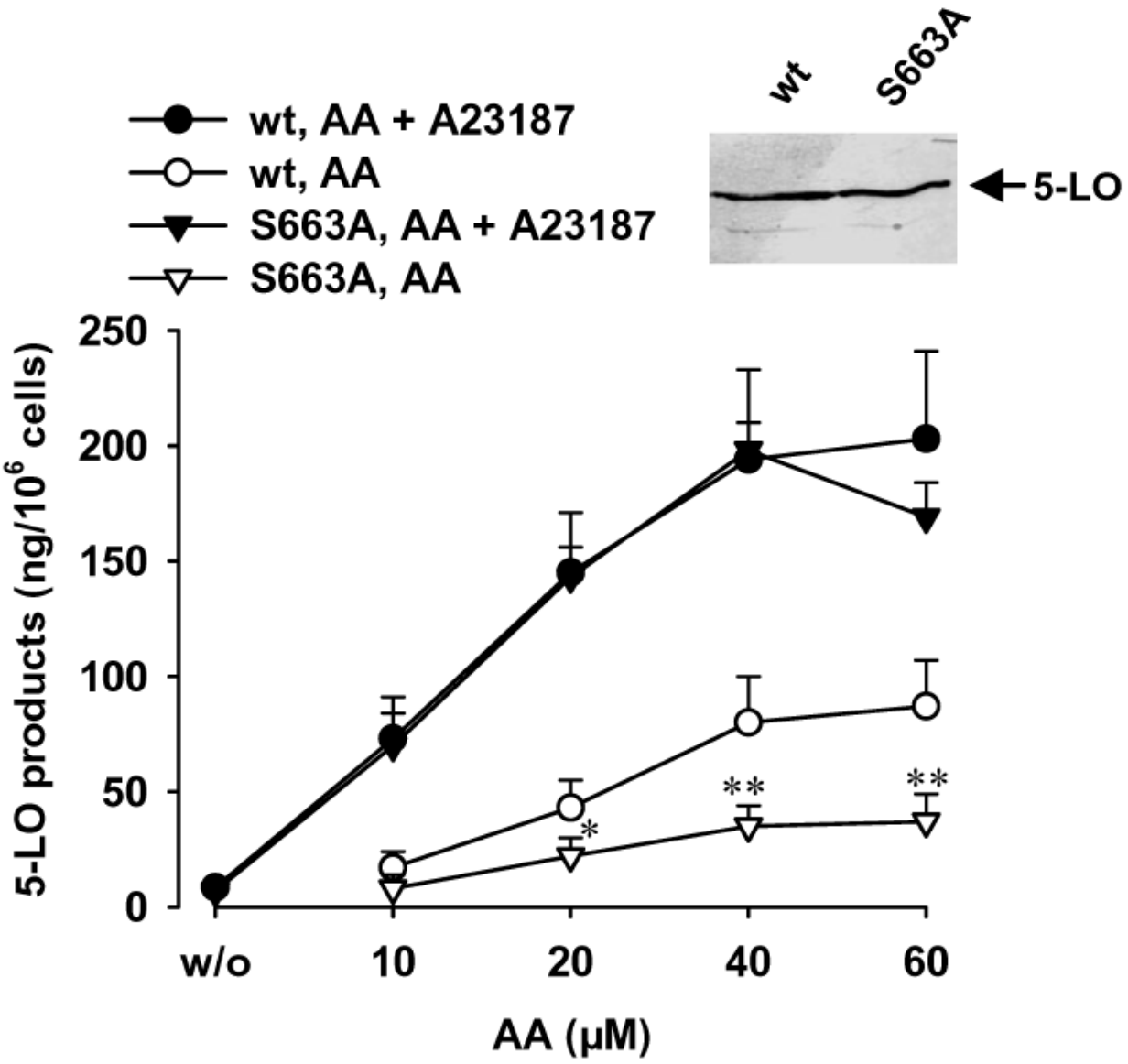


Figure 5. 5-LO product formation in transformed HeLa cells. 5-LO product formation of wt and S663A-5-LO. HeLa cells were transiently transformed with plasmid DNA (10 µg) encoding wt-5-LO or S663A-5-LO. Cells (2×10^6) were resuspended in 1 ml PGC buffer and stimulated with the indicated concentrations of AA in the absence or presence of 10 µM ionophore for 10 min at 37°C. 5-LO product formation was determined by HPLC. Results are given as mean +SE, n = 3. For one set of HeLa samples (total cell lysates corresponding to 0.1×10^6 cells), the expression of 5-LO proteins was analyzed by WB using anti-5-LO antiserum (AK7, 1551) (insert).

Fig. 6

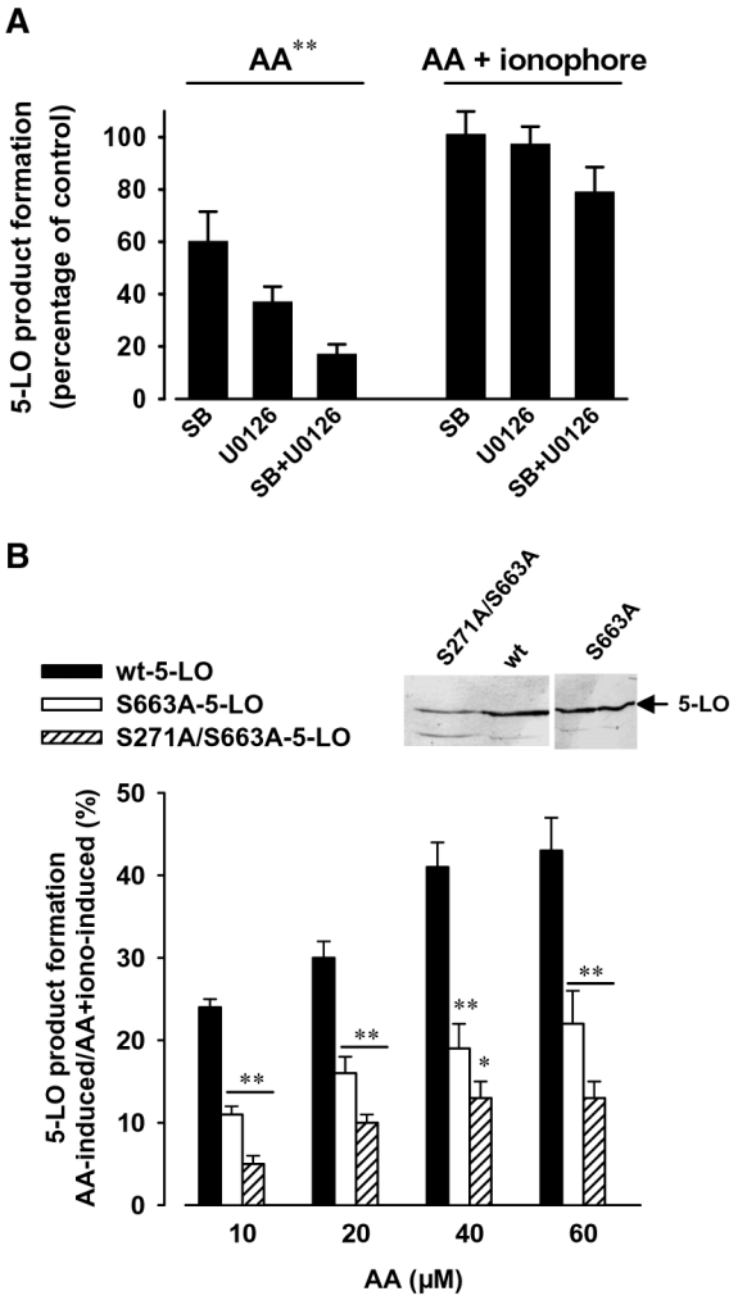


Figure 6. ERKs and p38 MAPK-regulated MK2 act in conjunction to stimulate 5-LO product formation. **(A)** Effects of U0126 and SB203580 on 5-LO product formation in PMNL. PMNL (5×10^6/ml) were preincubated with U0126 (3 µM) and SB203580 (10 µM) as indicated for 30 min at 37°C. For stimulation with 60 µM AA, cells were resuspended in 1 ml PBS containing 1 mM EDTA. For stimulation with 2.5 µM ionophore plus 10 µM AA, cells were resuspended in PGC buffer. Ten minutes after addition of stimuli, 5-LO product formation was determined by HPLC. Results are given as mean +SE, $n = 4–5$. Student t test: $^*P < 0.05$ and $^{**} P < 0.01$. One hundred percent of 5-LO product formation is defined as the amounts of 5-LO products formed in absence of inhibitors. **B)** Ratio of 5-LO product formation in transformed HeLa cells stimulated with AA and with AA plus ionophore. HeLa cells (2×10^6), transiently transformed with plasmid DNA (10 µg) encoding wt-5-LO, S663A-5-LO, or S271A-S663A-5-LO, were resuspended in 1 ml PGC buffer and stimulated with the indicated concentrations of AA in absence or presence of 10 µM ionophore. After 10 min at 37°C, 5-LO product formation was determined. Results are presented (the mean +SE, $n = 3$) as the quotient of AA-induced to AA plus ionophore-induced 5-LO product formation. Student t test: $^*P < 0.05$ and $^{**}P < 0.01$. The expression of 5-LO proteins was analyzed by WB (insert).

Fig. 7

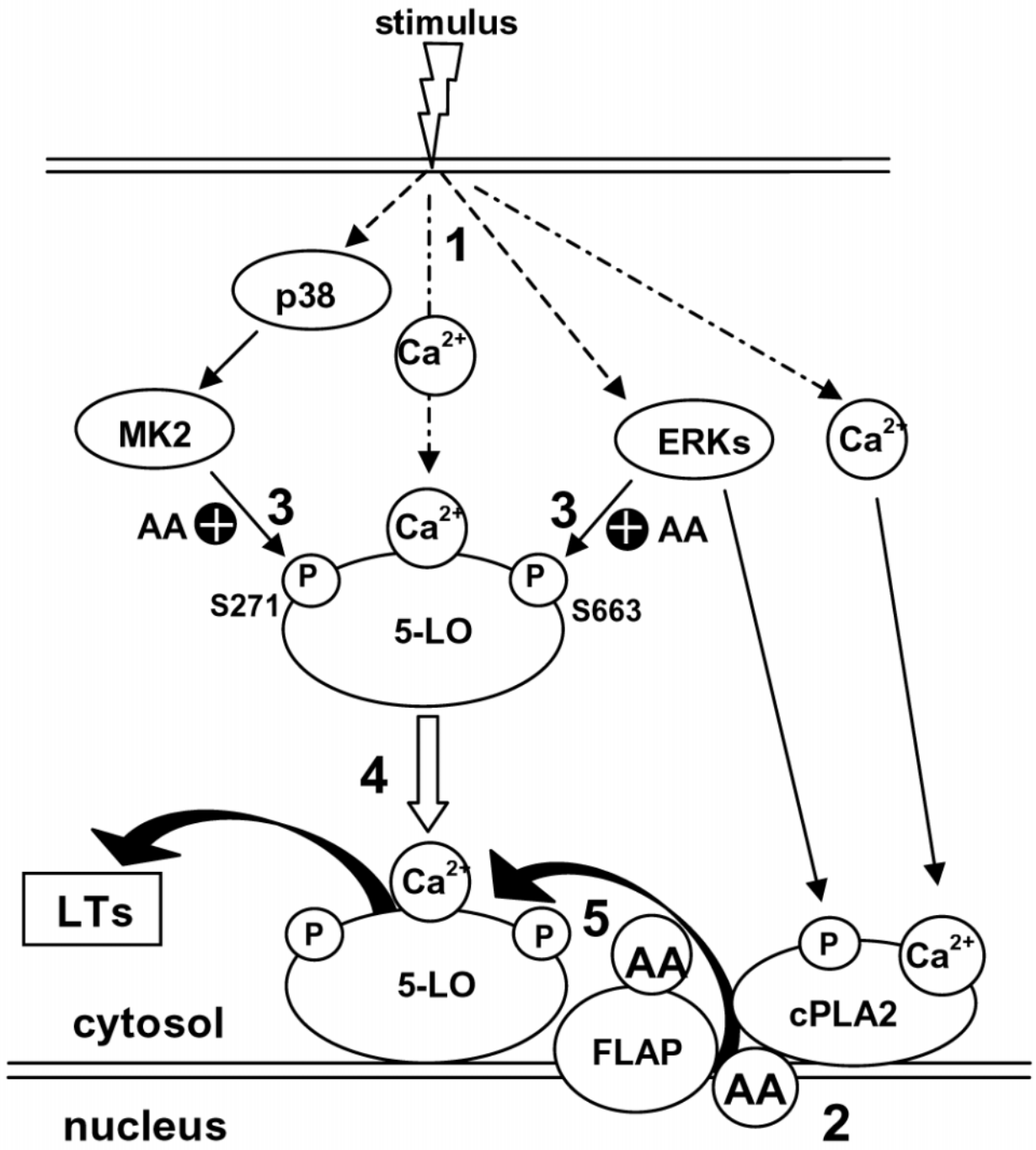


Figure 7. Schematic illustration of cellular 5-LO activation and AA metabolism. Extracellular stimuli lead to elevation of intracellular Ca^{2+} and activate MAPK pathways (1). These events stimulate translocation and activation of $cPLA_2$ that in turn releases AA from phospholipids at the nuclear membrane (2). Free AA may activate 5-LO phosphorylation by ERKs and MK2 (3). On activation, 5-LO translocates to the nuclear membrane and colocalizes with FLAP (4). Free AA is transferred via FLAP to 5-LO for conversion to leukotrienes (5). Depending on the stimulus and the cell type, phosphorylation and/or Ca^{2+} may be required for activation of 5-LO.

II

5-LIPOXYGENASE ACTIVATION BY MAPKAPK-2 AND ERKS

Oliver Werz[1], Eva Bürkert[1], Lutz Fischer[1], Dagmar Szellas[1], David Dishart[2], Bengt Samuelsson[2], Olof Rådmark[2], and Dieter Steinhilber[1]

[1]Institute of Pharmaceutical Chemistry and Microbiology, University of Frankfurt,Marie-Curie-Str. 9, 60439 Frankfurt, Germany. [2]Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II Karolinska Institutet, S-171 77 Stockholm, Sweden

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of proinflammatory leukotrienes from arachidonic acid (AA) (1). Ca^{2+}, phosphatidylcholine, ATP, and hydroperoxides stimulate the enzymatic activity of 5-LO *in vitro* (1). However, the mechanisms involved in the agonist-induced 5-LO activation in intact cells are less clear. Stimuli that cause an elevation of the intracellular Ca^{2+} levels activate 5-LO. Ca^{2+} binds 5-LO *in vitro* at the enzyme´s C2 domain (2) which is a prerequisite for association with membranes (3).

On the other hand, phosphorylation has a significant impact on cellular 5-LO activity (4-6). p38 MAPK-regulated MK-2 and –3 phosphorylate 5-LO *in vitro*, that is potently promoted by unsaturated fatty acids (e.g. AA), and a strong correlation between such phosphorylation events and 5-LO activity in intact cells was found (4, 5, 7). Intriguingly, activation of 5-LO in polymorphonuclear leukocytes (PMNL) by cell stress, involving p38 MAPK and MKs, is Ca^{2+}-independent (7).

5-LO was dose-dependently phosphorylated by active ERK2 *in vitro* (Fig. 1A). In analogy to MK2, 5-LO was only a poor substrate for ERK2 and AA increased 5-LO phosphorylation by ERK2 in a dose-dependent fashion up to 25-fold (Fig. 1B). Also oleic acid and linoleic acid increased ERK2 activity towards 5-LO, whereas saturated fatty acids failed to enhance 5-LO phosphorylation rates. Therefore, upon cell stimulation, efficient phosphorylation may occur only when free AA is provided.

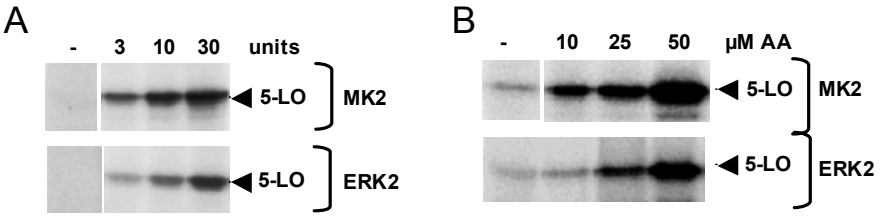


Figure 1: 5-LO is phosphorylated by MK-2 and ERK2; effects of AA. Protein phosphorylation was determined by in vitro kinase assay. (A) Purified recombinant 5-LO (3 µg) was incubated with the indicated amounts of kinases for 30 min at 30°C. (B) 5-LO was incubated with 10 mU kinase together with the indicated amounts of AA. Proteins were separated by SDS-PAGE and phosphorylated 5-LO (arrow) was detected by autoradiography of the dried gel.

Addition of exogenous AA to PMNL caused a dose-dependent induction of 5-LO activity that was accompanied by activation (phosphorylation) of ERK2 and p38 MAPK (Fig. 2A). This AA-induced 5-LO activity was partially reduced by U0126, an ERK pathway inhibitor, but also by the p38 MAPK inhibitor SB203580. Combination of both inhibitors gave additive effects, indicating that ERK and p38 MAPK pathways act in conjunction to stimulate 5-LO. Importantly, 5-LO activity induced by AA plus Ca^{2+}-ionophore (leading to high levels of intracellular Ca^{2+} that directly activates 5-LO) was hardly affected by U0126 and SB203580 (not shown).

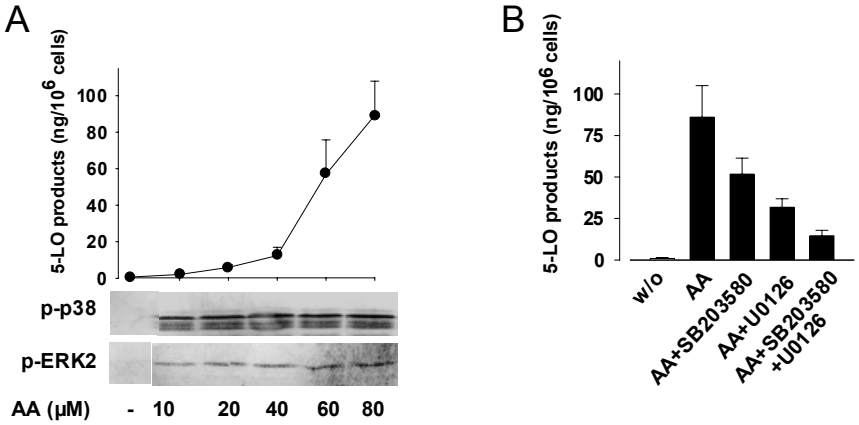


Figure 2: Activation of p38 MAPK and ERKs correlates with 5-LO activity. Human isolated PMNL were incubated with the indicated amounts of AA at 37°C (A) or preincubated with SB203580 (10 µM) and/or U0126 (3 µM) and then stimulated with 60µM AA (B). 5-LO products were analyzed by HPLC 10 min after addition of AA. ERK and p38 MAPK activation was determined 2.5 min after addition of AA by Western blotting using phospho-specific antibodies against the kinases.

The 5-LO sequence reveals putative phosphorylation motifs for MK-2 (LERQLS; 266-271) as well as for ERK2 (YLSP; 661-664), with Ser271 and Ser663 as potential phosphorylation sites. Mutation of these residues to alanine leads to 5-LO mutant proteins that were no longer substrates for the respective kinases. HeLa cells were transfected with wt or the 5-LO mutants and cellular 5-LO activity was determined. Almost identical amounts of 5-LO products were formed from wt and mutated 5-LO enzymes, when cells had been treated with AA plus ionophore, whereas significantly less 5-LO products as compared to wt-5-LO were formed, when cells had been challenged with AA alone (Fig. 3). Finally, deletion of both phosphorylation sites resulted in a even more pronounced reduction of AA-induced 5-LO activity. Together it appears that phosphorylation of 5-LO at Ser271 and Ser663 is of importance for 5-LO activation at low Ca^{2+} levels. In this scenario AA may play a pivotal regulatory role on several levels in as much as it (1) serves as substrate for 5-LO, (2) activates 5-LO kinases, and (3) may promote the phosphorylation of 5-LO by ERKs and MKs.

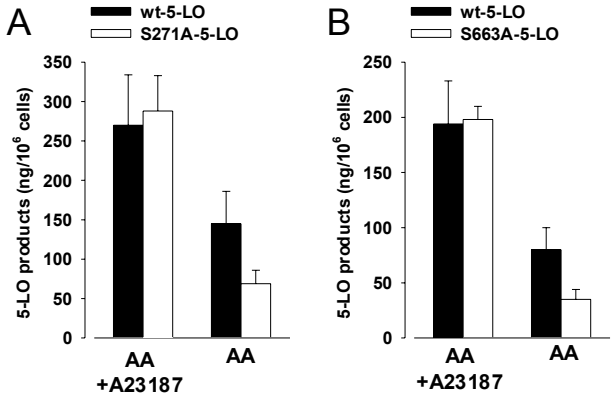


Figure 3: Effects of deletion of 5-LO phosphorylation sites. HeLa cells were transiently transfected with wt-5-LO, S271A-5-LO (A), or S663A-5-LO (B), stimulated with 40 µM AA with or without 10 µM ionophore A23187 and after 10 min at 37°C, 5-LO products were analyzed by HPLC.

ACKNOWLEDGEMENTS

This study was supported by grants from EU (QLG1-CT-2001-01521) and the Deutsche Pharmazeutische Gesellschaft. We thank Astrid Neuss and Agneta Nordberg for expert technical assistance.

REFERENCES

1 Rådmark, O. (2000) Am J Respir Crit Care Med 161, S11-5.
2 Hammarberg, T., Provost, P., Persson, B. and Radmark, O. (2000) Journal of Biological Chemistry 275, 38787-38793.
3 Kulkarni, S., Das, S., Funk, C.D., Murray, D. and Cho, W. (2002) J Biol Chem 277, 13167-13174.
4 Werz, O., Szellas, D., Steinhilber, D., and Rådmark, O. (2002) J Biol Chem 277, 14793-14800.
5 Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2000) Proc. Natl. Acad. Sci. 97, 5261-5266.
6 Lepley, R.A., Muskardin, D.T. and Fitzpatrick, F.A. (1996) J Biol Chem 271, 6179-6184.
7 Werz, O., Buerkert, E., Samuelsson, B., Rådmark, O., and Steinhilber, D. (2002) Blood 99, 1044-1052.

III

The FASEB Journal express article10.1096/fj.02-0815fje. Published online March 28, 2003.

Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors

Lutz Fischer,[†] Dagmar Szellas,[†] Olof Rådmark,[‡] Dieter Steinhilber,[†] and Oliver Werz[†]

[†]Institute of Pharmaceutical Chemistry, University of Frankfurt, D-60439 Frankfurt, Germany; [‡]Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, S-171 77 Stockholm, Sweden

Corresponding author: Oliver Werz, Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie Strasse 9, D-60439 Frankfurt, Germany. E-mail: o.werz@pharmchem.uni-frankfurt.de

ABSTRACT

Nonredox-type 5-lipoxygenase (5-LO) inhibitors such as ZM230487 or L-739.010 potently suppress leukotriene biosynthesis at low cellular peroxide tone. Here, we show that inhibition of 5-LO product formation by nonredox-type 5-LO inhibitors in human isolated polymorphonuclear leukocytes (PMNL) depends on the activation pathway of 5-LO. Thus, compared with 5-LO product synthesis induced by the Ca^{2+}-mobilizing agent ionophore A23187, cell stress-induced 5-LO product formation involving 5-LO kinase pathways required ~10- to 100-fold higher concentrations of ZM230487 or L-739.010 for comparable 5-LO inhibition. No such differences were observed for the iron ligand-type 5-LO inhibitor BWA4C or the novel-type 5-LO inhibitors hyperforin and 3-*O*-acetyl-11-keto-boswellic acid. Experiments using purified 5-LO revealed that Ca^{2+} is no prerequisite for potent enzyme inhibition by ZM230487, and exposure of PMNL to the combination of ionophore and cell stress did not restore potent 5-LO suppression. Intriguingly, a significant difference in the potency of nonredox-type inhibitors (but not of BWA4C) was determined between wild-type 5-LO and the mutant S271A/S663A-5-LO (lacking phosphorylation sites for ERK1/2 and MAPKAPK-2) in HeLa cells. Collectively, our data suggest that compared with Ca^{2+}-mediated 5-LO product formation, enzyme activation involving 5-LO phosphorylation events specifically and strongly alters the susceptibility of 5-LO toward nonredox-type inhibitors in intact cells.

Key words: leukotriene • polymorphonuclear leukocytes • inflammation • MAPK • calcium

5-Lipoxygenase (5-LO), the key enzyme in the biosynthesis of leukotrienes (LTs), metabolizes liberated arachidonic acid (AA) to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and subsequently to LTA_4, which is further converted to LTB_4 or LTC_4, depending on the enzymes present (1). These bioactive lipids have pivotal functions in the pathophysiology of inflammation and allergy (2) but also regulate carcinogenesis and survival of various cell types and tissues (3–6) as well as the metabolism of bone (7, 8). Recently, 5-LO was identified as a major gene contributing to atherosclerosis susceptibility in mice (9).

Enzymatic activity of 5-LO *in vitro* can be modulated by Ca^{2+}, ATP, phosphatidylcholine, and lipid hydroperoxides (for review, see refs. 10 and 11). *In vivo*, a rise of intracellular Ca^{2+} can activate cellular 5-LO, leading to 5-LO redistribution and colocalization with 5-LO-activating

protein (FLAP) and cytosolic phospholipase A_2 ($cPLA_2$) at the nuclear membrane (12). Such an activation of 5-LO can be induced by ligation of specific receptors for naturally occurring soluble and particulate stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), cytokines, immune complexes, and microbes, but also by Ca^{2+}-mobilizing agents such as ionophores, as well as by urate or phosphate crystals (13–18). Besides Ca^{2+}, 5-LO can also be activated via phosphorylation by p38 mitogen-activated protein kinase (MAPK)-regulated MAPK-activated protein kinases (MAPKAPKs, MKs) and/or extracellular signal-regulated kinases (ERKs) (19–24). Particularly in isolated human polymorphonuclear leukocytes (PMNL), 5-LO product formation was induced by cell stress in a p38 MAPK-dependent manner, which occurred without elevation of intracellular Ca^{2+} levels (22).

LTB_4 is one of the most potent chemoattractants and activators of leukocytes and has been shown to be involved in inflammatory processes (2). The cysteinyl-LTs C_4, D_4, and E_4 cause bronchoconstriction and mucus secretion and induce plasma exudation, thus it is generally accepted that cys-LTs play an important role in asthma (for review, see ref 25). 5-LO inhibitors revealed therapeutic value in asthma and some benefit in other inflammatory diseases (11). Based on the recently discovered effects of 5-LO metabolites to promote proliferation and survival of malignant cells, bone resorption and atherosclerosis, 5-LO inhibitors, may also possess potential for prevention and therapy of cancer (4, 26, 27), osteoporosis (28), and vascular diseases (9).

The nonredox-type 5-LO inhibitors, such as ZM230487, are highly potent, orally active inhibitors of LT biosynthesis in several *ex vivo* and *in vitro* models, capable of significantly reducing acute inflammatory responses (29, 30). However, they failed to inhibit more chronic inflammatory processes (31, 32), and it was found that elevated peroxide levels decrease the potency of ZM230487 (33). Here, we show that the potencies of the nonredox-type 5-LO inhibitors ZM230487 and L-739.010 depend on the cell stimulus and the 5-LO activation pathway, respectively. Our data suggest that phosphorylation events specifically alter the susceptibility of 5-LO toward nonredox-type inhibitors in intact cells.

MATERIALS AND METHODS

ZM230487 was a gift from Dr. R. M. McMillan (Zeneca Pharmaceuticals, Macclesfield, UK). BWA4C was a generous gift from Dr. L. G. Garland (Wellcome Research Laboratories, Beckenham, UK). Hyperforin was kindly provided by Dr. S. S. Chatterjee. We obtained these materials from the stated sources: Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Life Technologies, Rockville, MD); fetal calf serum (FCS), bovine insulin, Ca^{2+}-ionophore A23187, arachidonic acid, and bovine glutathione peroxidase-1 (G6137) (Sigma, Deisenhofen, Germany); activated (rat, recombinant) ERK2 isoform (Biomol, Plymouth Meeting, PA); activated GST-MK2 (Upstate Biotechnology, Lake Placid, NY); BAPTA/AM (Calbiochem, Bad Soden, Germany); 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR); [γ-^{32}P]ATP (110 TBq/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, UK); 3-*O*-acetyl-11-keto-boswellic acid (AKBA) (ChromaDex, Laguna Hills, CA); high-performance liquid chromatography (HPLC) solvents (Merck, Darmstadt, Germany); and oligonucleotides (Cyber Gene, Huddinge, Sweden).

Cells and transient transfections

Human PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation at 4000*g* for 20 min at 20°C for preparation of leukocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously. PMNL (5×10^6 cells/ml; purity >96–97%) were finally resuspended in phosphate-buffered saline (PBS), pH 7.4, plus 1 mg/ml glucose (PG buffer) or alternatively in PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated.

HeLa cells were maintained in DMEM, supplemented with 10% FCS, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO_2 incubator. Plasmid DNA (pcDNA3.1–5LO, 1 µg/ml) was transiently transfected into HeLa cells, using the calcium phosphate method (34), cultured for 48 h, and assayed for 5-LO product formation.

Site-directed mutagenesis

The codons for Ser-271 and Ser-663 in the plasmid pcDNA3.1–5LO were mutated using the QuikChange kit from Stratagene (Amsterdam, The Netherlands) as previously described (35). The mutated DNA was confirmed using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech), followed by analysis on an Applied Biosystem (Foster City, CA) PRISM 377 sequencer (performed by KISeq, Core Facilities at Karolinska Institutet).

Determination of 5-lipoxygenase product formation in intact cells

For assays of intact cells in the presence of Ca^{2+}, 5×10^6 freshly isolated PMNL or 1×10^6 HeLa cells were resuspended in 1 ml PGC buffer. When 5-LO product formation was determined in the absence of Ca^{2+}, PMNL were finally resuspended in 1 ml PG buffer, and 1 mM EDTA or 1 mM EDTA plus 30 µM BAPTA/AM were added. After preincubation with the indicated compounds at 37°C, 5-LO product formation was started by addition of the indicated stimuli plus exogenous AA as indicated. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol and 30 µl of 1 N HCl, and 200 ng prostaglandin B_1 and 500 µl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as previously described (36). 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells, which includes LTB_4 and its all-trans isomers 5(*S*),12(*S*)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid [5(*S*),12(*S*)-DiHETE] and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(p)ETE). 5-HETE and 5-HpETE coelute as one major peak; integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected, and oxidation products of LTB_4 were not determined.

Expression and purification of 5-LO from *Escherichia coli*

Expression of 5-LO was performed in *E. coli* JM 109 cells, transfected with pT3–5LO, and purification of 5-LO was performed as described previously (37). In brief, cells were lysed by incubation in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 µg/ml), 1 mM phenylmethysulphonyl fluoride, and lysozyme (500 µg/ml); homogenized by sonication (3×15 s); and centrifuged at 19,000*g* for 15 min. Proteins, including 5-LO, were

precipitated with 50% saturated ammonium sulfate during stirring on ice for 40 min. The precipitate was collected by centrifugation at 16,000*g* for 25 min, and the pellet was resuspended in 20 ml PBS containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 100,000*g* for 70 min at 4°C, the supernatant was applied to an ATP-agarose column (Sigma A2767), and the column was eluted as described previously (38). Purified 5-LO was immediately used for *in vitro* phosphorylation and for *in vitro* activity assay.

In vitro phosphorylation of 5-LO

Purified recombinant 5-LO (10 µg) was incubated with 50 µM oleic acid in the presence of 100 mU activated MK2 and/or 100 mU activated ERK2 in kinase buffer (25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 100 µM ATP). Final volume was 100 µl, and incubation time was 30 min at 30°C. Then, 5-LO was immediately used for 5-LO activity assay.

Activity assay of purified 5-LO

Purified 5-LO (0.5 µg in 5 µl) was added to 1 ml 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP, and 20 µg/ml phosphatidylcholine) containing the test compounds. After 5–10 min at 4°C, 1 mM glutathione (GSH) and 20 mU bovine glutathione peroxidase-1 (GPx-1) were added, the samples were prewarmed at 37°C for 30 s, and the reaction was started by the addition of 40 µM AA with or without 2 mM $CaCl_2$. After incubation at 37°C for 10 min, the reaction was stopped with 1 ml of methanol, and formed 5-LO metabolites were extracted and analyzed by HPLC as described previously.

In-gel kinase assay

PMNL (5×10^7 in 1 ml PGC buffer) were stimulated with 2.5 µM ionophore for the indicated periods at 37°C. Incubations were stopped by addition of the same volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoesis (SDS-PAGE) sample loading buffer (SDS-b) and heated for 6 min at 95°C. Total cell lysates corresponding to 0.5×10^6 PMNL were loaded on 10% SDS-PAGE gels. A Mini Protean system (Bio-Rad, Hercules, CA) was used, and separation gels contained 0.2 mg/ml purified recombinant human 5-LO. After electrophoresis, 5-LO phosphorylation by activated kinases was analyzed by in-gel kinase assay as described previously (20).

Determination of cellular peroxide formation

Measurement of peroxides was conducted using the peroxide-sensitive fluorescence dye DCF-DA. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with DCF-DA (1 µg/ml) for 2 min at 37°C in a thermally controlled fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring. The fluorescence emission at 530 nm was measured after excitation at 480 nm. The mean (±SE) fluorescence data measured 5 min after stimulus addition are expressed as fold increase over unstimulated cells (n=4) (see Table 1).

RESULTS

Inhibition of 5-LO product formation in PMNL by nonredox-type 5-LO inhibitors is stimulus-dependent

Standard test systems for screening and evaluation of 5-LO inhibitors are generally based on leukocytes challenged by the nonphysiological stimulus ionophore A23187. We have tested different types of potent 5-LO inhibitors for suppression of 5-LO product formation in human isolated PMNL, stimulated with either 2.5 μM ionophore A23187 (leading to prominent cellular Ca^{2+} influx) or by cell stress, using sodium arsenite (SA) or hyperosmotic NaCl (300 mM) that activates 5-LO in a p38 MAPK/MK-dependent manner. All incubations were performed in the presence of 1 mM Ca^{2+}, and 40 μM AA was added as exogenous substrate. Furthermore, cells were stimulated with 60 μM exogenous AA alone after chelation of extracellular Ca^{2+} by EDTA, conditions that also activate 5-LO by MAPK-dependent pathways (23, 24, 39).

A striking difference in 5-LO inhibition by the nonredox-type 5-LO inhibitors (ZM230487 and L-739.010) was observed for PMNL stimulated with ionophore compared with cells activated by cell stress. ZM230487 (Fig. 1A) and L-739.010 (Fig. 1B) potently suppressed 5-LO product formation in ionophore-stimulated PMNL (IC_{50}=20 nM and 10 nM, respectively). However, 10- to 100-fold higher concentrations of inhibitors were required for similar 5-LO inhibition when product formation was induced by cell stress (SA, hyperosmotic NaCl). Similarly, stimulation of PMNL with 60 μM AA alone caused a significant shift of the IC_{50} of ZM230487 and L-739.010 to higher values (0.4 and 0.8 μM, respectively). Interestingly, also for PMNL stimulated by the natural ligands fMLP or PAF (in the presence of 40 μM AA, respectively), which activate 5-LO by Ca^{2+} and by phosphorylation events (22, 23), the IC_{50} values of ZM230487 (≈110 and 180 nM, respectively) were also significantly higher as compared with ionophore-stimulated PMNL (data not shown).

In contrast, BWA4C, an iron ligand-type 5-LO inhibitor, showed no such differences (Fig. 1C), and the IC_{50} values (≈70–90 nM) were comparable in cells stimulated with either ionophore or cell stress (or 60 μM AA alone). Also the potent 5-LO inhibitor hyperforin, acting in a noncompetitive fashion (40), as well as the novel-type 5-LO inhibitor AKBA (41) suppressed 5-LO product synthesis in ionophore- and cell stress-stimulated PMNL with equal potencies (data not shown).

The role of Ca^{2+} on 5-LO inhibition by nonredox-type inhibitors

We addressed the role of Ca^{2+} regarding the sensitivity of 5-LO toward nonredox-type inhibitors. Extracellular Ca^{2+} was removed by 1 mM EDTA, and depletion of extra- and intracellular Ca^{2+} was achieved by chelation with 1 mM EDTA plus 30 μM BAPTA/AM. 5-LO product formation was almost not detectable in Ca^{2+}-depleted PMNL, which had been stimulated with ionophore plus AA (3.7±1.7 ng/10^6 cells), whereas prominent product formation was obtained from PMNL exposed to SA plus AA (31.1±5.8 ng/10^6 cells; compare with ref 22). As shown in Figure 2A, removal of Ca^{2+} further impaired the efficacy of ZM230487 (and L-739.010, data not shown) in SA-stimulated PMNL about fivefold, compared with cells receiving Ca^{2+}. Also, inhibition of 5-LO product formation induced by 300 mM NaCl required slightly higher inhibitor concentrations in the absence of Ca^{2+} (data not shown). No such clear differences were seen for BWA4C (Fig.

2B). Thus, the presence or absence of Ca^{2+} hardly affected the efficacy of the 5-LO iron ligand-type inhibitor.

To check whether elevated concentrations of Ca^{2+}, generated by cell stimulation with ionophore, are sufficient for potent inhibition of 5-LO by the nonredox inhibitors, 5-LO inhibition was determined in PMNL stimulated with SA added 3 min before ionophore to ensure high intracellular Ca^{2+} concentrations. As shown in Figure 3, the IC_{50} value of ZM230487 in such incubations was 200 nM, consistent with the IC_{50} value obtained from cells that received only SA. Thus, elevated Ca^{2+} is apparently not the reason for the high efficacy of ZM230487 when PMNL are stimulated with ionophore.

The effects of Ca^{2+} on 5-LO inhibition *in vitro* were investigated using purified human recombinant 5-LO. For efficient inhibition of 5-LO, low hydroperoxide levels are important (33), accordingly, GSH (1 mM) and GPx (20 mU) were included in the assay. ZM230487 was equipotent in the presence and in absence of Ca^{2+} (Fig. 4), with IC_{50} values of 60 and 65 nM, respectively, which are in the range of those obtained for intact PMNL stimulated with ionophore.

Kinetic analysis of 5-LO product formation and 5-LO kinase activation

As reported previously, the kinetics of p38 MAPK/5-LO kinase activation correlates to 5-LO product formation in PMNL stimulated with SA (compare with ref 22). Notably, exposure of PMNL to ionophore also results in the activation of 5-LO kinases (20, 42), which could activate 5-LO by phosphorylation, similar to SA. However, when the kinetics of 5-LO product formation and 5-LO kinase activation were determined in PMNL stimulated with ionophore, a significant discrepancy was apparent. 5-LO product formation was half-maximal after 30 s, and after 1 min, ~80% of maximal 5-LO product formation was achieved (Fig. 5, upper panel). However, significant activation of 5-LO kinases (bands at 40 [MK3], 47, and 55 kD [MK2]) occurred first after 1 min, and maximal kinase activity was detected after ~2 min (Fig. 5, lower panel). Thus, most of the 5-LO products have been formed within a period in which 5-LO kinases were not markedly active and 5-LO was not phosphorylated, supporting previous findings that in ionophore-stimulated PMNL, 5-LO kinase activity does not primarily contribute to 5-LO activation (22–24, 43). Consequently, 5-LO phosphorylation events should not counteract inhibition of ionophore-induced 5-LO activity by nonredox inhibitors.

The role of peroxides on 5-LO inhibition by nonredox-type inhibitors under cell stress conditions

An increased peroxide tone was found to impair the efficacy of ZM230487 in ionophore-stimulated PMNL approximately six- to eightfold (33). Therefore, it appeared possible that cell stress could impair the efficacy of ZM230487 by increasing the cellular peroxide level. Thus, we determined the cellular formation of peroxides in PMNL by using the peroxide-sensitive fluoresence dye DCF-DA. Stimulation of PMNL with 10 µM SA or with 0.3 M NaCl could not significantly enhance the release of peroxides compared with unstimulated PMNL, whereas ionophore or phorbol-12-myristate-13-acetate (PMA) (positive controls) strongly augmented the peroxide generation (Table 1). Moreover, pretreatment of PMNL for 20 min with 5 mM n-acetylcysteine (NAC) before cell stress in order to scavenge peroxides caused no change in 5-LO

inhibition (Table 2). Thus, an elevated peroxide tone is apparently not the reason for impaired efficacy of nonredox 5-LO inhibitors in cell stress-treated PMNL.

Deletion of putative 5-LO phosphorylation sites increases the efficacy of nonredox-type inhibitors

Previously, we showed that Ser-271 and Ser-663 are phosphorylation sites in 5-LO for MKs and ERKs, respectively, and mutation of these residues reduces the capacity of the mutated 5-LO enzymes for 5-LO product formation in HeLa cells stimulated with exogenous AA (23, 24). Here, we found significant differences between wild-type (wt) and S271A/S663A-5-LO regarding the susceptibility toward nonredox-type inhibitors but not toward the iron ligand-type 5-LO inhibitor BWA4C. After stimulation with 40 µM AA, ZM230487 (Fig. 6A) and L-739.010 (Fig. 6B) suppressed 5-LO product formation with IC_{50} values of 630 nM and 510 nM, respectively, in HeLa cells expressing wt-5-LO (where 5-LO enzyme phosphorylation may occur). Mutation of Ser-271 and Ser-663 to alanine, resulted in a protein that gave low product formation upon AA stimulation (compare with ref 23). Interestingly, ~6- to 10-fold lower concentrations of ZM230487 and L-739.010 were required for inhibition of this nonphosphorylatable 5-LO mutant, with IC_{50} values of 70 nM for ZM230487 and 90 nM for L-739.010. However, for BWA4C, the IC_{50} values were practically the same for both wt-5-LO and the mutated enzyme (IC_{50}=45 and 50 nM, respectively) (Fig. 6C).

Effect of *in vitro* phosphorylation of 5-LO on the sensitivity toward ZM230487

We examined the susceptibility of purified phosphorylated and nonphosphorylated 5-LO toward ZM230487 *in vitro*. Human recombinant 5-LO was purified and phosphorylated by MK2 and/or ERK2 in the presence of 50 µM oleic acid (which promotes 5-LO phosphorylation). Then, 5-LO product formation was determined in the presence of 1 mM GSH and 20 mU GPx (in order to remove hydroperoxides from the incubation mixture).

In agreement with previous findings (23, 24), enzyme phosphorylation by MK2 or ERK2 (or both) did not significantly alter the enzymatic activity of 5-LO *in vitro* (data not shown). As depicted in Figure 7, there was also no significant shift of the IC_{50} value of ZM230487 for phosphorylated 5-LO compared with unphosphorylated enzyme. Therefore, phosphate incorporation alone does not protect crude 5-LO catalytic activity against ZM230487 in cell-free systems, suggesting that the intact cellular environment or putative cellular constituents might be operative.

DISCUSSION

Here, we provide evidence that the efficacy of nonredox-type 5-LO inhibitors in isolated human PMNL depends on the 5-LO activation pathway. Thus, ~10- to 100-fold higher concentrations of ZM230487 or L-739.010 are required for cell stress-induced 5-LO product formation involving 5-LO kinase pathways, compared with 5-LO product synthesis induced by ionophore, leading to a prominent cellular Ca^{2+} influx. In contrast, no such discrepancies were observed for the iron ligand 5-LO inhibitor BWA4C or the novel-type 5-LO inhibitors hyperforin and AKBA. Studies using a mutated 5-LO protein lacking potential phosphorylation sites for 5-LO kinases indicate that enzyme phosphorylation might impair the efficacy of nonredox-type inhibitors (but not of BWA4C) in intact cells. Our data suggest that compared with Ca^{2+}-mediated 5-LO activation,

cell stress specifically alters the susceptibility of 5-LO in intact cells toward nonredox-type inhibitors, putatively involving enzyme phosphorylation events.

The search for potent orally active and specific 5-LO inhibitors led to the development of nonredox-type 5-LO inhibitors such as ZD2138 and its ethyl analog ZM230487, which possess high efficacy in several *in vitro* and *ex vivo* systems (29, 30, 44). However, significantly higher doses were required for the same effects in functional *in vivo* assays, and ZD2138 failed to strongly suppress LT formation at sites of chronic inflammation (see ref 33 and references therein). Moreover, 5-LO inhibition by nonredox-type inhibitors was strikingly different in (ionophore-stimulated) intact cells compared with purifed 5-LO or crude enzyme in cell homogenates (30, 33, 45). Detailed studies showed that ZM230487 and L-739.010 require GPx activity for efficient 5-LO inhibition (33). No such redox-dependent effects were observed with other types of 5-LO inhibitors (BWA4C or AKBA).

Activation of cellular 5-LO may occur by at least two different pathways (which may act in conjunction): either by elevation of intracellular Ca^{2+} (10) or by Ca^{2+}-independent pathways involving ERK- and/or p38 MAPK-mediated 5-LO phosphorylation (20–24). p38 MAPK is activated particularly in response to cell stress as well as after cell exposure to chemotactic factors, lipopolysaccharide or inflammatory cytokines, and is considered a pivotal signaling kinase in inflammation (46, 47). Also, AA activates p38 MAPK as well as ERKs in PMNL, and many priming agents (PMA, GM-CSF) and natural ligands (fMLP, PAF) that enhance or induce LT synthesis stimulate the activation of ERKs (see ref 23 and references therein). Thus, activation of 5-LO by these kinases might be of relevance for LT synthesis *in vivo* under inflammatory conditions, associated with cell stress (oxidative and osmotic stress, heat shock) or with the release of inflammatory cytokines (TNFα, IL-1) or chemotactic factors (fMLP, PAF, or C5a). Consequently, such phosphorylation-dependent 5-LO catalysis may be less susceptible toward nonredox-type inhibitors, which might explain the reported loss of efficacy of ZM230487 in chronic inflammatory diseases (see ref 33 and references therein).

5-LO product formation induced by the nonphysiological ionophore is mediated by the massive influx of Ca^{2+} that rapidly activates the enzyme. It appeared possible that elevated Ca^{2+} is required to give 5-LO a conformation that will lead to the efficient noncompetitive inhibition by nonredox-type inhibitors. However, elevation of intracellular Ca^{2+} by coaddition of ionophore could not improve the efficacy of ZM230487 in SA-treated PMNL (Fig. 3). Moreover, under reducing conditions, purified 5-LO showed the same sensitivity toward ZM230487 *in vitro* in the presence or absence of 1 mM Ca^{2+} (Fig. 4), suggesting that solely the presence of Ca^{2+} cannot confer 5-LO highly susceptible to ZM230487.

Similar as cell stress, treatment of PMNL with ionophore leads to a strong activation of MKs which can phosphorylate 5-LO (20). However, ionophore-induced MK activation succeeds the rapid Ca^{2+}-mediated 5-LO product formation, indicating that phosphorylation events are not primarily determinants for ionophore-induced 5-LO activation, compare also (20, 22). Thus, under these conditions the nonredox-type inhibitors interfere with Ca^{2+}-stimulated catalysis of non-phosphorylated 5-LO. On the other hand, when 5-LO kinases were activated 3 min before addition of ionophore, the effect of ZM230487 was reduced (Fig. 3), implying that SA-induced phosphorylation events may impair the susceptibility of 5-LO against ZM230487. For fMLP- or PAF-induced 5-LO catalysis, both Ca^{2+} and phosphorylation seem to contribute (22, 23), and

indeed we found reduced efficacy of the nonredox inhibitors under these conditions, similarly as for cell stress-induced 5-LO activation.

Hydroperoxides have been shown to impair the potency of ZM230487 (33). Therefore, elevation of the cellular redox tone could be a simple explanation for the impaired efficacy of nonredox-type inhibitors in PMNL subjected to cell stress. However, exposure of PMNL to SA or hyperosmotic NaCl caused no significant generation of peroxides (Table 1), and preincubation of PMNL with NAC, a reducing agent that quenches peroxides, did not restore the high potency of ZM230487 (Table 2). In this respect, it was found that hyperosmotic NaCl even suppresses the agonist-induced release of superoxide from PMNL (48). Therefore, other mechanisms, in addition to elevated hydroperoxide tone observed in our previous study (33), may mediate low efficacy of nonredox 5-LO inhibitors under cell stress conditions. It is possible that increased peroxide tone leads to 5-LO kinase activation (compare with ref 21), thus impairing the effect of ZM230487.

Our transfection experiments with wt-5-LO and the mutated S271A/S663A-5-LO further support the hypothesis that in the intact cell, incorporation of phosphate into 5-LO impairs the efficacy of nonredox-type inhibitors. Thus, in HeLa cells, the nonphosphorylatable S271A/S663A-5-LO mutant was much more sensitive toward the nonredox-type inhibitors as compared with the phosphorylatable wt-5-LO. However, *in vitro* studies using purified enzyme in a cell-free system demonstrate that solely phosphate incorporation into 5-LO by MK2 and/or ERK is not sufficient to alter the sensitivity of 5-LO toward ZM230487 (Fig. 7). Therefore, the cellular integrity or cellular components are needed to mediate effects on 5-LO related to phosphorylation. Along these lines, we found in previous studies that *in vitro,* product formation of phosphorylated and unphosphorylated 5-LO was quantitatively the same (23, 24), and there was also no difference in the specific catalytic activity between the crude wt and mutated enzymes in which phosphorylation sites (S271, S663) have been deleted. Thus, *in vitro,* phosphorylation does not affect enzymatic properties of 5-LO. However, deletion of phosphorylation sites caused significant loss of 5-LO product formation in intact HeLa cells stimulated with AA (23, 24), supporting the finding that phosphorylation of 5-LO affects catalytic properties of 5-LO in the intact cell only. It appears that cellular conditions or components are operative and might act in conjunction with the phosphorylated form of 5-LO to stimulate product formation. Indeed, 5-LO interacts with several cellular proteins (49, 50), and such interactions might be affected by the phosphorylation status of 5-LO. Consequently, it is not surprising that phosphorylation events alter 5-LO inhibition by ZM230487 and L-739.010 also solely in intact cells, but not *in vitro*.

The precise mode of action of ZM230487 and L-739.010 is unclear. Initially, it was suggested that these compounds act in a competitive manner by binding at a fatty acid substrate binding pocket distal to the active site of the enzyme. However, in cell-free systems under reducing conditions or in intact cells, ZM230487 acts a noncompetitive inhibitor (33). Thus, ZM230487 may bind to a regulatory domain, probably at a fatty acid (hydroperoxide) binding site, and this interference is seemingly essential for the potent (noncompetitive) 5-LO inhibition. Phosphorylation of 5-LO by MK2 and by ERK is promoted by unsaturated fatty acids, such as AA (23, 24). It is conceivable that phosphate incorporation in close proximity to such a regulatory fatty acid (hydroperoxide) binding site allows the enzyme to interfere with a cellular protein as outlined previously. Such protein/protein interactions could hamper the interference of ZM230487 (and L-739.010) with 5-LO by facilitating the binding or formation of activating fatty acid hydroperoxides, which in turn compete with nonredox-type inhibitors at this regulatory

site. Such a model would also be compatible with the fact that BWA4C, presumably interacting at a distant binding site, does not discriminate between inhibition of wt-5-LO and of S271A/S663A-5-LO (Fig. 6) and that its efficacy in PMNL is equal for 5-LO activated by phosphorylation using cell stress as the stimulus as for Ca^{2+}-activated 5-LO using ionophore as the agonist (Fig. 1).

Collectively, our data provide evidence that 5-LO phosphorylation leads to reduced efficacy of nonredox-type 5-LO inhibitors in intact cells. These findings could be of relevance for the development of 5-LO inhibitors, in particular for the establishment of alternative test systems suitable for the screening of LT synthesis inhibitors. An increased 5-LO phosphorylation status, associated with inflammation, malignant cell proliferation, increased bone resorption, and macrophage activation at atherosclerotic sites, should be taken into account for pharmacological targeting of the 5-LO pathway in order to control inflammatory and allergic reactions, cancer, osteoporosis, and vascular diseases.

ACKNOWLEDGMENTS

We thank Astrid Neuß and Agneta Nordberg for expert technical assistance. This study was supported by grants from the Fonds der Chemischen Industrie, the EU (LEUCHRON, QLRT-2000–01521), the Swedish Medical Research Council (03X-217), and the Deutsche Pharmazeutische Gesellschaft.

REFERENCES

1. Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C. A., Serhan, C. N. (1987) Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* **237,** 1171–1176

2. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220,** 568–575

3. Ohd, J. F., Wikstrom, K., Sjolander, A. (2000) Leukotrienes induce cell-survival signaling in intestinal epithelial cells. *Gastroenterology* **119,** 1007–1018

4. Avis, I., Hong, S. H., Martinez, A., Moody, T., Choi, Y. H., Trepel, J., Das, R., Jett, M., Mulshine, J. L. (2001) Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. *FASEB J.* **15,** 2007–2009

5. Romano, M., Catalano, A., Nutini, M., D'Urbano, E., Crescenzi, C., Claria, J., Libner, R., Davi, G., Procopio, A. (2001) 5-lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. *FASEB J.* **15,** 2326–2336

6. Gupta, S., Srivastava, M., Ahmad, N., Sakamoto, K., Bostwick, D. G., Mukhtar, H. (2001) Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer* **91,** 737–743

7. Chen, X. S., Sheller, J. R., Johnson, E. N., Funk, C. D. (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature* **372,** 179–182

8. Garcia, C., Boyce, B. F., Gilles, J., Dallas, M., Qiao, M., Mundy, G. R., Bonewald, L. F. (1996) Leukotriene B4 stimulates osteoclastic bone resorption both in vitro and in vivo. *J. Bone Miner. Res.* **11,** 1619–1627

9. Mehrabian, M., Allayee, H., Wong, J., Shi, W., Wang, X. P., Shaposhnik, Z., Funk, C. D., Lusis, A. J., Shih, W. (2002) Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ. Res.* **91,** 120–126

10. Rådmark, O. (2000) The molecular biology and regulation of 5-lipoxygenase. *Am. J. Respir. Crit. Care Med.* **161,** S11–S15

11. Steinhilber, D. (1999) 5-lipoxygenase: a target for antiinflammatory drugs revisited. *Curr. Med. Chem.* **6,** 69–83

12. Peters-Golden, M., Brock, T. G. (2000) Intracellular compartmentalization of leukotriene biosynthesis. *Am. J. Respir. Crit. Care Med.* **161,** S36–S40

13. Borgeat, P., Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA* **76,** 2148–2152

14. Chilton, F. H., O'Flaherty, J. T., Walsh, C. E., Thomas, M. J., Wykle, R. L., DeChatelet, L. R., Waite, B. M. (1982) Platelet activating factor. Stimulation of the lipoxygenase pathway in polymorphonuclear leukocytes by 1-O-alkyl-2-O-acetyl-sn-glycero-3- phosphocholine. *J. Biol. Chem.* **257,** 5402–5407

15. Claesson, H. E., Lundberg, U., Malmsten, C. (1981) Serum-coated zymosan stimulates the synthesis of leukotriene B4 in human polymorphonuclear leukocytes. Inhibition by cyclic AMP. *Biochem. Biophys. Res. Commun.* **99,** 1230–1237

16. Clancy, R. M., Dahinden, C. A., Hugli, T. E. (1983) Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by N-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. *Proc. Natl. Acad. Sci. USA* **80,** 7200–7204

17. Poubelle, P. E., De Medicis, R., Naccache, P. H. (1987) Monosodium urate and calcium pyrophosphate crystals differentially activate the excitation-response coupling sequence of human neutrophils. *Biochem. Biophys. Res. Commun.* **149,** 649–657

18. Serhan, C. N., Lundberg, U., Weissmann, G., Samuelsson, B. (1984) Formation of leukotrienes and hydroxy acids by human neutrophils and platelets exposed to monosodium urate. *Prostaglandins* **27,** 563–581

19. Lepley, R. A., Fitzpatrick, F. A. (1996) Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells. *Arch. Biochem. Biophys.* **331,** 141–144

20. Werz, O., Klemm, J., Samuelsson, B., Rådmark, O. (2000) 5-Lipoxygenase is phosphorylated by p38 kinase dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. USA* **97,** 5261–5266

21. Werz, O., Klemm, J., Rådmark, O., Samuelsson, B. (2001) p38 MAP kinase mediates stress-induced leukotriene synthesis in a human B-lymphocyte cell line. *J Leuk Biol* **70,** 830–838

22. Werz, O., Buerkert, E., Samuelsson, B., Rådmark, O., Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood* **99**, 1044–1052

23. Werz, O., Buerkert, E., Fischer, L., Szellas, D., Dishart, D., Rådmark, O., Samuelsson, B., Steinhilber, D. (2002) Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.* **16,** 1441–1443

24. Werz, O., Szellas, D., Steinhilber, D., Rådmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPKAP kinase-2. *J. Biol. Chem.* **277,** 14793–14800

25. Claesson, H. E., Dahlen, S. E. (1999) Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.* **245,** 205–227

26. Ghosh, J., Myers, C. E. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. USA* **95,** 13182–13187

27. Ding, X. Z., Iversen, P., Cluck, M. W., Knezetic, J. A., Adrian, T. E. (1999) Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.* **261,** 218–223

28. Alanko, J., Sievi, E., Lahteenmaki, T., Mucha, I., Vapaatalo, H., Parantainen, J. (1998) Catechol estrogens as inhibitors of leukotriene synthesis. *Biochem. Pharmacol.* **55,** 101–104

29. McMillan, R. M., Walker, E. R. H. (1992) Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.* **13,** 323–330

30. Smith, W. G., Shaffer, A. F., Currie, J. L., Thompson, J. M., Kim, S., Rao, T., Isakson, P. C. (1995) Characterization of 5-lipoxygenase inhibitors in biochemical and functional in vivo assays. *J. Pharmacol. Exp. Ther.* **275,** 1332–1338

31. Nasser, S. M., Bell, G. S., Hawksworth, R. J., Spruce, K. E., MacMillan, R., Williams, A. J., Lee, T. H., Arm, J. P. (1994) Effect of the 5-lipoxygenase inhibitor ZD2138 on allergen-induced early and late asthmatic responses. *Thorax* **49,** 743–748

32. Turner, C. R., Smith, W. B., Andresen, C. J., Eggler, J. F., Watson, J. W. (1996) The effect of 5-lipoxygenase inhibition on Ascaris antigen (Ag)-induced responses in atopic monkeys. *Inflamm. Res.* **45,** 42–49

33. Werz, O., Szellas, D., Henseler, M., Steinhilber, D. (1998) Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.* **54,** 445–451

34. Chen, C. A., Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6,** 632–638

35. Hammarberg, T., Provost, P., Persson, B., Rådmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J. Biol. Chem.* **275,** 38787–38793

36. Werz, O., Steinhilber, D. (1996) Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells - The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. *Eur. J. Biochem.* **242,** 90–97

37. Hammarberg, T., Zhang, Y. Y., Lind, B., Rådmark, O., Samuelsson, B. (1995) Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. *Eur. J. Biochem.* **230,** 401–407

38. Brungs, M., Rådmark, O., Samuelsson, B., Steinhilber, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. USA* **92,** 107–111

39. Bürkert, E., Szellas, D., Rådmark, O., Steinhilber, D., Werz, O. (2003) Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. *J. Leuk. Biol.* **73,** 191-200

40. Albert, D., Zündorf, I., Dingermann, T., Müller, W. E., Steinhilber, D., Werz, O. (2002) Hyperforin is a dual inhibitor of 5-lipoxygenase and cyclooxygenase-1. *Biochem. Pharmacol.* **64,** 1767–1775

41. Safayhi, H., Mack, T., Sabieraj, J., Anazodo, M. I., Subramanian, L. R., Ammon, H. P. (1992) Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. *J. Pharmacol. Exp. Ther.* **261,** 1143–1146

42. Werz, O., Klemm, J., Samuelsson, B., Rådmark, O. (2001) Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood* **97,** 2487–2495

43. Bürkert, E., Radmark, O., Samuelsson, B., Steinhilber, D., Werz, O. (2002) Hypertonicity suppresses ionophore-induced product formation and translocation of 5-lipoxygenase in human leukocytes. *J Leuk Biol* **71,** 477–486

44. Crawley, G. C., Dowell, R. I., Edwards, P. N., Foster, S. J., McMillan, R. M., Walker, E. R., Waterson, D., Bird, T. G., Bruneau, P., Giroaeau, J. M. (1992) Methoxytetrahydropyrans. A new series of selective and orally potent 5-lipoxygenase inhibitors. *J. Med. Chem.* **35,** 2600–2609

45. Werz, O., Schneider, N., Brungs, M., Sailer, E. R., Safayhi, H., Ammon, H. P. T., Steinhilber, D. (1997) A test system for leukotriene synthesis inhibitors based on the in-vitro differentiation of the human leukemic cell lines HL-60 and Mono Mac 6. *Naunyn Schmied Arch Pharmacol* **356,** 441–445

46. Kyriakis, J. M., Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* **18,** 567–577

47. Herlaar, E., Brown, Z. (1999) p38 MAPK signalling cascades in inflammatory disease. *Mol. Med. Today* **5,** 439–447

48. Junger, W. G., Hoyt, D. B., Davis, R. E., Herdon-Remelius, C., Namiki, S., Junger, H., Loomis, W., Altman, A. (1998) Hypertonicity regulates the function of human neutrophils by modulating chemoattractant receptor signaling and activating mitogen-activated protein kinase p38. *J. Clin. Invest.* **101,** 2768–2779

49. Lepley, R. A., Fitzpatrick, F. A. (1994) 5-lipoxygenase contains a functional Src homology 3- binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins. *J. Biol. Chem.* **269,** 24163–24168

50. Provost, P., Samuelsson, B., Rådmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. USA* **96,** 1881–1885

Received December 6, 2002; accepted January 7, 2003.

Table 1

Generation of peroxides in PMNL

Stimulus	DCF-DA fluorescence (fold increase over untreated cells)
SA (10 µM)	1.2 ± 0.1
NaCl (0.3 M)	1.1 ± 0.3
Ionophore (2.5 µM)	7.3 ± 0.4
PMA (100 nM)	12.1 ± 1.4

Table 2

Effects of N-acetylcysteine (NAC) on 5-LO inhibition by ZM230487

	IC_{50} value (ZM230487) (μM)	
Stimulus	–NAC	+NAC
SA (10 μM)	0.35	0.38
NaCl (0.3 M)	0.6	0.7

Fig. 1

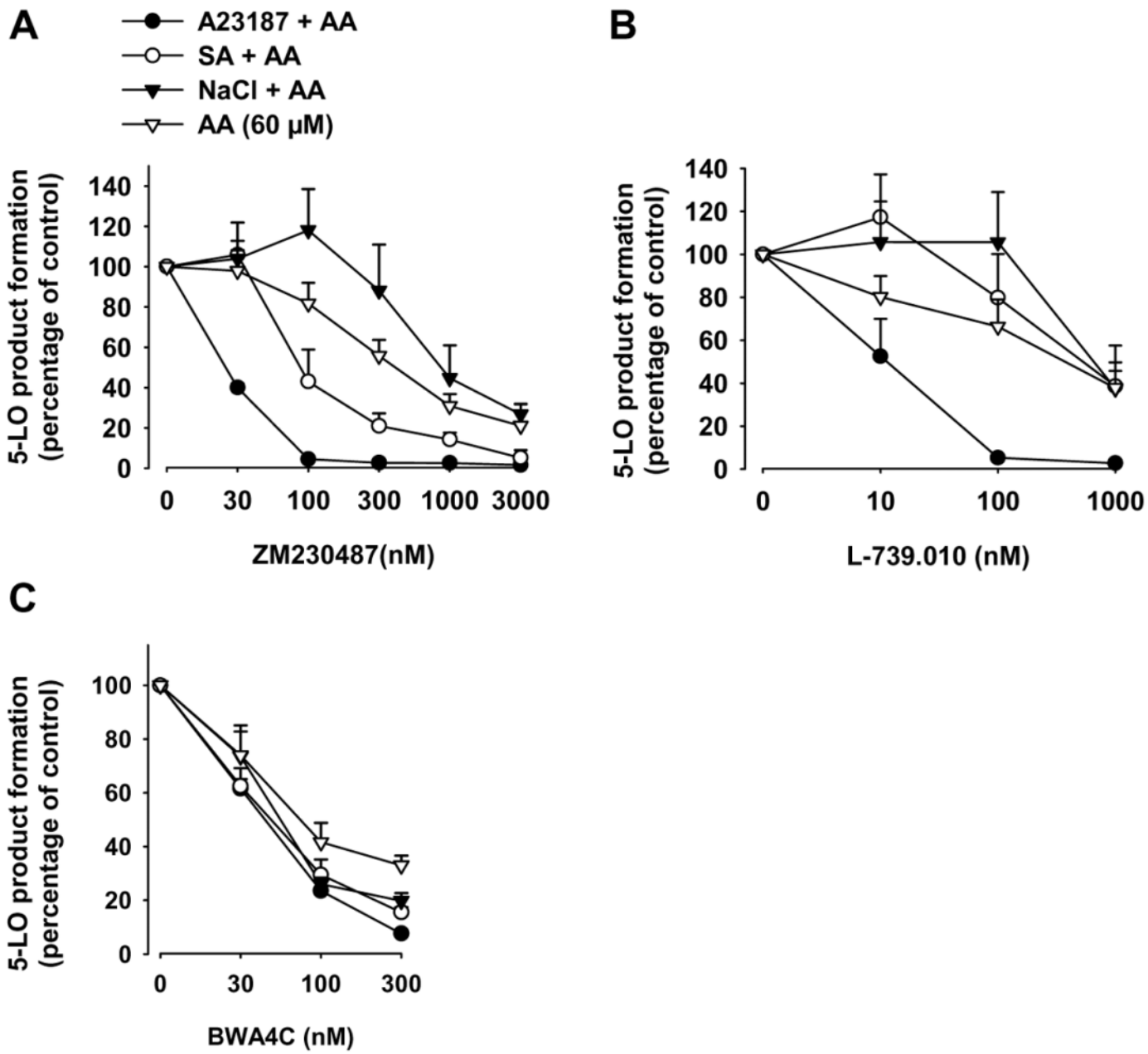


Figure 1. Efficacy of nonredox-type 5-LO inhibitors in PMNL is stimulus-dependent. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with ZM230487 (**A**), L-739.010 (**B**), or BWA4C (**C**) at the indicated concentrations for 15 min at 37°C. SA (10 µM) and NaCl (300 mM) were added 3 min before addition of 40 µM AA; ionophore A23187 (2.5 µM) was added together with 40 µM AA. Alternatively, PMNL resuspended in PG buffer containing 1 mM EDTA were stimulated with 60 µM AA alone. After 10 min at 37°C, 5-LO products were determined by HPLC. Results are given as mean ±SE, *n*=3–4. The control values (100%) in the absence of inhibitors were 107.2 ± 6.5, 37.6 ± 5.6, 48.8 ± 7.6, and 48.2 ± 9.8 ng/10^6 cells, stimulated with ionophore, SA, and NaCl (each in the presence of 40 µM AA) and 60 µM AA alone, respectively.

Fig. 2

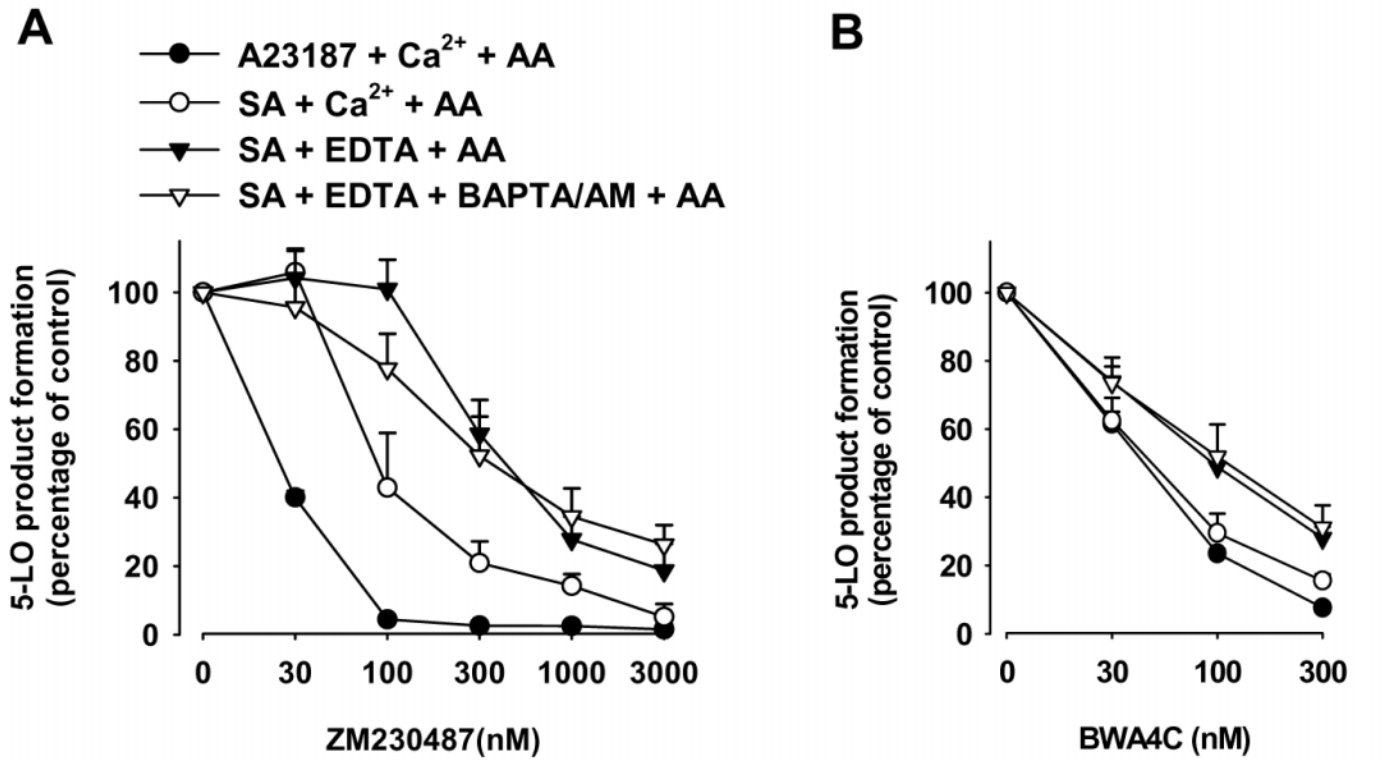


Figure 2. Ca^{2+} depletion impairs the efficacy of nonredox-type 5-LO inhibitors in SA-activated PMNL. $CaCl_2$ (1 mM), EDTA (1 mM), and BAPTA/AM (30 µM) were added to 5×10^6 freshly isolated PMNL in PG buffer as indicated, and ZM230487 (**A**) or BWA4C (**B**) were added at the indicated concentrations. After 15 min at 37°C, SA (10 µM) was added 3 min before addition of 40 µM AA. Ionophore A23187 (2.5 µM) was added together with AA. After another 10 min, 5-LO products were determined. Results are given as mean ±SE, *n*=3. The control values (100%) in the absence of inhibitors for cells stimulated with SA were 37.6 ± 5.6, 40.2 ± 2.1, and 31.1 ± 5.8 ng/10^6 cells in the presence of Ca^{2+}, EDTA, and EDTA plus BAPTA/AM, respectively.

Fig. 3

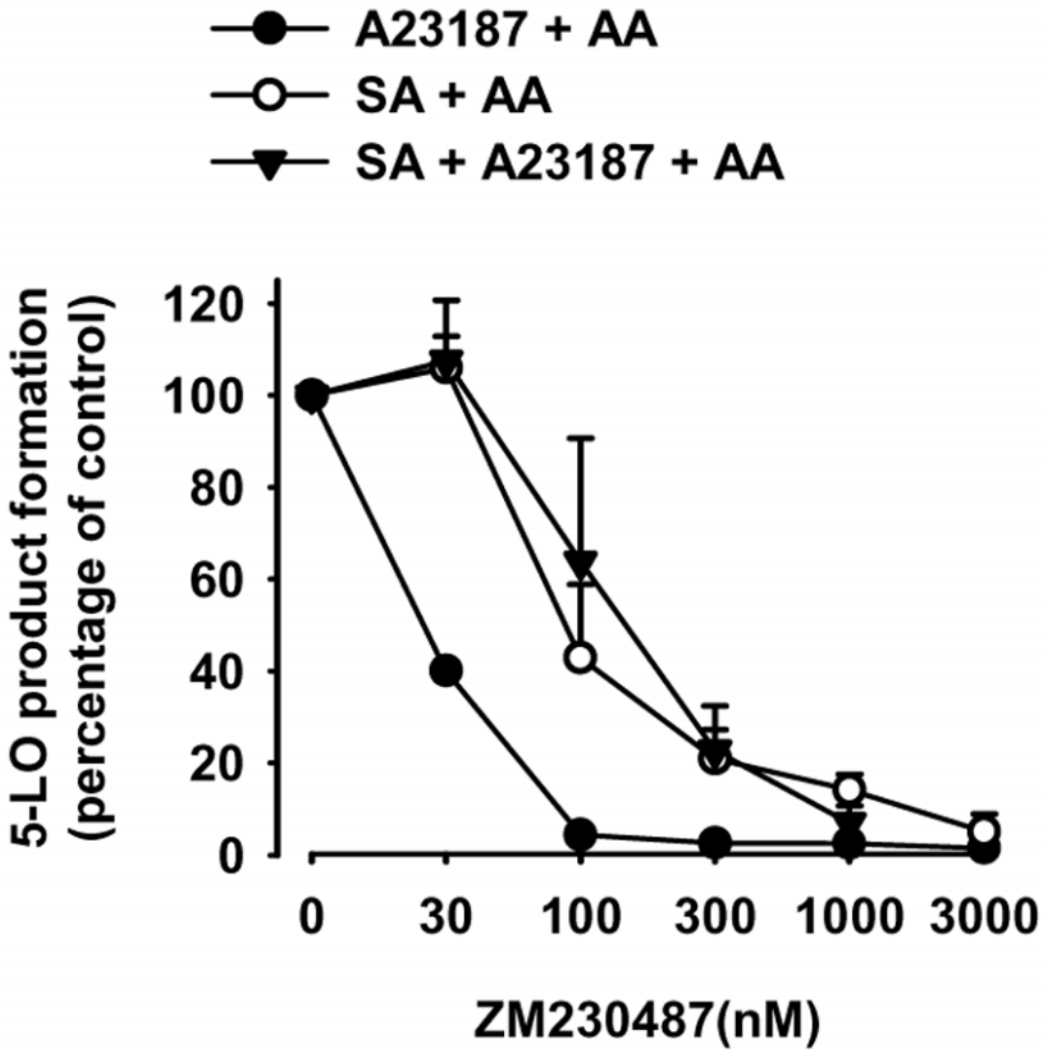


Figure 3. SA counteracts efficient 5-LO inhibition by ZM230487 in ionophore-stimulated PMNL. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with ZM230487 at the indicated concentrations for 15 min at 37°C. Cells were exposed to SA (10 µM) 3 min before addition of 40 µM AA as indicated; 2.5 µM ionophore A23187 was added together with AA. After 10 min at 37°C, 5-LO products were determined by HPLC. Results are given as mean ±SE, *n*=3–4.

Fig. 4

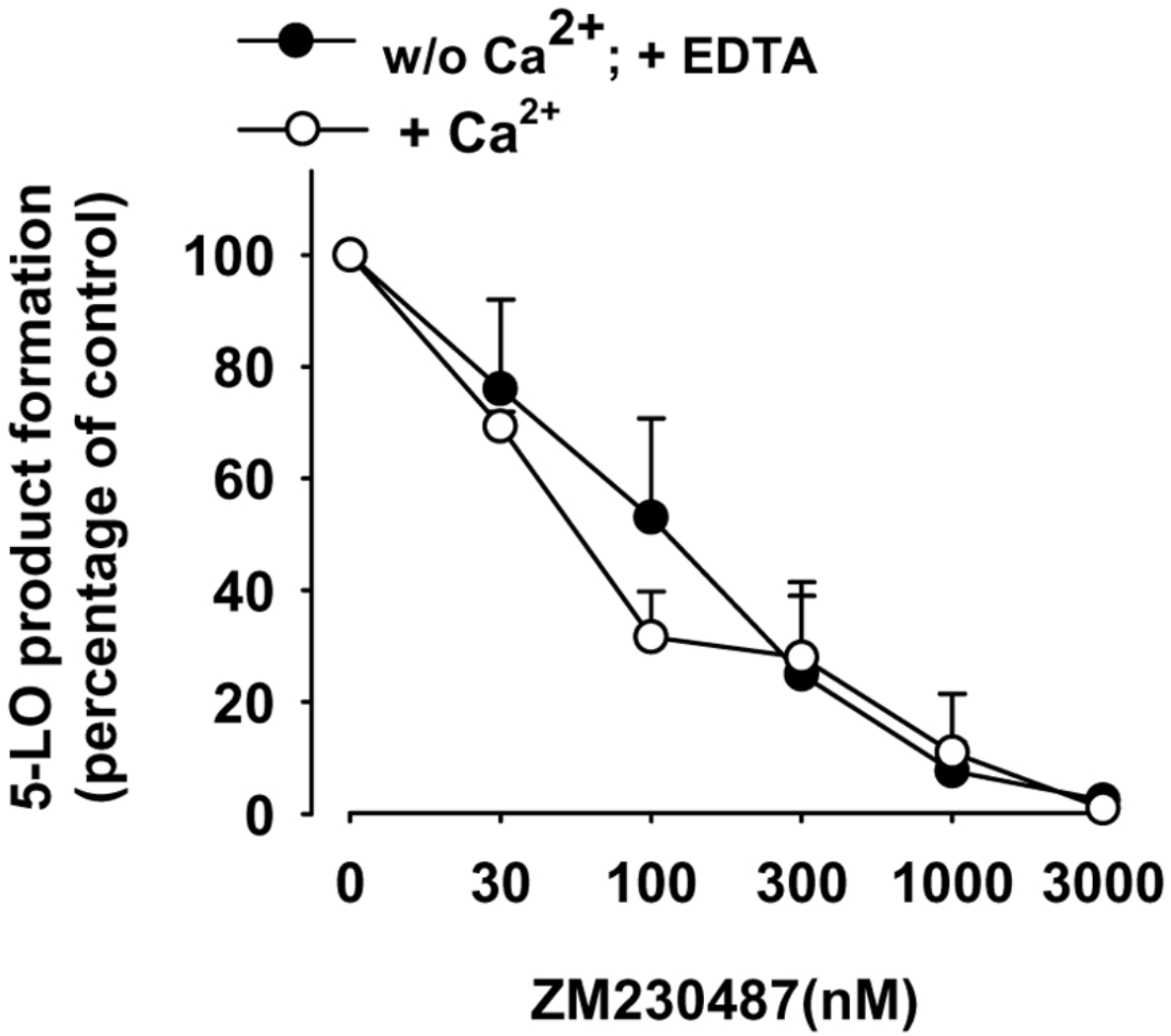


Figure 4. Ca^{2+} has no influence on inhibition of purified 5-LO by ZM230487. Human recombinant 5-LO was expressed in *Escherichia coli* and purified as described in Materials and Methods. Purified 5-LO was added to a 5-LO reaction mix containing the indicated amounts of inhibitor. After 5–10 min at 4°C, 1 mM GSH plus 20 mU GPx were added, the samples were prewarmed at 37°C for 30 sec, and the 5-LO reaction was started by addition of 40 µM AA in the absence or presence of $CaCl_2$ (2 mM). After 10 min at 37°C, 5-LO product formation was determined by HPLC as described. Results are given as mean ±SE, *n*=3.

Fig. 5

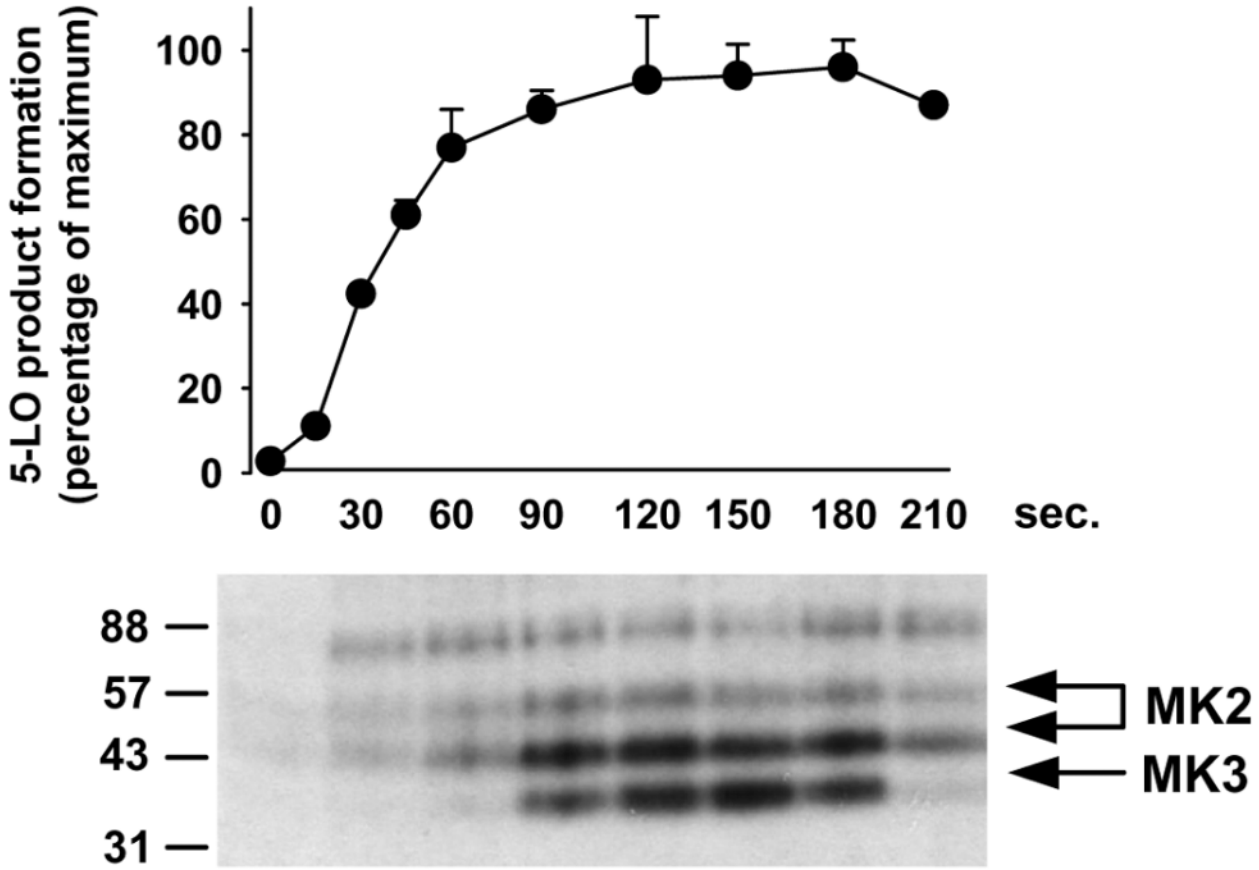


Figure 5. Kinetics of 5-LO product formation and 5-LO kinase activation. Freshly isolated PMNL (5×10^7 in 10 ml PGC buffer) were stimulated with 2.5 µM ionophore plus 40 µM AA. After the indicated times at 37°C, aliquots of these incubations were terminated by addition of the same volume of ice-cold methanol or SDS-b to stop 5-LO product formation or 5-LO kinase activation, respectively. 5-LO products were determined by HPLC. Values are given as mean ±SE, *n*=3. 5-LO kinase activation was determined by in-gel kinase assay, using 0.2 mg/ml 5-LO as substrate as described. Arrows indicate the positions of MK2 and MK3.

Fig. 6

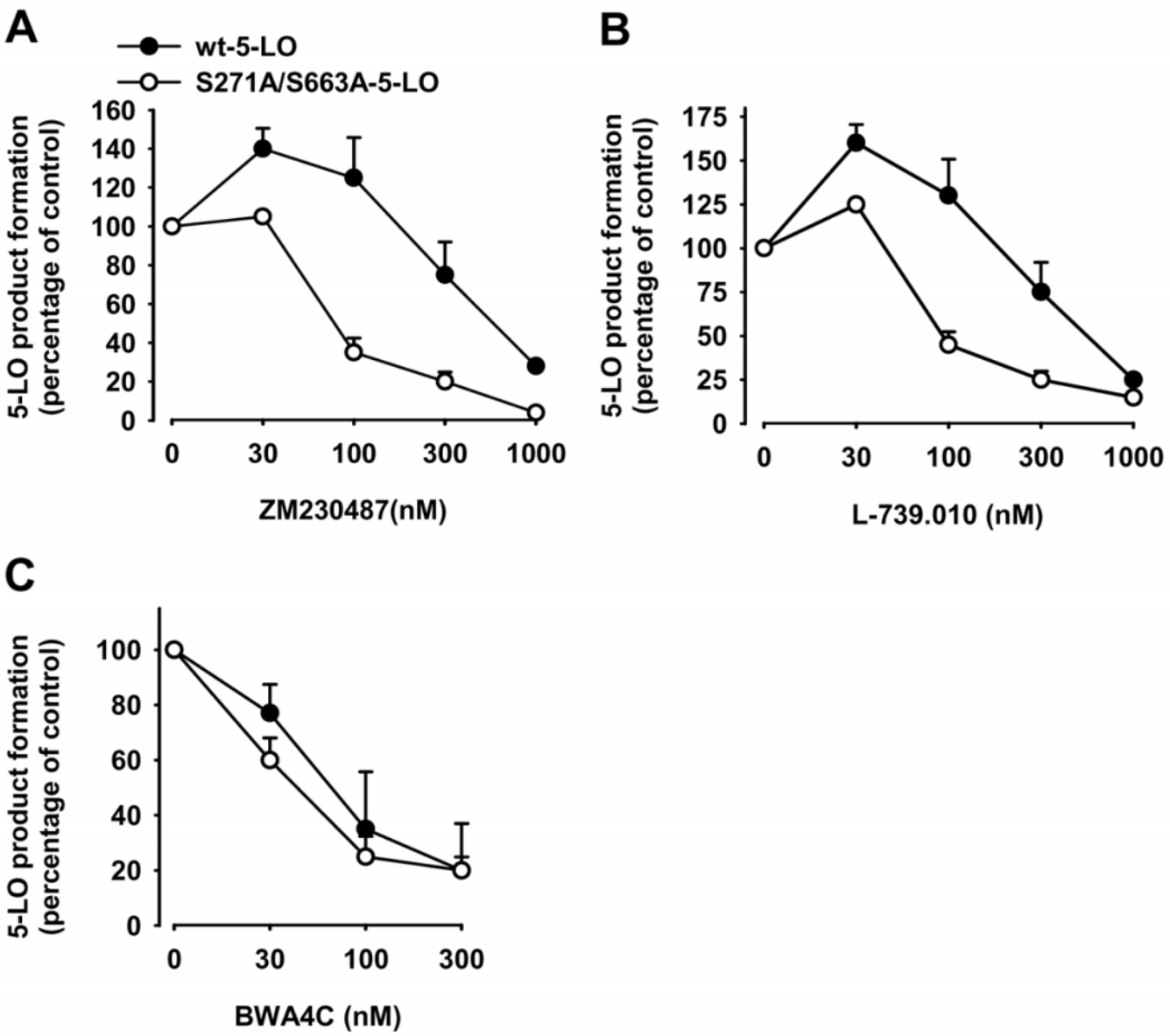


Figure 6. Effects of 5-LO inhibitors on 5-LO product formation in HeLa cells transformed with wt-5-LO or S271A/S663A-5-LO. HeLa cells were transiently transformed with plasmids pcDNA3.1-5LO or pcDNA3.1-5LO-S271A/S663A (1 µg/ml growth medium). Cells (1×10^6) were resuspended in 1 ml PGC buffer, and ZM230487 (**A**), L-739.010 (**B**), or BWA4C (**C**) were added at the indicated concentrations. After 15 min at 37°C, cells were stimulated with 40 µM AA for another 10 min. 5-LO product formation was determined by HPLC. Results are given as mean ±SE, *n*=4.

Fig. 7

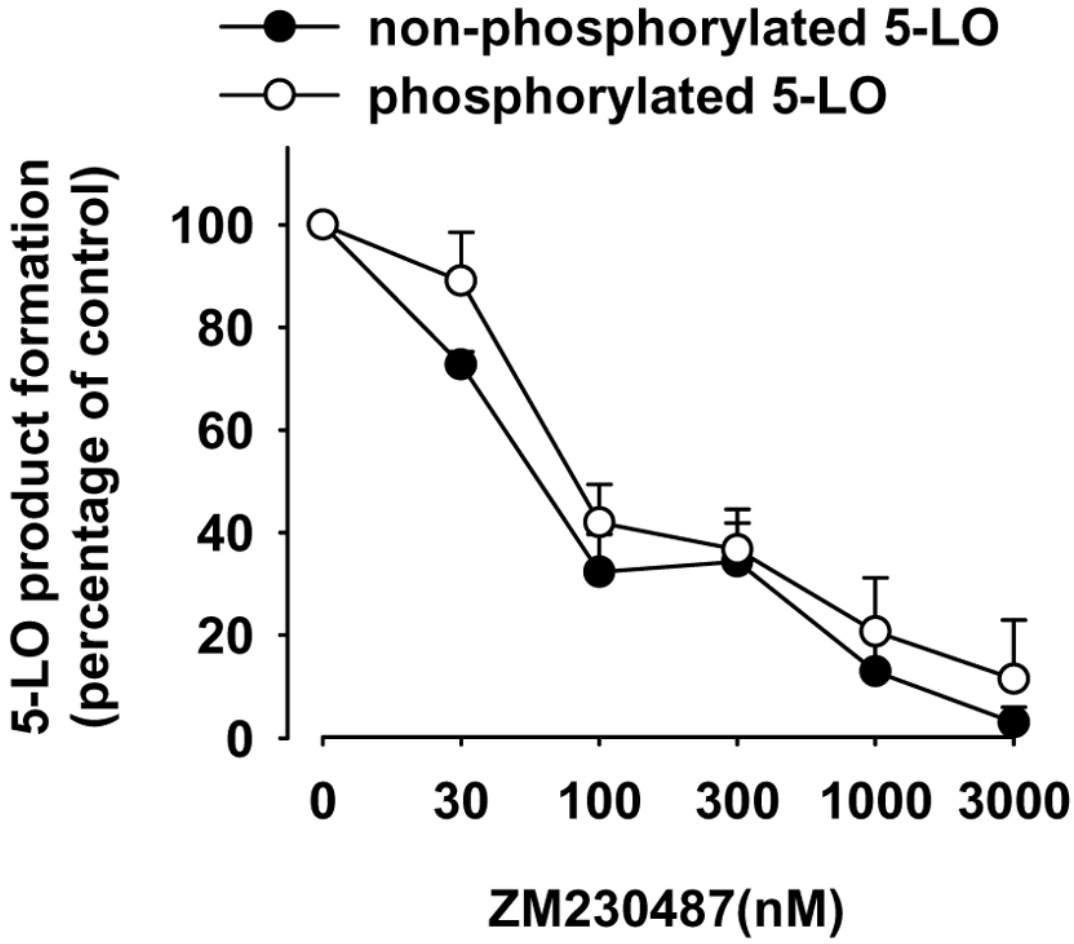


Figure 7. Effects of in vitro phosphorylation of 5-LO on the susceptibility toward ZM230487. Human recombinant 5-LO was expressed in *Escherichia coli*, purified, and phosphorylated by MK2 and ERK in vitro as described in Materials and Methods. Phosphorylated or nonphosphorylated 5-LO (0.5 µg) were added to a 5-LO reaction mix containing the indicated amounts of ZM230487. After 3 min at 4°C, 1 mM GSH plus 20 mU GPx were added, the samples were prewarmed at 37°C for 30 sec, and the 5-LO reaction was started by addition of 2 mM $CaCl_2$ and 40 µM AA. After 10 min at 37°C, 5-LO product formation was determined by HPLC as described. Results are given as mean ±SE, *n*=3.

Fig. 8

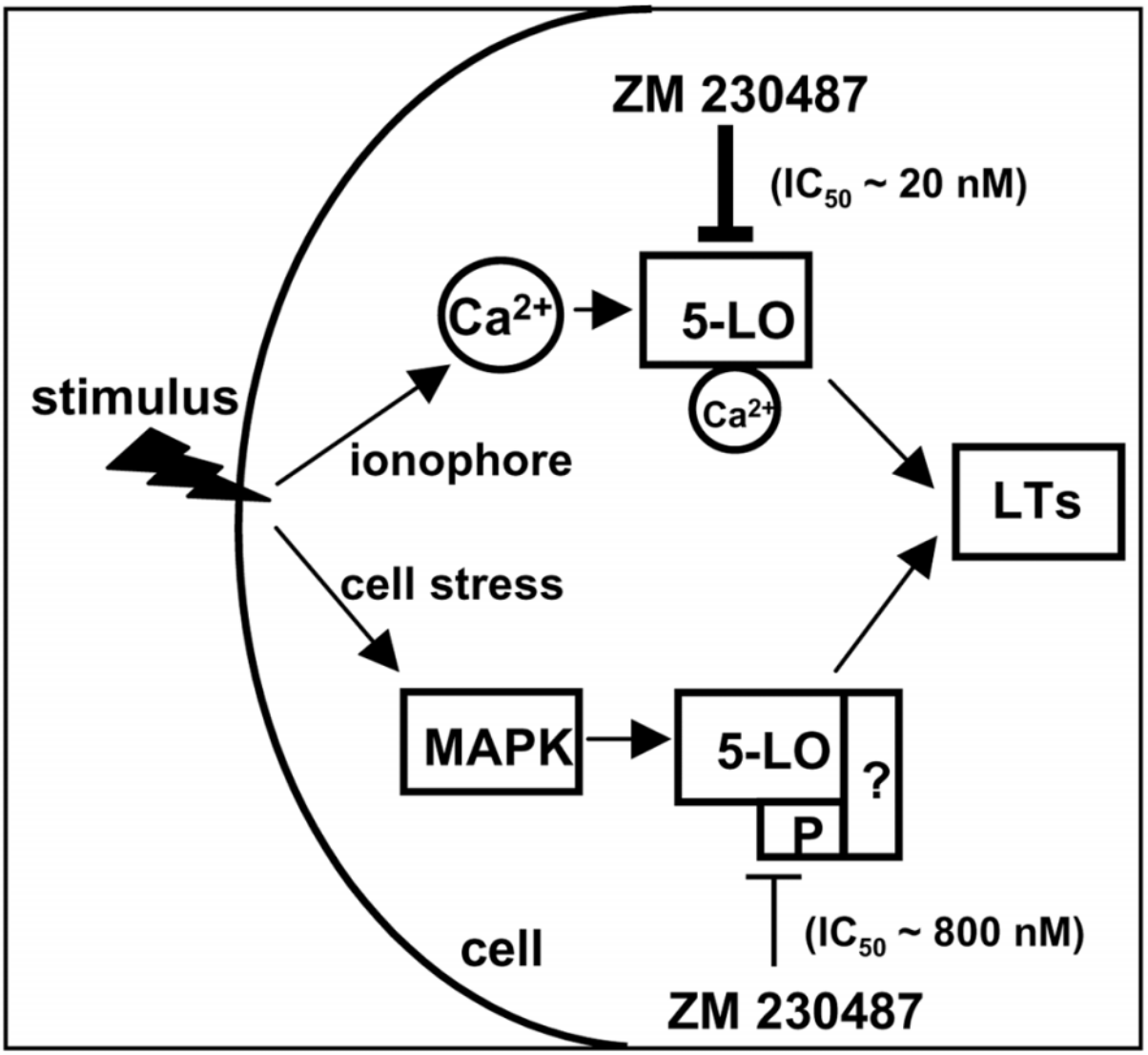


Figure 8. Mechanisms of 5-LO activation and effects on pharmacological inhibition. On cell stimulation, 5-LO can be activated by elevation of Ca^{2+} or via phosphorylation by members of the MAPK family, depending on the stimulus. Nonredox-type inhibitors, such as ZM230487, potently suppress Ca^{2+}-activated 5-LO ($IC_{50} \approx 20$ nM), whereas 5-LO activated by phosphorylation requires much higher inhibitor concentrations ($IC_{50} \approx 800$ nM). Apparently, an unknown cellular component is operative to impair the susceptibility of phosphorylated 5-LO against ZM230487.

IV

British Journal of Pharmacology (2004) 142, 861–868
www.nature.com/bjp

Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610

[1]Lutz Fischer, [1]Dieter Steinhilber & *,[1]Oliver Werz

[1]Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie-Str. 9, Frankfurt D-60439, Germany

1 5-Lipoxygenase (5-LO) is a crucial enzyme in the synthesis of the bioactive leukotrienes (LTs) from arachidonic acid (AA), and inhibitors of 5-LO are thought to prevent the untowarded pathophysiological effects of LTs.

2 In this study, we present the molecular pharmacological profile of the novel nonredox-type 5-LO inhibitor CJ-13,610 that was evaluated in various *in vitro* assays.

3 In intact human polymorphonuclear leukocytes (PMNL), challenged with the Ca^{2+}-ionophore A23187, CJ-13,610 potently suppressed 5-LO product formation with an $IC_{50} = 0.07\,\mu$M. Supplementation of exogenous AA impaired the efficacy of CJ-13,610, implying a competitive mode of action.

4 In analogy to ZM230487 and L-739.010, two closely related nonredox-type 5-LO inhibitors, CJ-13,610 up to 30 μM failed to inhibit 5-LO in cell-free assay systems under nonreducing conditions, but inclusion of peroxidase activity restored the efficacy of CJ-13,610 ($IC_{50} = 0.3\,\mu$M).

5 In contrast to ZM230487 and L-739.010, the potency of CJ-13,610 does not depend on the cell stimulus or the activation pathway of 5-LO. Thus, 5-LO product formation in PMNL induced by phosphorylation events was equally suppressed by CJ-13,610 as compared to Ca^{2+}-mediated 5-LO activation. In transfected HeLa cells, CJ-13,610 only slightly discriminated between phosphorylatable wild-type 5-LO and a 5-LO mutant that lacks phosphorylation sites.

6 In summary, CJ-13,610 may possess considerable potential as a potent orally active nonredox-type 5-LO inhibitor that lacks certain disadvantages of former representatives of this class of 5-LO inhibitors.

British Journal of Pharmacology (2004) **142,** 861–868. doi:10.1038/sj.bjp.0705860

Keywords: 5-Lipoxygenase; leukotriene; CJ-13,610; polymorphonuclear leukocyte; inflammation

Abbreviations: AA, arachidonic acid; ERK, extracellular signal-regulated kinase; FLAP, 5-lipoxygenase-activating protein; GPx, glutathione peroxidase; GSH, glutathione; LO, lipoxygenase; LOOH, lipid hydroperoxides; LT, leukotriene; MAPK, mitogen-activated protein kinase; MK, MAPKAPK, mitogen-activated protein kinase-activated protein kinase; PBS, phosphate-buffered saline pH 7.4; PG buffer, PBS containing 1 mg ml^{-1} glucose and 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocytes; SA, sodium arsenite

Introduction

Upon stimulation, certain inflammatory cell types, mainly granulocytes and monocytes/macrophages, possess the ability to release leukotrienes (LTs) and related lipid metabolites such as 5(*S*)-hydro(pero)xyeicosatetraenoic acid (5-H(P)ETE). These bioactive lipids are generated by the initial conversion of arachidonic acid (AA) to 5-HPETE and LTA_4 via the enzyme 5-lipoxygenase (5-LO). The subsequent conversion of LTA_4 by LTA_4 hydrolase gives LTB_4, whereas metabolism by LTC_4 synthase leads to the cysteinyl-LTs C_4, D_4, and E_4 (Samuelsson *et al*., 1987; Funk, 2001). LTB_4 is a potent chemotactic and chemokinetic mediator that activates granulocytes (Claesson & Dahlen, 1999) and augments phagocytosis of macrophages (Mancuso *et al*., 1998). The cysteinyl-LTs cause smooth muscle contraction, mucus secretion, plasma extravasation, vasoconstriction, and recruitment of eosinophils (Funk, 2001). Aside of these functions, more recent studies suggest a role of 5-LO products also in carcinogenesis and cell survival (Romano & Claria, 2003), in the metabolism of bone (Chen *et al*., 1994; Garcia *et al*., 1996), and finally in atherosclerotic processes (Mehrabian & Allayee, 2003; Dwyer *et al*., 2004). Accordingly, anti-LT therapy is beneficial in the treatment of asthma and other inflammatory diseases (Steinhilber, 1999), but may have potential also in the treatment of pancreatic and prostate cancer (Ghosh & Myers, 1998; Ding *et al*., 1999; Romano & Claria, 2003), osteoporosis (Alanko *et al*., 1998), and atherosclerosis (Mehrabian & Allayee, 2003; Dwyer *et al*., 2004).

5-LO contains a nonheme iron in the active site. In the resting state of the enzyme the iron is in the ferrous (Fe^{2+}) form, whereas the ferric (Fe^{3+}) form is necessary to enter the catalytic cycle (for a review see Rådmark, 2002). Accordingly, reducing compounds and iron-ligand inhibitors (such as zileuton, which is approved for the therapy of asthma in the U.S.) are direct 5-LO inhibitors acting at the active site iron. Despite their high potency *in vitro*, these compounds exhibit only poor bioavailability and only modest selectivity for 5-LO (Steinhilber, 1999).

*Author for correspondence;
E-mail: o.werz@pharmchem.uni-frankfurt.de
Advance online publication: 14 June 2004

In search of potent and selective 5-LO inhibitors, nonredox-type 5-LO inhibitors such as ZD2138 and its ethyl analogue ZM230487 were identified as highly potent, orally active inhibitors of LT biosynthesis that specifically and enantioselectively interact with 5-LO. Although these compounds significantly reduced a number of acute inflammatory responses (McMillan & Walker, 1992; Smith *et al*., 1995), they failed to inhibit more chronic inflammatory processes (Nasser *et al*., 1994; Turner *et al*., 1996). It was found that an elevated peroxide tone decreases the potency of ZM230487 that may limit the therapeutic value under chronic inflammatory conditions, connected to increased peroxide levels (Werz *et al*., 1998). Moreover, the potency of ZM230487 depends on the 5-LO activation pathway (Fischer *et al*., 2003). Thus, the sensitivity of 5-LO for ZM230487 in polymorphonuclear leukocytes (PMNL) was about 10- to 100-fold lower for 5-LO activated by phosphorylation as compared to Ca^{2+}-mediated enzyme activation. Finally, due to poor aqueous solubility and moderate oral absorption, the compounds exhibit unsatisfactory bioavailability.

Recently, novel ionizable imidazolylphenyl analogues of ZD2138 with improved solubility and better oral absorption were developed, with an *in vitro* activity comparable to that of ZD2138 (Mano *et al*., 2003). Structural modification led to improved pharmacokinetic and toxicological characteristics, exemplified by CJ-13,454, a practical lead for orally active nonredox-type 5-LO inhibitors (Mano *et al*., 2004). In this study, we evaluated the molecular pharmacology of CJ-13,610, a recently developed analogue of these imidazolylphenyl compounds, using various *in vitro* test systems with relevance for the *in vivo* pharmacology.

Methods

Materials

CJ-13,610 was provided by Glaxo Smith Kline (Stevenage, U.K.). The materials and sources were: Dulbecco's modified Eagle's medium (DMEM), GibcoBRL, Life Technologies (Rockville, MD, U.S.A.); fetal calf serum, bovine insulin, Ca^{2+}-ionophore A23187, arachidonic acid, dithiothreitol (DTT), glutathione peroxidase (GPx), sodium arsenite (SA), Sigma (Deisenhofen, Germany); HPLC solvents, Merck (Darmstadt, Germany); 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(*S*)-HPODE), Cayman; oligonucleotides, Cyber Gene (Huddinge, Sweden).

Cell culture, plasmids, and transient transfections

Human PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation at $4000 \times g$ for 20 min at 20°C for preparation of leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously (Werz *et al*., 2002a). PMNL (7.5×10^6 cells ml^{-1}; purity >96–97%) were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) plus 1 mg ml^{-1} glucose (PG buffer), or alternatively in PBS plus 1 mg ml^{-1} glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated.

HeLa cells were maintained in DMEM, supplemented with 10% fetal calf serum and 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin at 37°C in a 5% CO_2 incubator. Plasmid DNA (pcDNA3.1-5LO (Provost *et al*., 2001) or pcDNA3.1-5LO-S271A-S663A (Werz *et al*., 2002b), 10 μg each) was transiently transfected into HeLa cells using the calcium phosphate method, cultured for 48 h, and assayed for 5-LO product formation as described elsewhere (Fischer *et al*., 2003).

Expression and purification of 5-LO from Escherichia coli

Expression of 5-LO was performed in *E. coli* JM 109 cells, transfected with pT3-5LO, and purification of 5-LO was performed as described previously (Fischer *et al*., 2003). In brief, cells were lysed by incubation in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg ml^{-1}), 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysozyme (500 μg ml^{-1}), homogenized by sonication (3×15 s) and centrifuged at $19{,}000 \times g$ for 15 min. Proteins including 5-LO were precipitated with 50% saturated ammonium sulfate during stirring on ice for 60 min. The precipitate was collected by centrifugation at $16{,}000 \times g$ for 25 min and the pellet was resuspended in 20 ml PBS containing 1 mM EDTA and 1 mM PMSF. After centrifugation at $100{,}000 \times g$ for 70 min at 4°C, the $100{,}000 \times g$ supernatant was applied to an ATP-agarose column (Sigma A2767), and the column was eluted as described previously (Brungs *et al*., 1995). Partially purified 5-LO was immediately used for *in vitro* activity assays.

Determination of 5-LO product formation in intact cells

For assays of intact cells, 7.5×10^6 freshly isolated PMNL or 2×10^6 HeLa cells were finally resuspended in 1 ml PGC buffer. After preincubation with the indicated compounds at 37°C, 5-LO product formation was started by the addition of the indicated stimuli plus exogenous AA as indicated. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol and 30 μl of 1 N HCl, 200 ng prostaglandin B_1, and 500 μl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described (Werz & Steinhilber, 1996). 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells that includes LTB_4 and its all-*trans* isomers, 5(*S*),12(*S*)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(*S*),12(*S*)-DiHETE), and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected and oxidation products of LTB_4 were not determined.

Determination of 5-LO product formation in cell-free systems

For the determination of 5-LO activity in cell homogenates, 7.5×10^6 freshly isolated PMNL were resuspended in PBS containing 1 mM EDTA, sonicated (3×10 s) at 4°C, and 1 mM ATP was added. For determination of the activity of recombinant isolated 5-LO, partially purified 5-LO (0.5 μg in 5 μl) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 25 μg ml^{-1} phosphatidylcholine, 1 mM ATP, and 20 μg ml^{-1} γ-globulin). Samples of either cell homogenates or

partially purified 5-LO were supplemented with DTT (1 mM), GSH (1 mM), GPx-1 (30 mU), and CJ-13,610 as indicated. After 5–10 min at 4°C, samples were prewarmed for 30 s at 37°C and 2 mM $CaCl_2$ and AA at the indicated concentrations were added to start 5-LO product formation. The reaction was stopped after 10 min at 37°C by the addition of 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells.

Subcellular localization of 5-LO

Subcellular localization of 5-LO was investigated as described previously (Werz *et al*., 2002a). In brief, freshly isolated PMNL (3×10^7) in 1 ml PGC buffer were incubated at 37°C for 10 min with the indicated stimuli and chilled on ice. Nuclear and non-nuclear fractions were obtained after cell lysis by 0.1% NP-40. Aliquots of these fractions were analyzed for 5-LO protein by SDS–PAGE and immunoblotting using anti-5-LO antiserum (AK7, 1551; affinity purified on a 5-LO column). Proteins were visualized by alkaline phosphatase-conjugated IgGs (Sigma) using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) as substrates.

Statistics

The program 'GraphPad PRISM 3.0' was used for statistical comparisons. Statistical evaluation of the data was performed using Student's *t*-test for unpaired observations. A *P*-value of <0.05 was considered significant.

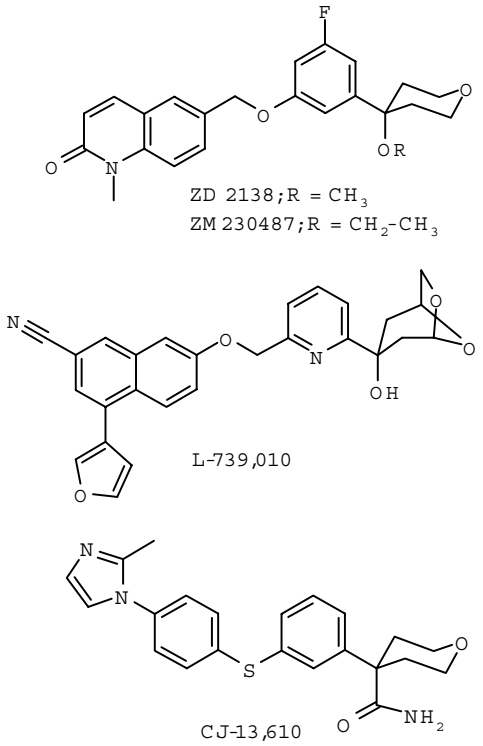


Figure 1 Chemical structures of nonredox-type 5-LO inhibitors. In comparison to ZD2138 or ZM230487, for CJ-13,610 the dihydroquinolinone pharmacophore was replaced by a imidazolylphenyl moiety, the alkoxy group by a carboxamide group, and the methyl ether was replaced by a sulfide group.

Results

CJ-13,610 suppresses 5-LO product formation in intact human PMNL

Replacement of the dihydroquinolinone by an imidazolylphenyl of ZM230487 as well as replacement of the ethoxy group of the tetrahydro[2H]pyran by a carboxamide moiety led to a novel series of nonredox-type 5-LO inhibitors (Mano *et al*., 2003; 2004), exemplified by CJ-13,610 (Figure 1). Freshly isolated human PMNL were preincubated with CJ-13,610 and stimulated with 2.5 μM ionophore A23187 in the absence or presence of exogenous AA. As can be seen in Figure 2a, CJ-13,610 dose dependently suppressed 5-LO product formation in ionophore A23187-stimulated PMNL in the absence of exogenous AA with an IC_{50} of about 70 nM. Supplementation of AA impaired the efficacy of CJ-13,610 and shifted the IC_{50} to higher values. Thus, at 2 μM of exogenously added AA, the IC_{50} was determined as 280 nM and further increase of the substrate concentration (10 or 100 μM) caused further impaired efficacy of CJ-13,610 (IC_{50} at 100 μM AA was approx. 900 nM). As can be seen from the Lineweaver–Burke plot in Figure 2b, kinetic analysis confirm competitive inhibition of 5-LO by CJ-13,610 in intact PMNL. Moreover, at low AA concentrations, CJ-13,610 completely blocked 5-LO product synthesis, whereas at 100 μM AA, some basal 5-LO activity, even at 100 μM CJ-13,610, still remained. Together, these data indicated that CJ-13,610 is a potent inhibitor of 5-LO product formation in intact cells, apparently acting in a competitive manner.

Inhibition of 5-LO in cell-free systems by CJ-13,610 requires GPx activity

In a previous study, we found that ZM230487 and L739.010 exhibit only low efficacy in cell-free assay systems, but replenishment of peroxidase activity by the addition of GPx-1 plus GSH to purified 5-LO enzyme or by supplementation of thiols (DTT or GSH) to cell homogenates leads to potent 5-LO inhibition (Werz *et al*., 1997; 1998). CJ-13,610 was assayed for 5-LO inhibition in whole homogenates of human PMNL. As can be see from Figure 3a, CJ-13,610 (up to 30 μM) failed to suppress 5-LO under standard assay conditions, regardless of the AA concentration. However, replenishment of GPx activity by the addition of 1 mM DTT renders CJ-13,610 a potent 5-LO inhibitor. Thus, at 4, 10, and 40 μM AA, similar IC_{50} values (approx. 280, 320, and 700 nM, respectively) were determined as compared to intact PMNL. Again, as observed in intact cells, elevation of the AA concentration impairs the potency of CJ-13,610 in homogenates, and kinetic analysis (Figure 3b) indicate competitive properties of CJ-13,610.

Next, human recombinant 5-LO was expressed in *E. coli*, partially purified, and CJ-13,610 was tested for enzyme inhibition under reducing (presence of GPx and GSH) and nonreducing conditions. In the absence of GPx, 5-LO activity was not significantly inhibited by CJ-13,610 up to 30 μM (Figure 4). However, inclusion of 30 mU GPx-1 and 1 mM GSH caused 5-LO enzyme inhibition with an $IC_{50} = 5\,\mu$M, when the AA concentration was adjusted to 4 μM. In contrast, at 40 μM AA, addition of GPx-1 and GSH could not confer

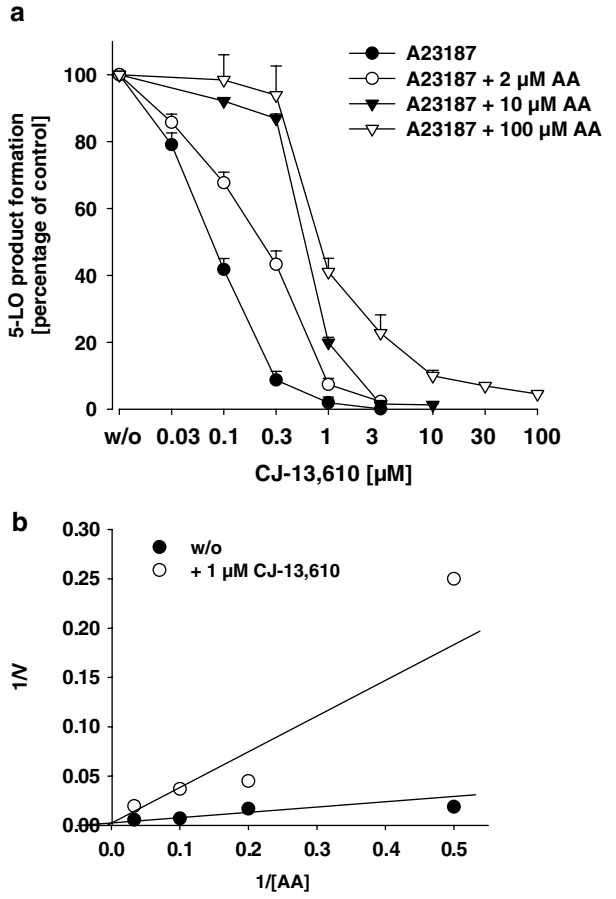


Figure 2 CJ-13,610 suppresses 5-LO product formation in intact human PMNL. (a) Freshly isolated PMNL (7.5×10^6 in 1 ml PGC buffer) were preincubated with CJ-13,610 at the indicated concentrations for 15 min at 37°C. Cells were stimulated with ionophore A23187 (2.5 μM) with or without AA at the indicated concentrations. After 10 min at 37°C, 5-LO products were extracted and determined by HPLC as described in the Methods section. Results are given as mean ± s.e., $n = 4$. (b) Kinetic analysis of 5-LO product inhibition by 1 μM CJ-13,610 is given as Lineweaver–Burke plot. The AA concentrations were 2, 5, 10, and 30 μM.

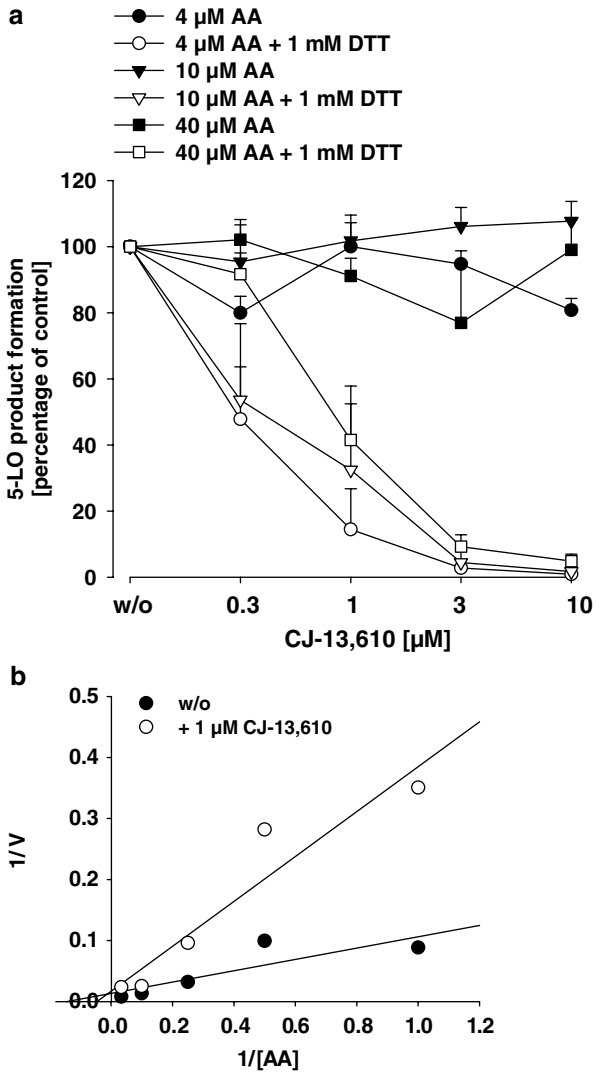


Figure 3 Inhibition of 5-LO by CJ-13,610 in whole-cell homogenates of human PMNL. (a) Whole homogenates of human PMNL were prepared as described, preincubated with the indicated concentrations of CJ-13,610 in the presence or absence of 1 mM DTT for 5–10 min on ice. Samples were prewarmed at 37°C for 30 s, then AA (4, 10, or 40 μM) and 2 mM $CaCl_2$ were added. After another 10 min at 37°C, 5-LO activity was determined as described in the Methods section. Results are given as mean ± s.e., $n = 3$–4. (b) Kinetic analysis of 5-LO product inhibition by 1 μM CJ-13,610 is given as Lineweaver–Burke plot. The AA concentrations were 1, 2, 4, 10, and 20 μM.

CJ-13,610 potent 5-LO inhibitory activity. Thus, also under these experimental settings, AA impairs the potency of CJ-13,610 under reducing conditions in cell-free assay systems.

Exogenous lipid hydroperoxides (LOOH) reduce the efficacy of CJ-13,610 in intact cells

In intact cells, the level of peroxides is controlled by GPx isoenzymes (Ursini *et al*., 1995). Elevation of the cellular peroxide tone, for example, by exogenous addition of 13(*S*)-HPODE or diamide, reduced the potency of ZM230487 and L-739.010 (Werz *et al*., 1998). As shown in Figure 5a, the potency of CJ-13,610 in intact PMNL, stimulated with ionophore A23187 and AA, was considerably impaired when cells had been pretreated with 13(*S*)-HPODE. This is visualized by a shift of the IC_{50} value of CJ-13,610 for untreated cells (0.55 μM) to an about six-fold higher value (3.1 μM) in cells exposed to 3 μM 13(*S*)-HPODE. When the concentration of 13(*S*)-HPODE was further increased to 10 μM, 5-LO inhibition by CJ-13,610 (up to 10 μM) was completely abolished. Interestingly, in the absence of exogenous AA, 3 μM 13(*S*)-HPODE led to the same reduced efficacy of CJ-13,610 in PMNL stimulated with ionophore A23187 ($IC_{50} = 2.8$ μM, Figure 5b). This suggests that LOOH are superior to AA in competing with CJ-13,610 and that AA may have no (or at least only modest) additional competitive effects on top of LOOH.

Efficacy of CJ-13,610 in intact cells is stimulus independent

Recently, we could show that the efficacy of ZM230487 and L-739.010 is stimulus dependent and that phosphorylation events

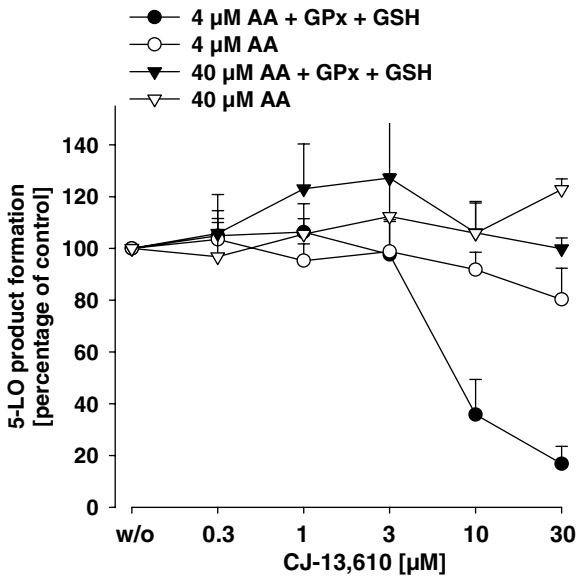


Figure 4 Inhibition of purified recombinant 5-LO by CJ-13,610. Human recombinant 5-LO was expressed in *E. coli* and partially purified as described. 5-LO (0.5 μg) was added to a 5-LO reaction mix containing the indicated amounts of CJ-13,610, 1 mM GSH, and 30 mU GPx. After 5–10 min on ice, the samples were prewarmed for 30 s at 37°C and 2 mM $CaCl_2$ and AA (4 or 40 μM) was added. After 10 min at 37°C, formed 5-LO products were extracted and determined by HPLC. Results are given as mean ± s.e., $n=3$.

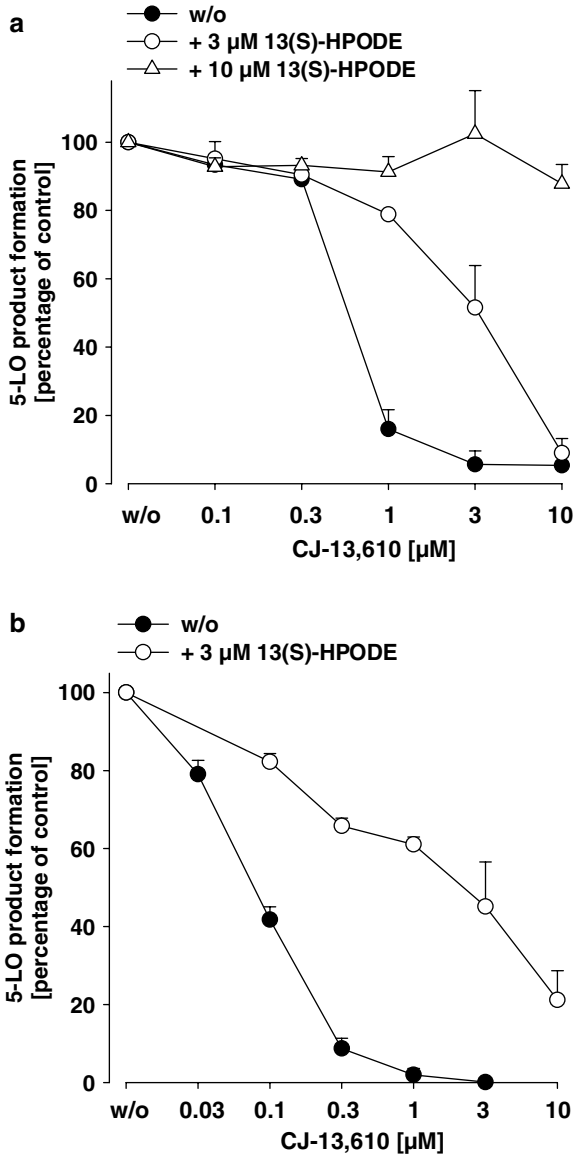


Figure 5 Elevation of the cellular peroxide tone impairs the potency of CJ-13,610 in intact PMNL. Freshly isolated PMNL (7.5×10^6 in 1 ml PGC buffer) were preincubated with CJ-13,610 at the indicated concentrations at 37°C. After 15 min, 13(*S*)-HPODE was added as indicated and cells were subsequently stimulated with (a) 2.5 μM ionophore A23187 plus 10 μM AA or with (b) 2.5 μM ionophore A23187 alone. After 10 min at 37°C, 5-LO products were extracted and determined by HPLC. Results are given as mean ± s.e., $n=3$.

of 5-LO may impair the sensitivity of the enzyme against these inhibitors (Fischer *et al*., 2003). As shown in Figure 6, the potency of CJ-13,610 in PMNL stimulated with 10 μM SA or 300 mM NaCl, which activate 5-LO by phosphorylation events in a Ca^{2+}-independent manner, is comparable to that obtained in cells challenged by ionophore A23187. Cell stress alone (in contrast to ionophore) is not capable of providing sufficient amounts of free AA as 5-LO substrate in intact cells. Therefore, 20 μM AA was included in all experiments in order to ensure equal substrate availability. The results demonstrate that the efficacy of CJ-13,610 is independent of the stimulus used to evoke 5-LO activation and product synthesis, suggesting that 5-LO phosphorylation events have no major impact on the interference of the compound with the enzyme.

CJ-13,610 does not inhibit translocation of 5-LO to the nuclear membrane

Activation of 5-LO in the cell is accompanied by a rapid translocation of the enzyme to the nuclear membrane, which may stimulate 5-LO product synthesis (Peters-Golden & Brock, 2001). We tested if CJ-13,610 could interfere with the 5-LO translocation process. 5-LO protein was assessed in the nuclear and non-nuclear fraction after subcellular fractionation of PMNL. As shown in Figure 7, in resting PMNL, 5-LO was exclusively in the non-nuclear fraction, whereas the addition of A23187 caused enrichment of 5-LO in the nuclear fraction and a concomitant loss in the non-nuclear fraction. Preincubation of PMNL with CJ-13,610 did not suppress this A23187-induced 5-LO translocation.

Effects of CJ-13,610 on 5-LO product synthesis in transfected HeLa cells

In order to confirm the hypothesis that 5-LO inhibition by CJ-13,610 does not depend on the 5-LO phosphorylation status, HeLa cells were transiently transfected with phosphorylatable wild-type 5-LO (WT-5-LO) or with nonphosphorylatable mutant S271A/S663A-5-LO (lacking the phosphorylation sites for MKs and extracellular signal-regulated kinase (ERKs), respectively), preincubated with CJ-13,610, stimulated with 20 μM AA (5-LO activation conditions that are based on enzyme phosphorylation) and 5-LO product formation was

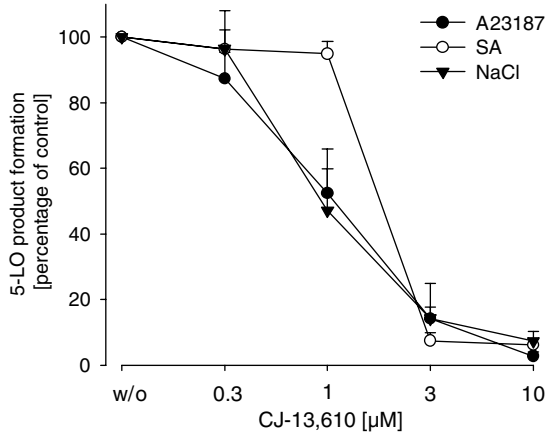


Figure 6 Cell stress does not affect the efficacy of CJ-13,610 in intact cells. Freshly isolated PMNL (7.5×10^6 in 1 ml PGC buffer) were preincubated with CJ-13,610 at the indicated concentrations for 15 min at 37°C. SA (10 μM) and NaCl (300 mM) were added 3 min prior to the addition of 20 μM AA, ionophore A23187 (2.5 μM) was added together with 20 μM AA. After 10 min at 37°C, 5-LO products were extracted and determined by HPLC. Results are given as mean ± s.e., $n = 3$.

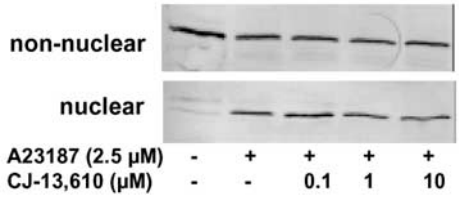


Figure 7 Effects of CJ-13,610 on nuclear 5-LO translocation in intact PMNL. Freshly isolated PMNL (3×10^7 in 1 ml PGC buffer) were preincubated with CJ-13,610 at the indicated concentrations for 15 min at 37°C. Then, 2.5 μM A23187 was added to the samples and incubated for another 5 min at 37°C. 5-LO was detected in nuclear and non-nuclear fractions by immunoblotting after subcellular fractionation. Similar results were obtained in two additional independent experiments.

determined. In comparison to intact PMNL, we found significant differences in HeLa cells with respect to 5-LO inhibition by CJ-13,610 (Figure 8). Thus, at low concentrations (1 and 3 μM), CJ-13,610 significantly increased (up to 1.4-fold) product synthesis of WT-5-LO, and the IC_{50} value of CJ-13,610 was about 10-fold higher in HeLa (approx. 7 μM) as compared to PMNL. Phosphorylation-mediated 5-LO product formation (of WT-5-LO) required about two-fold higher inhibitor concentrations for efficient inhibition as compared to 5-LO product formation by S271A/S663A-5-LO. Notably, the nonphosphorylatable enzyme did not exhibit increased 5-LO product synthesis at low inhibitor concentrations.

Discussion

In the present study, we have evaluated the molecular pharmacology of the novel nonredox-type 5-LO inhibitor CJ-13,610 using various *in vitro* test systems, relevant for the *in vivo* pharmacology. In analogy to ZM230487 or L-739.010, two structurally related nonredox-type 5-LO inhibitors, CJ-13,610 potently suppressed 5-LO product formation in intact cells as well as in cell-free assay systems under reducing conditions, whereas an elevated peroxide tone strongly

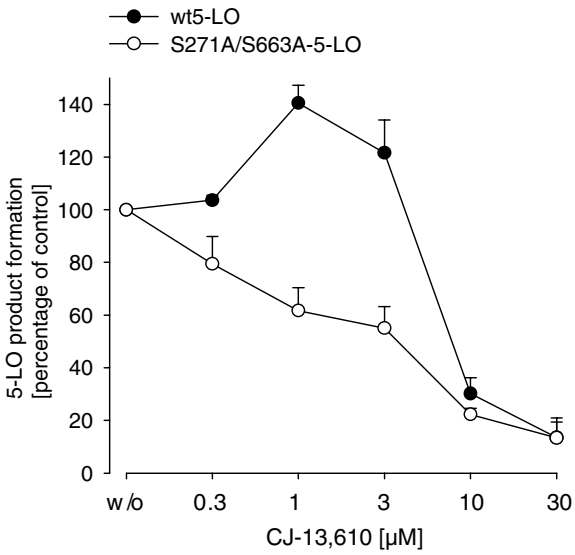


Figure 8 Effects of CJ-13,610 on 5-LO product formation in HeLa cells transformed with WT-5-LO and S271A/S663A-5-LO. HeLa cells were transiently transformed with plasmids pcDNA3.1-5LO or pcDNA3.1-5LO-S271A-S663A (10 μg). Cells (2×10^6) were resuspended in 1 ml PGC buffer and CJ-13,610 was added at the indicated concentrations. After 15 min at 37°C, cells were stimulated with 20 μM AA for another 10 min. 5-LO products were extracted and determined by HPLC. Results are given as mean ± s.e., $n = 4$.

impaired the efficacy of CJ-13,610. However, in contrast to ZM230487 and L-739.010, CJ-13,610 shows competitive kinetics with respect to AA also under reducing conditions, and the efficacy of CJ-13,610 is cell stimulus independent and less affected by the phosphorylation status of 5-LO. Therefore, the pharmacological profile of CJ-13,610 is in part distinct from that of other nonredox 5-LO inhibitors and may possess therapeutic potential as an orally active 5-LO inhibitor, lacking certain disadvantages of former representatives of this inhibitor class. Recently, the efficacy, safety, and tolerability of 6 weeks treatment by oral dosing with CJ-13,610 in adults with chronic obstructive pulmonary disease was evaluated in a phase II multicenter, randomized, double-blind placebo-controlled parallel group study.

Nonredox-type 5-LO inhibitors have been initially designed as active site-directed inhibitors devoid of redox and iron ligand properties that compete with AA at a (hypothetical) substrate binding cleft of 5-LO (McMillan & Walker, 1992). In fact, biochemical characterization of ZM230487 and L-739.010 revealed evidence for competitive kinetics with low affinity under nonreducing conditions, whereas efficient 5-LO inhibition under reducing conditions showed noncompetitive kinetics (Werz *et al*., 1998). It was found that LOOH or an elevated peroxide tone strongly counteract the high affinity of nonredox 5-LO inhibitors and efficient inhibition of 5-LO product formation required low LOOH levels (Werz *et al*., 1998). Nevertheless, the precise mode of action and the binding site(s) of nonredox 5-LO inhibitors have not been elucidated yet.

In order to initialize 5-LO catalysis, LOOH are important for the conversion of the active site iron from the ferrous to the ferric state (Rouzer & Samuelsson, 1986; Hammarberg *et al*., 2001). However, no discrete locale where LOOH bind to 5-LO has been identified. In cell-free systems, CJ-13,610 up to the highest concentration (30 μM) tested was unable to suppress

5-LO, unless peroxide levels were decreased by replenishment of GPx activity. Moreover, in intact PMNL, elevation of the cellular peroxide tone by exogenous addition of 13(*S*)-HPODE abolished the inhibitory effects of CJ-13,610. Thus, in analogy to ZM230487 or L-739.010 (Werz *et al*., 1998), also CJ-13,610 requires a low peroxide tone for efficient 5-LO inhibition. Such a pattern is unique for nonredox-type inhibitors since the potencies of iron-ligand 5-LO inhibitors (BWA4C) or 5-lipoxygenase-activating protein (FLAP) inhibitors (MK886) are not affected by the peroxide tone (Werz *et al*., 1998). Apparently, both CJ-13,160 as well as ZM230487 and L-739.010 compete with activating LOOH at a putative regulatory LOOH-binding site with high affinity, thereby preventing 5-LO catalysis.

Experimental data indicate that 5-LO has two distinct fatty acid-binding sites, one regulatory that may bind LOOH and one at the catalytic site where transformation of AA takes place (Sailer *et al*., 1998; Burkert *et al*., 2003). In contrast to ZM230487, the potency of CJ-13,610 under reducing conditions is impaired by AA, suggesting that CJ-13,610 may not only potently interfere with the regulatory LOOH- but also with the arachidonate(substrate)-binding cleft at the active site. Interference of CJ-13,610 at such a substrate-binding cleft could be visualized in this study by different experimental settings: First, in intact PMNL, elevated AA concentrations clearly impaired the efficacy of CJ-13,610; second, under cell-free assay conditions elevated AA concentrations significantly decreased inhibition of crude 5-LO in homogenates supplemented with DTT; and third, inhibition of partially purified 5-LO in the presence of GPx and GSH was apparent at 4 μM AA, but not at 40 μM AA. This pattern is in sharp contrast to ZM230487 and L-739.010, whose high affinities under reducing conditions were not altered by variation of the AA concentration, although competitive kinetics with low affinities were evident under nonreducing conditions (Werz *et al*., 1997; 1998). Of interest, LOOH are superior to AA in competing with CJ-13,610, and AA may have no additional competitive effects on top of LOOH. Thus, the reduction in the efficacy of CJ-13,610 by 3 μM 13(*S*)-HPODE was quantitatively almost the same in PMNL stimulated in the absence or in the presence of AA. Together, these findings imply that under certain (more chronic) inflammatory situations, which are associated with elevated cellular levels of LOOH and free AA, a possibly reduced efficacy of CJ-13,610 should be taken into account. In this regard, it was shown that ZD2138 failed to protect against allergen-induced asthmatic responses in asthmatic subjects (Nasser *et al*., 1994), and did not prevent pulmonary inflammation or the development of airway hyper-responsiveness (Turner *et al*., 1996).

It should be noted that the effects of CJ-13,610 determined in HeLa cells differed from that obtained with PMNL. For example, in HeLa cells, CJ-13,610 significantly increased 5-LO product synthesis at low ($<3\,\mu$M) concentrations, a phenomenon that cannot be readily explained. Compared to PMNL, the IC_{50} value was about 10-fold higher in HeLa cells at same substrate concentrations (20 μM AA). The reason for this difference is also not clear. Presumably, the machinery necessary for 5-LO product formation and the existence of regulating cofactors (e.g. FLAP, LOOH and Ca^{2+} levels, GPx activity) are different in HeLa cells. For example, it is conceivable that CJ-13,610 could interfere with the fatty acid-binding FLAP, necessary for efficient 5-LO product synthesis in intact cells. However, in experiments with HeLa cells (not expressing FLAP (own unpublished observations)), transiently transfected with FLAP, CJ-13,610 was equipotent as compared to MOCK-transfected cells (not shown). Therefore, FLAP should not affect the potency of CJ-13,610, supported by the finding that CJ-13,610 did also not interfere with 5-LO translocation to the nucleus in PMNL, which may be FLAP dependent (Peters-Golden & Brock, 2001). Nevertheless, the potency of CJ-13,610 may depend on the cellular environment, that is, the presence of certain 5-LO regulatory mechanisms or cofactors, which remain to be identified.

Activation of cellular 5-LO in response to external stimuli involves 5-LO translocation from a soluble locale to the nuclear membrane where the enzyme colocalizes with FLAP (Peters-Golden & Brock, 2001), and is mediated by elevation of the intracellular Ca^{2+} levels and/or phosphorylation of 5-LO by mitogen-activated protein kinase-activated protein kinase (MAPKAP) kinases at Ser-271 and by ERKs 1/2 at Ser-663 (Werz *et al*., 2000; 2002a, b). It was shown that the activation of 5-LO by stimuli that induce phosphorylation of 5-LO is Ca^{2+}-independent in certain cell types (Werz *et al*., 2002a; Burkert *et al*., 2003). The efficacies of the nonredox-type 5-LO inhibitors ZM230487 and L-739.010 depend on the stimulus and activation pathway utilized to induce 5-LO product synthesis (Fischer *et al*., 2003). Whereas Ca^{2+}-mediated 5-LO activation in PMNL is efficiently suppressed by ZM230487 and L-739.010, 10- to 100-fold higher inhibitor concentrations are required to suppress 5-LO product synthesis induced by phosphorylation (Fischer *et al*., 2003). In contrast, the efficacy of CJ-13,610 in intact PMNL is not reduced when 5-LO is activated by phosphorylation events. Also, CJ-13,610 exhibited no pronounced difference in the potencies towards phosphorylatable wt- or nonphosphorylatable mutated S217A/S663A-5-LO in HeLa cells. Notably, several diseases related or connected to elevated levels of 5-LO products such as inflammatory reactions, allergic asthma, various types of cancer, and atherosclerosis are associated with an increased phosphorylation status of the cell (Hajjar & Pomerantz, 1992; Johnson & Lapadat, 2002), which determine the susceptibility of 5-LO towards nonredox inhibitors. Owing to the fact that the efficacy of CJ-13,610 does not depend on the 5-LO phosphorylation status, targeting of 5-LO by such a drug could indeed be beneficial for the therapy of these diseases. Although the therapeutic potential of CJ-13,610 may be somewhat limited by reduced efficacy due to an elevated peroxide tone, the compound obviously possesses advantages over former representatives of this class of 5-LO inhibitors that could not attain regular approval due to poor clinical benefits.

We thank Astrid Neuß for expert technical assistance and Dr Patrick Provost and David Dishart for providing the plasmid pcDNA3.1-5LO and pcDNA3.1-5LO-S271A-S663A, respectively. This study was supported by grants from the Fonds der Chemischen Industrie, the EU (LEUCHRON, QLRT-2000-01521), and the Deutsche Pharmazeutische Gesellschaft.

References

ALANKO, J., SIEVI, E., LAHTEENMAKI, T., MUCHA, I., VAPAATALO, H. & PARANTAINEN, J. (1998). Catechol estrogens as inhibitors of leukotriene synthesis. *Biochem. Pharmacol.,* **55,** 101–104.

BRUNGS, M., RÅDMARK, O., SAMUELSSON, B. & STEINHILBER, D. (1995). Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 107–111.

BURKERT, E., SZELLAS, D., RADMARK, O., STEINHILBER, D. & WERZ, O. (2003). Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. *J. Leukocyte Biol.,* **73,** 191–200.

CHEN, X.S., SHELLER, J.R., JOHNSON, E.N. & FUNK, C.D. (1994). Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature,* **372,** 179–182.

CLAESSON, H.E. & DAHLEN, S.E. (1999). Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.,* **245,** 205–227.

DING, X.Z., IVERSEN, P., CLUCK, M.W., KNEZETIC, J.A. & ADRIAN, T.E. (1999). Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.,* **261,** 218–223.

DWYER, J.H., ALLAYEE, H., DWYER, K.M., FAN, J., WU, H., MAR, R., LUSIS, A.J. & MEHRABIAN, M. (2004). Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.,* **350,** 29–37.

FISCHER, L., SZELLAS, D., RÅDMARK, O., STEINHILBER, D. & AND WERZ, O. (2003). Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB J.,* **17,** 949–951.

FUNK, C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science,* **294,** 1871–1875.

GARCIA, C., BOYCE, B.F., GILLES, J., DALLAS, M., QIAO, M., MUNDY, G.R. & BONEWALD, L.F. (1996). Leukotriene B4 stimulates osteoclastic bone resorption both *in vitro* and *in vivo*. *J. Bone Miner Res.,* **11,** 1619–1627.

GHOSH, J. & MYERS, C.E. (1998). Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 13182–13187.

HAJJAR, D.P. & POMERANTZ, K.B. (1992). Signal transduction in atherosclerosis: integration of cytokines and the eicosanoid network. *FASEB J.,* **6,** 2933–2941.

HAMMARBERG, T., KUPRIN, S., RADMARK, O. & HOLMGREN, A. (2001). Epr investigation of the active site of recombinant human 5-lipoxygenase: inhibition by selenide. *Biochemistry,* **40,** 6371–6378.

JOHNSON, G.L. & LAPADAT, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science,* **298,** 1911–1912.

MANCUSO, P., STANDIFORD, T.J., MARSHALL, T. & PETERS-GOLDEN, M. (1998). 5-Lipoxygenase reaction products modulate alveolar macrophage phagocytosis of *Klebsiella pneumoniae*. *Infect. Immun.,* **66,** 5140–5146.

MANO, T., OKUMURA, Y., SAKAKIBARA, M., OKUMURA, T., TAMURA, T., MIYAMOTO, K. & STEVENS, R.W. (2004). 4-[5-Fluoro-3-[4-(2-methyl-1H-imidazol-1-yl)benzyloxy]phenyl]-3,4,5,6-tetrahydro-2H-pyran-4-carboxamide, an orally active inhibitor of 5-lipoxygenase with improved pharmacokinetic and toxicology characteristics. *J. Med. Chem.,* **47,** 720–725.

MANO, T., STEVENS, R.W., ANDO, K., NAKAO, K., OKUMURA, Y., SAKAKIBARA, M., OKUMURA, T., TAMURA, T. & MIYAMOTO, K. (2003). Novel imidazole compounds as a new series of potent, orally active inhibitors of 5-lipoxygenase. *Bioorg. Med. Chem.,* **11,** 3879–3887.

MCMILLAN, R.M. & WALKER, E.R.H. (1992). Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.,* **13,** 323–330.

MEHRABIAN, M. & ALLAYEE, H. (2003). 5-Lipoxygenase and atherosclerosis. *Curr. Opin. Lipidol.,* **14,** 447–457.

NASSER, S.M., BELL, G.S., HAWKSWORTH, R.J., SPRUCE, K.E., MACMILLAN, R., WILLIAMS, A.J., LEE, T.H. & ARM, J.P. (1994). Effect of the 5-lipoxygenase inhibitor ZD2138 on allergen-induced early and late asthmatic responses. *Thorax,* **49,** 743–748.

PETERS-GOLDEN, M. & BROCK, T.G. (2001). Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett.,* **487,** 323–326.

PROVOST, P., DOUCET, J., HAMMARBERG, T., GERISCH, G., SAMUELSSON, B. & RÅDMARK, O. (2001). 5-Lipoxygenase interacts with coactosin-like protein. *J. Biol. Chem.,* **276,** 16520–16527.

RÅDMARK, O. (2002). Arachidonate 5-lipoxygenase. *Prostaglandins Other Lipid Mediat,* **68–69,** 211–234.

ROMANO, M. & CLARIA, J. (2003). Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. *FASEB J.,* **17,** 1986–1995.

ROUZER, C.A. & SAMUELSSON, B. (1986). The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS Lett.,* **204,** 293–296.

SAILER, E.R., SCHWEIZER, S., BODEN, S.E., AMMON, H.P.T. & SAFAYHI, H. (1998). Characterization of an acetyl-11-keto-beta-boswellic acid and arachidonate-binding regulatory site of 5-lipoxygenase using photoaffinity labeling. *Eur. J. Biochem.,* **256,** 364–368.

SAMUELSSON, B., DAHLÉN, S.-E., LINDGREN, J.-Å., ROUZER, C.A. & SERHAN, C.N. (1987). Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science,* **237,** 1171–1176.

SMITH, W.G., SHAFFER, A.F., CURRIE, J.L., THOMPSON, J.M., KIM, S., RAO, T. & ISAKSON, P.C. (1995). Characterization of 5-lipoxygenase inhibitors in biochemical and functional *in vivo* assays. *J. Pharmacol. Exp. Ther.,* **275,** 1332–1338.

STEINHILBER, D. (1999). 5-lipoxygenase: a target for antiinflammatory drugs revisited. *Curr. Med. Chem.,* **6,** 69–83.

TURNER, C.R., SMITH, W.B., ANDRESEN, C.J., EGGLER, J.F. & WATSON, J.W. (1996). The effect of 5-lipoxygenase inhibition on *Ascaris* antigen (Ag)-induced responses in atopic monkeys. *Inflamm. Res.,* **45,** 42–49.

URSINI, F., MAIORINO, M., BRIGELIUSFLOHE, R., AUMANN, K.D., ROVERI, A., SCHOMBURG, D. & FLOHE, L. (1995). Diversity of glutathione peroxidases. *Biothiols, Pt B,* **252,** 38–53.

WERZ, O., BUERKERT, E., SAMUELSSON, B., RÅDMARK, O. & STEINHILBER, D. (2002a). Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood,* **99,** 1044–1052.

WERZ, O., BURKERT, E., FISCHER, L., SZELLAS, D., DISHART, D., SAMUELSSON, B., RADMARK, O. & STEINHILBER, D. (2002b). Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.,* **16,** 1441–1443.

WERZ, O., KLEMM, J., SAMUELSSON, B. & RADMARK, O. (2000). 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 5261–5266.

WERZ, O., SCHNEIDER, N., BRUNGS, M., SAILER, E.R., SAFAYHI, H., AMMON, H.P.T. & STEINHILBER, D. (1997). A test system for leukotriene synthesis inhibitors based on the *in-vitro* differentiation of the human leukemic cell lines HL-60 and Mono Mac 6. *Naunyn Schmied. Arch. Pharmacol.,* **356,** 441–445.

WERZ, O. & STEINHILBER, D. (1996). Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells – the presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. *Eur. J. Biochem.,* **242,** 90–97.

WERZ, O., SZELLAS, D., HENSELER, M. & STEINHILBER, D. (1998). Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.,* **54,** 445–451.

(Received February 18, 2004
Revised April 16, 2004
Accepted April 30, 2004)

V

Biochemical and Biophysical Research Communications **290,** 185–190 (2002)
doi:10.1006/bbrc.2001.6153, available online at http://www.idealibrary.com on IDEAL®

Boswellic Acids Activate p42^{MAPK} and p38 MAPK and Stimulate Ca^{2+} Mobilization[1]

Anja Altmann, Lutz Fischer, Manfred Schubert-Zsilavecz, Dieter Steinhilber, and Oliver Werz[2]
Institute of Pharmaceutical Chemistry, University of Frankfurt, D-60439 Frankfurt, Germany

Received November 7, 2001

Here we show that extracts of *Boswellia serrata* gum resins and its constituents, the boswellic acids (BAs), activate the mitogen-activated protein kinases (MAPK) p42^{MAPK} and p38 in isolated human polymorphonuclear leukocytes (PMNL). MAPK activation was rapid and transient with maximal activation after 1–2.5 min of exposure and occurred in a dose-dependent manner. The keto-BAs (11-keto-β-BA and 3-*O*-acetyl-11-β-keto-BA) gave substantial kinase activation at 30 μM, whereas other BAs lacking the 11-keto group were less effective. Moreover, 11-keto-BAs induced rapid and prominent mobilization of free Ca^{2+} in PMNL. Inhibitor studies revealed that phosphatidylinositol 3-kinase (PI 3-K) is involved in BA-induced MAPK activation, whereas a minor role was apparent for protein kinase C. MAPK activation by 3-*O*-acetyl-11-β-keto-BA was partially inhibited when Ca^{2+} was removed by chelation. Our results suggest that 11-keto-BAs might function as potent activators of PMNL by stimulation of MAPK and mobilization of intracellular Ca^{2+}.

Key Words: **boswellic acids; polymorphonuclear leukocyte; p38 MAPK; p44/42^{MAPK}; Ca^{2+}; phosphatidylinositol 3-kinase; protein kinase C.**

Abbreviations used: AB, antibody; A-β-BA, 3-*O*-acetyl-β-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-β-boswellic acid; KBA, 11-keto-β-boswellic acid; β-BA, β-boswellic acid; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; JNK, c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; PAF, platelet-activating factor; PBS, phosphate-buffered saline, pH 7.4; PG buffer, PBS containing 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PI 3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMNL, polymorphonuclear leukocytes; SDS-b, 2× SDS–PAGE sample loading buffer; WB, Western blotting.

[1] This study was supported by grants from the EU (QLG1-CT-2001-01521).

[2] To whom correspondence and reprint requests should be addressed at Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie Strasse 9, D-60439 Frankfurt, Germany. Fax: +69-798 29323. E-mail: o.werz@pharmchem.uni-frankfurt.de.

Boswellic acids (BAs) are pentacyclic triterpenes that have been identified as the active principles of Frankincense, the gum resin of *Boswellia* species. BA derivatives inhibit human leukocyte elastase *in vitro* (1) and suppress the biosynthesis of proinflammatory leukotrienes by direct inhibition of leukocyte 5-lipoxygenase (2), which could explain the anti-inflammatory properties of Frankincense stated in several experimental animal models and clinical trials, for review see (3). In addition, BAs inhibit topoisomerases in myeloid cells and have been shown to induce differentiation and apoptosis in leukemia cell lines (4–6) as well as apoptosis in malignant glioma cells (7). However, little is known about the effects of BAs on biochemical and cellular signaling pathways in leukocytes.

Transduction of extracellular signals leading to a diverse array of cellular responses includes the activation of specific kinases. The mammalian mitogen-activated protein kinase (MAPK) superfamily consists of at least four distinct groups, organized in signaling modules (MEKK/MEK/MAPK) that transmit extracellular signals by sequential phosphorylation and activation of the components of a respective cascade: the extracellular signal-regulated kinases 1 and 2 (ERK1/2, also termed p44/42^{MAPK}), the p38 MAPKs, the c-Jun NH_2 terminal kinases (JNKs), and the Big MAPK 1 (also termed ERK5) (8, 9). Although structurally related, these kinases can act on different molecular substrates (transcription factors, protein kinases) and their actions may lead to distinct and sometimes opposite biological functions (10). Whereas p44/42^{MAPK} are mainly activated in response to mitogenic stimuli, such as growth factors and G-protein-coupled receptor agonists, p38 MAPK, JNKs and ERK5 are activated in response to various forms of cell stress or cytokines (8, 11). The p44/42^{MAPK} pathway may play a pivotal role in cell growth, differentiation and cellular transformation, but also can regulate cellular events, such as secretion and cell motility (10). In neutrophils, the p44/42^{MAPK} pathway has been proposed to play a certain role in neutrophil functions, in response to appropriate external stimuli (12–14). p38 MAPK activation

0006-291X/02 $35.00

results in the production of TNFα and IL-1 and has been implicated in granulocyte apoptosis, adhesion, degranulation, chemotaxis and oxidative burst [reviewed in (15)]. In this study we addressed the effects of BAs on various signaling pathways important for cellular responses of neutrophils, particularly the activation of MAPK pathways and the mobilization of Ca^{2+}.

MATERIALS AND METHODS

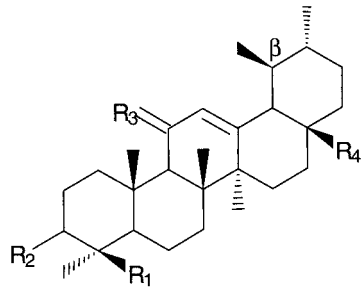
Materials. Ethanolic extracts of *B. serrata* were obtained from Engelhard Arzneimittel GmbH (Niederdorfelden, Germany); A-β-BA, β-BA, AKBA, and KBA were purchased from ChromaDex (Laguna Hills, CA). α-Amyrin and ursolic acid were from Extrasynthèse (Genay, France). Activated (rat, recombinant) $p42^{MAPK}$ isoform was from Biomol; [γ-^{32}P]ATP (110 TBq/mmol) was purchased from Amersham–Pharmacia Biotech (Freiburg, Germany). Materials and reagents: Nycoprep, PAA Laboratories (Linz, Austria); Ca^{2+}-ionophore A23187, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), and myelin basic protein (MBP) were from Sigma (Deisenhofen, Germany). BAPTA/AM and Fura-2/AM were from Calbiochem (Bad Soden, Germany). RO-31-8425 was from Alexis, Switzerland, and GF109203x and wortmannin, from Biotrend (Colonia, Germany).

Cells. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation for preparation of leukocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously (16). PMNL (5×10^6 cells/ml; purity > 96–97%) were finally resuspended in PBS plus 1 mg/ml glucose (PG buffer), or alternatively in PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated.

Measurement of intracellular Ca^{2+} levels. Freshly isolated PMNL (1×10^7 in 1 ml PGC buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C. Cells were washed, resuspended in 1 ml PGC buffer and transferred into a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Aminco–Bowman Series 2) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al.* (17). F_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

MAPK activation. Freshly isolated PMNL (5×10^6) were resuspended in PGC buffer or in PG buffer containing 1 mM EDTA and/or 30 μM BAPTA/AM, final volume was 100 μl. After addition of the indicated stimuli, samples were incubated at 37°C and the reaction was stopped by addition of 100 μl of ice-cold 2× SDS–PAGE sample loading buffer [SDS-b: 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol], vortexed, and heated for 6 min at 95°C. Twenty microliters of total cell lysates was analyzed for activated MAPK by SDS–PAGE and Western blotting (WB) or by in-gel kinase assay.

SDS–PAGE and Western blotting. Total cell lysates (20 μl) were mixed with 4 μl of glycerol/0.1% bromophenol blue (1:1, v/v) and analyzed by SDS–PAGE on a 10% gel. After electroblot to nitrocellulose membrane (Amersham–Pharmacia), blocking with 5% nonfat dry milk for 1 h at RT, membranes were washed and incubated with primary antibody for overnight at 4°C. Phospho-specific antibodies (AB) recognizing $p44/42^{MAPK}$ (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), and JNK (Thr183/Tyr185) were obtained from New England Biolabs, Inc., and used as 1:2000 dilution. The membranes were washed and incubated with 1:1000 dilution of alkaline phosphatase conjugated IgGs (Sigma) for 2 h at RT. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).



R_1=COOH	R_2= α-OAc	R_3=O	R_4=H	AKBA
R_1=COOH	R_2= α-OH	R_3=O	R_4=H	KBA
R_1=COOH	R_2= α-OAc	R_3=H_2	R_4=H	Acetyl-β-BA
R_1=COOH	R_2= α-OH	R_3=H_2	R_4=H	β-BA
R_1=H	R_2= β-OH	R_3=H_2	R_4=H	α-Amyrin
R_1=H	R_2= β-OH	R_3=H_2	R_4=COOH	β-BA

FIG. 1. Structures of boswellic acids and derivatives.

In-gel kinase assay. Total cell lysates of PMNL corresponding to 0.5×10^6 cells were analyzed for $p42^{MAPK}$ activity by in-gel kinase assay using MBP (0.5 mg/ml) as substrate as described (18). Phosphorylated proteins were visualized using a Fuji Phosphorimager FLA-3000.

RESULTS

Boswellic Acids Activate p38 MAPK and $p42^{MAPK}$ in Human Isolated PMNL

Ethanolic extracts of *B. serrata* resin as well as the four major pentacyclic triterpenic acids present, namely β-boswellic acid (β-BA), 3-*O*-acetyl-β-boswellic acid (A-β-BA), 11-keto-β-boswellic acid (KBA), and 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) (Fig. 1), were assayed for activation of MAPK in freshly isolated PMNL from human peripheral blood. Ca^{2+}-ionophore A23187 was utilized as positive control (18). After stimulation, cells were lysed and total cell lysates were subjected to WB using phospho-specific AB against $p44/42^{MAPK}$, p38 MAPK, and JNKs. In addition, MAPK activities of total cell lysates were assessed by in-gel kinase assay using MBP as substrate.

As can be seen from Fig. 2A, exposure of PMNL to crude extracts of *B. serrata* (0.38 to 13 μg/ml) for 3 min led to a dose-dependent activation of $p42^{MAPK}$ and p38 MAPK, whereas $p44^{MAPK}$ and JNKs (not shown) seemed not to be activated. Similarly, KBA and AKBA (30 μM each, corresponding to $\approx$3.8 μg/ml *B. serrata* extracts) caused activation of $p42^{MAPK}$ and p38 MAPK 1.5 min after addition, whereas β-BA and A-β-BA (30 μM for 1.5 min, each) did not activate $p42^{MAPK}$ and caused only slight activation of p38 MAPK (Fig. 2B). AKBA also slightly activated $p44^{MAPK}$ and was more efficient in

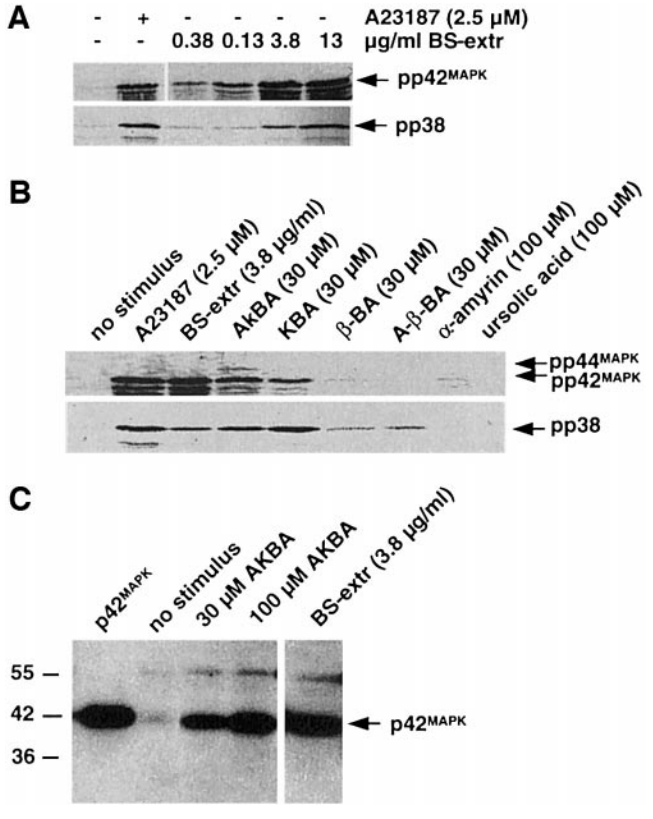


FIG. 2. Activation of MAPK by extracts of *B. serrata* and isolated BAs. To determine activation of p38 MAPK and p44/42^{MAPK}, freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with the indicated amounts of *B. serrata* extracts (BS-extr) for 3 min at 37°C (A) or with BS-extr., and the compounds indicated in the figure (B) for 1.5 min at 37°C. After addition of the same volume of ice-cold SDS-b, samples were analyzed for dually phosphorylated p38 MAPK (pp38) or p44/42^{MAPK} (pp44/42^{MAPK}) by WB. (C) Samples from above were assayed for p42^{MAPK} activity by in-gel kinase assay using MBP (0.5 mg/ml) as substrate. Purified recombinant active 42^{MAPK} (10 mU) was used as positive control. The position of p42^{MAPK} (42 kDa) in the gel is indicated. Results are representative of at least three separate experiments.

p42^{MAPK} activation than KBA. However, KBA was virtually equally effective as AKBA in activation of p38 MAPK. The pentacyclic triterpenes α-amyrin and ursolic acid up to 100 μM gave no MAPK activation (Fig. 2B).

Activation of p42^{MAPK} by BAs was confirmed by in-gel kinase assays. Thus in agreement with the results from above (Fig. 2A), cell stimulation by extracts of *B. serrata* resin (3.8 μg/ml) and by AKBA (30 and 100 μM) led to prominent kinase activity (Fig. 2C). Kinase activities were rather low for cells treated with KBA, β-BA and A-β-BA (100 μM each, not shown).

The dose responses for MAPK activation by KBA and AKBA were determined. Both, p42^{MAPK} and p38 MAPK became clearly activated in cells treated with 30 μM AKBA for 1.5 min (Fig. 3A). In contrast, 100 μM KBA was needed for a clear activation of p42^{MAPK}. However, 10 to 30 μM KBA gave substantial activation of p38 MAPK (Fig. 3A). Again, for β-BA and A-β-BA (100 μM each) only weak MAPK activation was obtained (not shown), indicating that the 11-keto group is a structural requirement for substantial activation of MAPKs. Activation of both p42^{MAPK} and p38 MAPK by BAs was rapid and occurred within 30 s, peaking around 1 to 2.5 min and declining after about 10 min (Fig. 3B). In this respect, no appreciable differences between the various BAs were observed.

Boswellic Acids Induce Ca^{2+} Mobilization in PMNL

We considered the possibility that BAs could induce Ca^{2+} mobilization in PMNL, one of the hallmarks of PMNL activation in response to various agonists. As shown in Fig. 4, exposure of freshly isolated PMNL to AKBA or KBA (30 μM, each) caused a rapid (within 10 to 15 s) and prominent elevation of intracellular Ca^{2+}, that was comparable to the effect of chemotactic fMLP, used as a positive control. Thus, intracellular Ca^{2+} levels of unstimulated cells were about 40–50 nM, rising to about 240 nM upon stimulation with 1 μM fMLP. Upon exposure to 10 and 30 μM AKBA, intracellular Ca^{2+} increased to about 120 and 230 nM, respectively. Similarly, 10 and 30 μM KBA raised intracellular Ca^{2+} to 105 and 145 nM. In contrast, β-BA, A-β-BA, α-amyrin or ursolic acid (30 μM each) were virtually not effective (not shown).

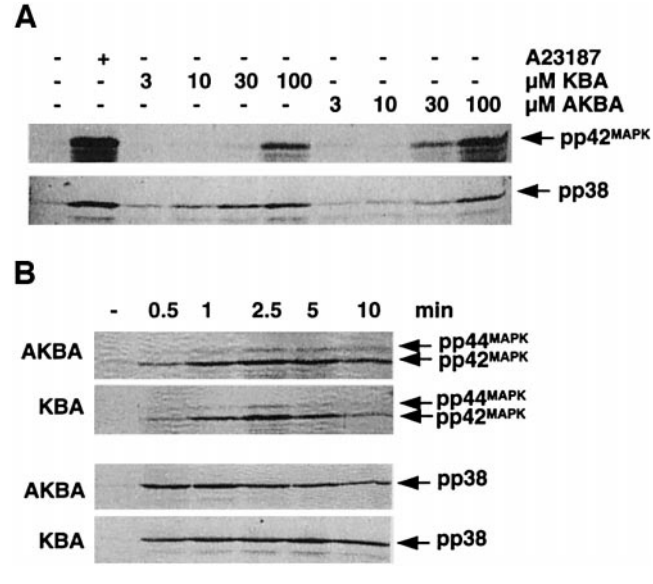


FIG. 3. Dose response and time course of MAPK activation by AKBA and KBA. (A) Dose response. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with the indicated amounts of AKBA or KBA for 1.5 min at 37°C, ionophore A23187 (2.5 μM) was used as positive control. (B) Time course. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were stimulated with 30 μM AKBA or 100 μM KBA for the indicated times at 37°C. All incubations were terminated by addition of the same volume of ice-cold SDS-b and analyzed for dually phosphorylated p38 MAPK or p44/42^{MAPK} by WB.

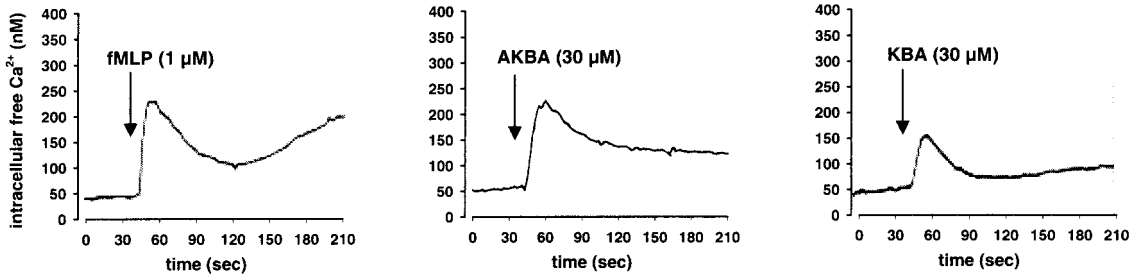


FIG. 4. BAs induce the mobilization of Ca^{2+}. To Fura-2-loaded PMNL (1×10^7/ml PGC buffer) the indicated stimuli were added and the fluorescence was measured. Intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment of three or four.

Role of Ca^{2+}, PKC, and PI 3-K in AKBA-Induced MAPK Activation

We attempted to elucidate the upstream signaling pathways leading to activation of p38 MAPK and $p42^{MAPK}$ induced by AKBA. Activation of MAPK by various agonists [fMLP, platelet-activating factor (PAF)] was shown to depend in part on Ca^{2+}. Removal of extracellular Ca^{2+} by EDTA and/or depletion of intracellular Ca^{2+} by cell-permeable BAPTA/AM only partially suppressed AKBA-induced activation of $p42^{MAPK}$ (Fig. 5A). Thus Ca^{2+} seems to play a minor role in AKBA-induced MAPK activation.

Next, the involvement of protein kinase C (PKC) and PI 3-K in MAPK activation was investigated. As depicted from Fig. 5B (WB) and C (in-gel kinase assay), inhibition of PKC by RO-31-8425 (1 μM) slightly attenuated activation of $p42^{MAPK}$ and p38 MAPK induced by 30 μM AKBA, whereas GF109203x (1 μM) rather enhanced $p42^{MAPK}$ activation. Thus particular isoforms of PKC (affected by RO-31-8425) could be partially involved in activation of $p42^{MAPK}$ and p38 MAPK. Wortmannin, an inhibitor of PI 3-K, was used to determine a possible participation of PI 3-K in the upstream activation of MAPKs. As shown in Figs. 5B and 5C, wortmannin strongly attenuated $p42^{MAPK}$ activation evoked by 30 μM AKBA and reduced p38 MAPK activation, indicating an involvement of PI 3-K in the upstream cascades required for AKBA-induced MAPK signaling. Similar results were obtained when KBA (100 μM) was used as stimulus (not shown).

DISCUSSION

Here we demonstrate that BAs the major constituents of extracts of *B. serrata* gum can stimulate important cellular signaling pathways in isolated human PMNL. Thus, 11-keto-BAs caused substantial activation of $p42^{MAPK}$ and p38 MAPK, and induced the mobilization of intracellular Ca^{2+}. Recently, the tetracyclic triterpene 3-oxo-tirucallic acid was identified in *B. serrata* extracts as a component that enhanced 5-LO product formation, accompanied with moderate Ca^{2+} mobilization, and that increased phosphorylation of MEK-1/2 (19).

Activation of $p42^{MAPK}$ and p38 MAPK was determined by WB using specific AB that detect only the dually phosphorylated (active) form of the kinases, and was confirmed by in-gel kinase assays using MBP as substrate, a suitable method to detect $p44/42^{MAPK}$ activities in PMNL extracts (13). Activation of both MAPKs by BAs was rapid and transient, and concentrations of 10 to 100 μM of AKBA or KBA necessary to mobilize Ca^{2+} or to activate MAPKs, are in the range of the concentrations required for various biological effects observed by others (1, 4). The presence of the 11-keto-group appears important for the effects of BAs, since β-BA and A-β-BA were much less efficient than AKBA or KBA in MAPK activation, and only AKBA and KBA caused Ca^{2+} mobilization in PMNL. Notably, the keto group was also required for efficient inhibition of agonist-induced leukotriene formation in neutrophils (20) and for pronounced inhibitory effects on DNA, RNA and protein synthesis in HL60 cells (21). Although no receptors for BAs have been identified yet, it is possible that 11-keto-BAs may bind a certain cell surface receptor transducing the signal to the respective cellular target(s). The 11-keto group might be a structural determinant for interaction with such a putative receptor(s).

The effects of BAs elicited in PMNL in this study resemble those evoked by chemotactic factors. Thus, similar as 11-keto-BAs, fMLP and PAF induced the activation of $p42^{MAPK}$ and p38 MAPK [but not of JNKs (22)] and caused rapid mobilization of intracellular Ca^{2+} in PMNL (23–26). The characteristics of fMLP-induced MAPK activation, such as the time course, the partial dependence on Ca^{2+} as well as on PKC, and the involvement of PI 3-K, particularly in the activation of 42^{MAPK} (22, 25, 27, 28), are conform with those induced by AKBA or KBA. Therefore, BAs might share signal transduction pathways with fMLP in PMNL. G-proteins couple the fMLP-receptor interaction with subsequent biochemical and functional responses (29) and it was concluded that fMLP-induced activation of MAPK is a consequence of ligation of the

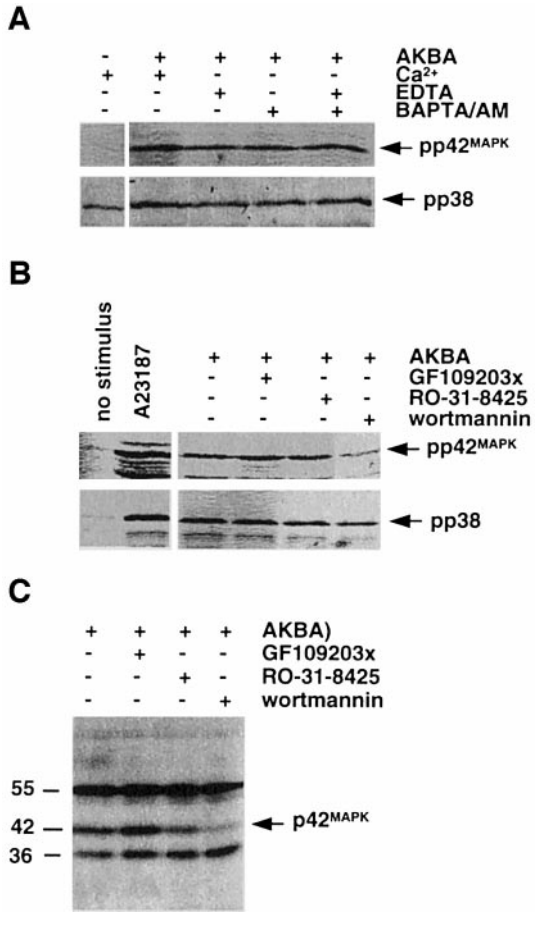


FIG. 5. Effects of Ca^{2+}-depletion, PKC and PI 3-K inhibitors on MAPK activation induced by AKBA. (A) Effects of Ca^{2+} chelation. $CaCl_2$ (1 mM), EDTA (1 mM), and BAPTA/AM (30 μM) were added to 5×10^6 freshly isolated PMNL in PG buffer as indicated. After 15 min at 37°C, 30 μM AKBA was added and the incubations were continued for another 1.5 min. Then, incubations were terminated by addition of the same volume of ice-cold SDS-b. (B, C) Effects of PKC and PI 3-K inhibition. GF109203x, RO-31-8425 (1 μM each), and wortmannin (0.2 μM) were added to 5×10^6 freshly isolated PMNL in 100 μl PG buffer as indicated. After 15 min at 37°C, AKBA (30 μM) was added and the incubations were continued for another 1.5 min. Then, incubations were terminated by addition of the same volume of ice-cold SDS-b and analyzed for dually phosphorylated p38 MAPK or $p44/42^{MAPK}$ by WB (B) or analyzed for $p42^{MAPK}$ by in-gel kinase assay (C). The position of $p42^{MAPK}$ is indicated, bands at 55 and 36 kDa may result from other unidentified kinases. Results are representative of at least three separate experiments.

fMLP receptor (30). Thus, it is reasonable to speculate that BAs could directly activate G-protein-coupled receptors. Alternatively, BAs might act indirectly by stimulation of mediator release that bind such receptors leading to activation of MAPK. Future work is necessary to elucidate such mechanisms.

PKC isoforms have been implicated as components in the signal transduction cascade resulting in activation of $p44/42^{MAPK}$ as well as of p38 MAPK in PMNL. RO-31-8524 only partially inhibited AKBA-induced MAPK activation, and GF109203x even increased the effects of AKBA for unknown reasons. Similarly, minor importance of PKC was proposed in the upstream regulation of fMLP-induced activation of $p44/42^{MAPK}$ and p38 MAPK in neutrophils (28). In PMNL, rapid activation of the PI 3-K occurs in response to various agonists like fMLP or arachidonic acid (31), which for example can regulate chemotaxis (32). It was shown that in PMNL, PI 3-K is partially involved in agonist-induced $p44/42^{MAPK}$ activation (25, 28), but apparently plays a minor role in the activation of p38 MAPK (28, 33). Wortmannin, a selective inhibitor of PI 3-K, considerably suppressed the AKBA-induced activation of $p42^{MAPK}$ and also reduced p38 MAPK activation (Figs. 5B and 5C). Thus, PI 3-K might be an important component in the signal transduction of $p42^{MAPK}$ activation induced by AKBA.

Rapid elevation of intracellular Ca^{2+} mediates many PMNL responses to receptor agonists (34). Ca^{2+} has been implicated in the regulation of protein phosphorylation (35), and Ca^{2+} selective ionophores (ionomycin, A23187) caused strong activation of $p44/42^{MAPK}$ and p38 MAPK in human neutrophils (36, 37). AKBA and KBA rapidly increased intracellular Ca^{2+} levels in PMNL, with comparable time courses and magnitudes as observed for fMLP (Fig. 4) and this might be the reason for AKBA-induced MAPK activation, as it was observed after stimulation with Ca^{2+} ionophores. In agreement with the results of Ferby *et al.* using PAF as neutrophil agonist (25), depletion of Ca^{2+} reduced the magnitude of AKBA-induced MAPK only partially, indicating that alternative mechanisms than elevated Ca^{2+} may contribute to MAPK activation by BAs.

In summary we could demonstrate, that extracts of *B. serrata* gum and isolated 11-keto-BAs can mobilize intracellular Ca^{2+} and stimulate the activation of $p42^{MAPK}$ and p38 MAPK in PMNL. Since these pivotal signaling events regulate numerous effectors of PMNL, such as superoxide production, phagocytosis of particles, release of lysosomal enzymes, and the release of lipid mediators, it remains a future challenge to investigate whether BAs are capable of stimulating such PMNL functions.

REFERENCES

1. Safayhi, H., Rall, B., Sailer, E. R., and Ammon, H. P. (1997) *J. Pharmacol. Exp. Ther.* **281,** 460–463.
2. Safayhi, H., Mack, T., Sabieraj, J., Anazodo, M. I., Subramanian, L. R., and Ammon, H. P. (1992) *J. Pharmacol. Exp. Ther.* **261,** 1143–1146.
3. Safayhi, H., and Sailer, E. R. (1997) *Planta Med.* **63,** 487–493.
4. Hoernlein, R. F., Orlikowsky, T., Zehrer, C., Niethammer, D., Sailer, E. R., Simmet, T., Dannecker, G. E., and Ammon, H. P. (1999) *J. Pharmacol. Exp. Ther.* **288,** 613–619.
5. Jing, Y., Nakajo, S., Xia, L., Nakaya, K., Fang, Q., Waxman, S., and Han, R. (1999) *Leuk. Res.* **23,** 43–50.
6. Syrovets, T., Buchele, B., Gedig, E., Slupsky, J. R., and Simmet, T. (2000) *Mol. Pharmacol.* **58,** 71–81.

7. Glaser, T., Winter, S., Groscurth, P., Safayhi, H., Sailer, E. R., Ammon, H. P., Schabet, M., and Weller, M. (1999) *Br. J. Cancer* **80,** 756–765.
8. Kyriakis, J. M., and Avruch, J. (1996) *Bioessays* **18,** 567–577.
9. Cobb, M. H. (1999) *Prog. Biophys. Mol. Biol.* **71,** 479–500.
10. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci.* **20,** 117–122.
11. Abe, J., Kusuhara, M., Ulevitch, R. J., Berk, B. C., and Lee, J. D. (1996) *J. Biol. Chem.* **271,** 16586–16590.
12. Kuroki, M., and O'Flaherty, J. T. (1997) *Biochem. Biophys. Res. Commun.* **232,** 474–477.
13. Capodici, C., Pillinger, M. H., Han, G., Philips, M. R., and Weissmann, G. (1998) *J. Clin. Invest.* **102,** 165–175.
14. Downey, G. P., Butler, J. R., Tapper, H., Fialkow, L., Saltiel, A. R., Rubin, B. B., and Grinstein, S. (1998) *J. Immunol.* **160,** 434–443.
15. Herlaar, E., and Brown, Z. (1999) *Mol. Med. Today* **5,** 439–447.
16. Böyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **97**(Suppl. 21), 77–89.
17. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
18. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5261–5266.
19. Boden, S. E., Schweizer, S., Bertsche, T., Dufer, M., Drews, G., and Safayhi, H. (2001) *Mol. Pharmacol.* **60,** 267–273.
20. Sailer, E. R., Schweizer, S., Boden, S. E., Ammon, H. P., and Safayhi, H. (1998) *Eur. J. Biochem.* **256,** 364–368.
21. Shao, Y., Ho, C. T., Chin, C. K., Badmaev, V., Ma, W., and Huang, M. T. (1998) *Planta Med.* **64,** 328–331.
22. Nick, J. A., Avdi, N. J., Young, S. K., Knall, C., Gerwins, P., Johnson, G. L., and Worthen, G. S. (1997) *J. Clin. Invest.* **99,** 975–986.
23. Naccache, P. H., Volpi, M., Showell, H. J., Becker, E. L., and Sha'afi, R. I. (1979) *Science* **203,** 461–463.
24. Ng, D. S., and Wong, K. (1989) *Res. Commun. Chem. Pathol. Pharmacol.* **64,** 351–354.
25. Ferby, I. M., Waga, I., Sakanaka, C., Kume, K., and Shimizu, T. (1994) *J. Biol. Chem.* **269,** 30485–30488.
26. Thompson, H. L., Marshall, C. J., and Saklatvala, J. (1994) *J. Biol. Chem.* **269,** 9486–9492.
27. Ferby, I., Waga, I., Kume, K., Sakanaka, C., and Shimizu, T. (1996) *Adv. Exp. Med. Biol.* **416,** 321–326.
28. Krump, E., Sanghera, J. S., Pelech, S. L., Furuya, W., and Grinstein, S. (1997) *J. Biol. Chem.* **272,** 937–944.
29. Lad, P. M., Olson, C. V., and Smiley, P. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 869–873.
30. Thompson, H. L., Shiroo, M., and Saklatvala, J. (1993) *Biochem. J.* **290,** 483–488.
31. Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. M., and Sklar, L. A. (1989) *J. Biol. Chem.* **264,** 15668–15673.
32. Wymann, M. P., Sozzani, S., Altruda, F., Mantovani, A., and Hirsch, E. (2000) *Immunol. Today* **21,** 260–264.
33. Chang, L. C., and Wang, J. P. (2000) *J. Pharm. Pharmacol.* **52,** 539–546.
34. O'Flaherty, J. T., Rossi, A. G., Jacobson, D. P., and Redman, J. F. (1991) *Biochem. J.* **277,** 705–711.
35. Rollet, E., Caon, A. C., Roberge, C. J., Liao, N. W., Malawista, S. E., McColl, S. R., and Naccache, P. H. (1994) *J. Immunol.* **153,** 353–363.
36. Elzi, D. J., Bjornsen, A. J., MacKenzie, T., Wyman, T. H., and Silliman, C. C. (2001) *Am. J. Physiol. Cell Physiol.* **281,** C350–C360.
37. Werz, O., Buerkert, E., Samuelsson, B., Radmark, O., and Steinhilber, D. (2001) *Blood,* in press.

VI

British Journal of Pharmacology (2004) 141, 223–232
www.nature.com/bjp

Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes

[1]Anja Altmann, [1]Daniel Poeckel, [1]Lutz Fischer, [1]Manfred Schubert-Zsilavecz, [1]Dieter Steinhilber & *,[1]Oliver Werz

[1]Institute of Pharmaceutical Chemistry, University of Frankfurt, Marie-Curie Strasse 9, Frankfurt D-60439, Germany

1 We have previously shown that 11-keto boswellic acids (11-keto-BAs), the active principles of *Boswellia serrata* gum resins, activate p38 MAPK and $p42/44^{MAPK}$ and stimulate Ca^{2+} mobilisation in human polymorphonuclear leucocytes (PMNL).

2 In this study, we attempted to connect the activation of MAPK and mobilisation of Ca^{2+} to functional responses of PMNL, including the formation of reactive oxygen species (ROS), release of arachidonic acid (AA), and leukotriene (LT) biosynthesis.

3 We found that, in PMNL, 11-keto-BAs stimulate the formation of ROS and cause release of AA as well as its transformation to LTs *via* 5-lipoxygenase.

4 Based on inhibitor studies, 11-keto-BA-induced ROS formation is Ca^{2+}-dependent and is mediated by NADPH oxidase involving PI 3-K and $p42/44^{MAPK}$ signalling pathways. Also, the release of AA depends on Ca^{2+} and $p42/44^{MAPK}$, whereas the pathways stimulating 5-LO are not readily apparent.

5 Pertussis toxin, which inactivates $G_{i/0}$ protein subunits, prevents MAPK activation and Ca^{2+} mobilisation induced by 11-keto-BAs, implying the involvement of a $G_{i/0}$ protein in BA signalling.

6 Expanding studies on differentiated haematopoietic cell lines (HL60, Mono Mac 6, BL41-E-95-A) demonstrate that the ability of BAs to activate MAPK and to mobilise Ca^{2+} may depend on the cell type or the differentiation status.

7 In summary, we conclude that BAs act *via* $G_{i/0}$ protein(s) stimulating signalling pathways that control functional leucocyte responses, in a similar way as chemoattractants, that is, *N*-formyl-methionyl-leucyl-phenylalanine or platelet-activating factor.
British Journal of Pharmacology (2004) **141,** 223–232. doi:10.1038/sj.bjp.0705604

Keywords: Boswellic acids; leucocytes; MAPK; Ca^{2+}; reactive oxygen species; lipoxygenase; arachidonic acid

Abbreviations: AA, arachidonic acid; AB, antibody; A-β-BA, 3-*O*-acetyl-β-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-β-boswellic acid; β-BA, β-boswellic acid; $cPLA_2$, cytosolic phospholipase A_2; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenyleneiodonium chloride; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GPCR, G protein-coupled receptor; KBA, 11-keto-β-boswellic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PG buffer, PBS pH 7.4 containing 1 mg ml^{-1} glucose; PGC buffer, PBS containing 1 mg ml^{-1} glucose and 1 mM $CaCl_2$; PI 3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; PMNL, polymorphonuclear leucocytes; PTX, pertussis toxin; ROS, reactive oxygen species; SDS-b, 2 × SDS–PAGE sample-loading buffer; TGFβ, transforming growth factor β; WB, Western blotting

Introduction

Extracts of *Boswellia serrata* gum resins have been traditionally used as folk medicine to cure inflammatory and arthritic diseases (Safayhi & Sailer, 1997). It was found that *B. serrata* extracts suppress the formation of proinflammatory leukotrienes (LTs), and boswellic acids (BAs) were identified as the active principles targeting 5-lipoxygenase (5-LO), the key enzyme in LT biosynthesis (Safayhi *et al*., 1992; 1995). In addition, human leucocyte elastase was found to be a target for BAs (Safayhi *et al*., 1997). Aside of these anti-inflammatory implications, BAs have been reported to influence the growth and differentiation of tumour cells. Thus, *B. serrata* extracts or isolated BAs induce the apoptosis of brain tumour cell lines (Glaser *et al*., 1999), meningioma cells (Park *et al*., 2002), rat gliomas (Winking *et al*., 2000), liver and colon cancer cell lines (Liu *et al*., 2002a), and also of leukaemic cells (Hoernlein *et al*., 1999). Caspase-8 (Liu *et al*., 2002a), topoisomerases (Hoernlein *et al*., 1999), and the $p42/44^{MAPK}$ pathway (Park *et al*., 2002) have been suggested as signalling molecules mediating the apoptotic effects of BAs.

With respect to inhibition of 5-LO, 3-acetyl-11-keto-BA (AKBA) was the most potent BA, whereas BAs lacking an 11-keto-group were weak 5-LO inhibitors (Safayhi *et al*., 1992).

*Author for correspondence;
E-mail: o.werz@pharmchem.uni-frankfurt.de
Advance online publication: 22 December 2003

The IC_{50} values of AKBA for inhibition of 5-LO differ between different groups and appear to depend also on the cell type. Thus, the IC_{50} values were determined in the range of 1.5 μM for 5-LO in rat neutrophils (Safayhi *et al*., 1992) and 12–15 μM in differentiated HL60 and MM6 cells, respectively (Werz *et al*., 1997). In cell-free systems, the IC_{50} values of AKBA for 5-LO inhibition were around 50 μM, implying that, in intact cells, cellular components or mechanisms improve the efficacy of AKBA (Werz *et al*., 1997). Finally, low concentrations of ethanolic extracts of *B. serrata* potentiated 5-LO product formation in PMNL induced by ionophore (Safayhi *et al*., 2000), and 3-oxo-tirucallic acid, isolated from *B. serrata* extracts, stimulated 5-LO product synthesis in resting and agonist-challenged PMNL (Boden *et al*., 2001). Therefore, until today, inhibition of 5-LO as the main principle for the anti-inflammatory effects of *B. serrata* extracts, determined in several *in vivo* models (Gupta *et al*., 1992; Krieglstein *et al*., 2001) and pilot clinical studies (Gerhardt *et al*., 2001; Gupta *et al*., 2001), remains a matter of debate.

Chemotactic agonists, such as platelet-activating factor (PAF), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), or LTB_4, bind to their specific G protein-coupled receptors (GPCR), leading to the activation of MAPK and the mobilisation of Ca^{2+}, which are pivotal signalling molecules that regulate a number of functional processes of PMNL, including chemotaxis, degranulation, formation of reactive oxygen species (ROS), release of arachidonic acid (AA), and LT biosynthesis (Herlaar & Brown, 1999; Belcheva & Coscia, 2002; Johnson & Druey, 2002). Recently, we showed that 11-keto-BAs (AKBA, KBA, in the range of 10–30 μM) are potent activators of $p42^{MAPK}$ and p38 MAPK, and stimulate Ca^{2+} mobilisation in PMNL (Altmann *et al*., 2002). Comparison of these actions of BAs with those of chemotactic agonists led us to conclude that BAs may possibly function as ligands of a certain GPCR. In this study, we investigated if BAs are able to induce the functional cellular responses in PMNL and if a $G_{i/0}$-coupled heterotrimeric G protein mediates BA-induced MAPK/Ca^{2+} signalling. Finally, we demonstrate that BAs induce signalling responses depending on the cell type.

Methods

Materials

A-β-BA, β-BA, AKBA, and KBA were purchased from ChromaDex (Laguna Hills, CA, U.S.A.). Nycoprep was from PAA Laboratories (Linz, Austria); diphenyleneiodonium chloride (DPI), phorbol 12-myristate 13-acetate (PMA), Ca^{2+}-ionophore A23187, and fMLP were from Sigma Chemical Co. (Deisenhofen, Germany); BAPTA/AM, LY-294002, SB203580, U0126, and Fura-2/AM were from Calbiochem (Bad Soden, Germany); RO-31-8425 was from Alexis (Grünberg, Germany); 2′,7′-dichlorofluorescein diacetate (DCF-DA) and lucigenin were from Molecular Probes European BV (Leiden, Netherlands); and wortmannin from Biotrend (Colonia, Germany). RPMI-1640 medium was from Gibco-BRL (Grand Island, NY, U.S.A.), and fetal calf serum was obtained from Boehringer Mannheim (Mannheim, Germany). Human TGF-β1 was purified from outdated platelets, as described (Werz *et al*., 1996). Calcitriol was kindly provided by Dr H. Wiesinger (Schering AG). [^{3}H]AA was from Biotrend (Colonia, Germany); and high-performance liquid chromatography (HPLC) solvents were from Merck (Darmstadt, Germany).

Cells

Human PMNL were freshly isolated from leucocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation for the preparation of leucocyte concentrates. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes, as described (Werz *et al*., 2002a). PMNL were finally resuspended in PBS plus 1 mg ml^{-1} glucose (PG buffer), or alternatively in PBS plus 1 mg ml^{-1} glucose and 1 mM $CaCl_2$ (PGC buffer), as indicated.

HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg ml^{-1} streptomycin, and 100 U ml^{-1} penicillin. For differentiation towards granulocytic cells, cells were seeded at a density of 2×10^5 cells ml^{-1} and cultured in the presence of 1.5% (v v^{-1}) dimethylsulphoxide, 500 pM calcitriol, and 2 ng ml^{-1} TGFβ for 4 days.

Mono Mac (MM) 6 cells were cultured and differentiated with TGFβ and calcitriol, as described (Werz *et al*., 1996).

Determination of release of [^{3}H]-labelled AA from PMNL

Freshly isolated PMNL were resuspended at 2×10^6 in 1 ml RPMI 1640 medium containing 4.8 nM [^{3}H]AA (corresponding to 0.25 μCi ml^{-1}, specific activity 200 Ci mmol^{-1}) and incubated for 120 min at 37°C in 5% CO_2 atmosphere. Thereafter, the cells were collected by centrifugation, washed once with PBS and twice with PBS containing 2 mg ml^{-1} fatty acid-free albumin, to remove unincorporated [^{3}H]AA. Labelled PMNL (5×10^6) were resuspended in 1 ml PGC buffer containing 2 mg ml^{-1} fatty acid-free albumin. The reaction was started by addition of the indicated stimuli. After 5 min at 37°C, the samples were placed on ice for 2 min and cells were centrifuged at $400 \times g$ for 5 min at RT. Aliquots (100 μl) of the supernatants were measured in a beta-counter (Micro Beta Trilux, Perkin Elmer, Foster City, CA, U.S.A.) to detect the amounts of [^{3}H]-labelled AA released into the medium.

Determination of cellular peroxide formation

Measurement of peroxides was conducted using the peroxide-sensitive fluorescence dye DCF-DA. Freshly isolated PMNL (1×10^7), HL 60 cells (1×10^7), or MM6 cells (5×10^6) were resuspended in 1 ml PGC buffer and preincubated with DCF-DA (1 μg ml^{-1} for PMNL and MM6; 10 μg ml^{-1} for HL 60 cells) for 2 min at 37°C in a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, U.S.A.) with continuous stirring. The fluorescence emission at 530 nm was measured after excitation at 485 nm. The mean fluorescence data measured 5 min after stimulus addition are given as arbitrary units.

Detection of superoxide anion

Freshly isolated PMNL (2×10^5) were resuspended in 200 μl of 100 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 20 mM HEPES, pH 7.4, and transferred to a 96-well plate. Lucigenin (25 μM, final concentration) and 1 mM $CaCl_2$ were added and cells were stimulated with the indicated stimuli. After stirring, samples were measured in duplicates at 37°C. The chemiluminescence (CL) was recorded using a Micro Luminat Plus LB 96 V (Berthold, Bad Wildbad, Germany) at intervals of 6 s (two cycles) in a total detection time of 3 min. The detected CL was summarised over two intervals and plotted *versus* blank values.

Measurement of intracellular Ca^{2+} levels

The determination of intracellular Ca^{2+} levels was performed as described previously (Werz, 2002a). In brief, freshly isolated PMNL (1×10^7), HL 60 cells (1×10^7 buffer), or MM6 cells (3×10^6) were resuspended in 1 ml PGC buffer and incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, cells were finally resuspended in 1 ml PGC buffer and transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, U.S.A.) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al.* (1985), whereas F_{max} (maximal fluorescence) was obtained by lysing the cells with 0.5% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Determination of MAPK activation by SDS–PAGE and Western blotting

Freshly isolated PMNL, HL60, or MM6 cells (5×10^6 each) were resuspended in PGC buffer; the final volume was 100 μl. After addition of the indicated stimuli, samples were incubated at 37°C and the reaction was stopped by addition of 100 μl of ice-cold 2× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample-loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS ($w\,v^{-1}$), 10% β-mercaptoethanol), vortexed and heated for 6 min at 95°C. Total cell lysates (20 μl) were mixed with 4 μl of glycerol/0.1% bromophenolblue (1 : 1, $v\,v^{-1}$) and analysed by SDS–PAGE using a Mini Protean system (Bio-Rad, Hercules, CA, U.S.A.) on a 10% gel. After electroblot to nitrocellulose membrane (Amersham Pharmacia, Little Chalfont, U.K.), membranes were blocked with 5% nonfat dry milk in 50 mM Tris/HCl, pH 7.4, and 100 mM NaCl (Tris-buffered saline (TBS)) plus 0.1% Tween 20 for 1 h at RT. Membranes were washed and then incubated with primary antibody (AB) overnight at 4°C. Phospho-specific ABs recognising $p44/42^{MAPK}$ (Thr202/Tyr204) and p38 MAPK (Thr180/Tyr182) were obtained from New England Biolabs (Beverly, MA, U.S.A.), and used as 1 : 2000 dilution. The membranes were washed and incubated with 1 : 1000 dilution of alkaline phosphatase-conjugated immunoglobulin G (Sigma Chemical Co.) for 2 h at RT. After washing with TBS and TBS plus 0.1% NP-40, proteins were visualised with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Determination of 5-LO product formation in PMNL

To determine 5-LO product formation in intact cells, 1×10^7 freshly isolated PMNL were finally resuspended in 1 ml PG buffer. When 5-LO product formation was assayed in the absence of Ca^{2+}, PMNL were finally resuspended in 1 ml PG buffer and 1 mM EDTA and 30 μM BAPTA/AM were added. The reaction was started by simultaneous addition of exogenous AA with the indicated BAs. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol, and 30 μl of 1 N HCl, and 200 ng prostaglandin B_1 and 500 μl of PBS were added. Formed AA metabolites were extracted using C-18 solid-phase extraction columns and analysed by HPLC as described (Werz *et al.*, 1997). 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans* isomers, 5(*S*),12(*S*)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(*S*),12(*S*)-DiHETE), 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), and 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE). 5-HETE and 5-HPETE coelute as one major peak; integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected and oxidation products of LTB_4 were not determined.

Statistics

The results are presented as mean ± s.e. The program 'GraphPad PRISM 3.0' was used for statistical comparisons. Statistical evaluation of the data was performed using Student's *t*-test for unpaired observations. A *P*-value of <0.05 was considered significant.

Results

11-Keto-BAs stimulate the release of superoxide anion and ROS in PMNL

In order to explore the effects of BAs on PMNL, we sought to determine whether BAs are capable of eliciting functional processes connected to Ca^{2+} mobilisation and MAPK activation. Phagocytes undergo an oxidative burst in response to different agonists, resulting in the release of ROS *via* the NADPH oxidase. PMNL, preloaded with the ROS-sensitive dye DCF-DA, were stimulated with BAs (30 μM, each) and for comparison with 1 μM fMLP, 2.5 μM ionophore A23187, and 100 nM PMA. After 5 min, ROS formation was determined by analysing the fluorescence of the oxidised dye. In agreement with the literature (Roos *et al.*, 1976; Simchowitz & Spilberg, 1979), fMLP, ionophore A23187, or PMA caused a rapid formation of ROS in PMNL (Figure 1). AKBA and KBA strongly upregulated the formation of ROS, whereas β-BA and A-β-BA had only moderate effects (Figure 1). Notably, the magnitude of AKBA- (or KBA-) induced ROS formation was much more pronounced as compared to fMLP, being in a close range of the efficacy of ionophore and PMA. Similar results were obtained when the formation of superoxide anion (O_2^-) was determined by measuring the chemiluminescence of the metabolised lucigenin. Thus, stimulation of PMNL with 30 μM AKBA resulted in a 12-fold increase in O_2^- formation compared to unstimulated cells, whereas fMLP (1 μM) and

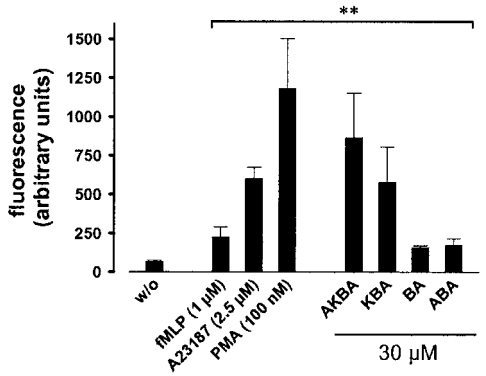


Figure 1 11-Keto-BAs induce the generation of ROS in PMNL. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with DCF-DA (1 μg ml^{-1}) for 2 min at 37°C, prior addition of the indicated stimuli. The generation of peroxides was measured as described. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units $\pm$s.e., $n=4$. Student's t-test; $^{**}P<0.01$.

PMA (100 nM) caused 4- and 20-fold elevations, respectively (not shown).

AKBA-induced ROS formation involves NADPH oxidase, PI 3-K, and p42/44^{MAPK}, and requires Ca^{2+}

To further dissect the signalling pathways leading to ROS formation, pharmacological inhibitors of potential signalling molecules were examined using the DCF-DA fluorescence assay. PMA, used as a control, binds to PKC isoenzymes, stimulating NADPH oxidase by multiple phosphorylations of p47^{phox} (Heyworth & Badwey, 1990). DPI, a direct inhibitor of NADPH oxidase (Hancock & Jones, 1987), almost completely abolished PMA- as well as AKBA-induced ROS formation (Figure 2a). Also, chelation of Ca^{2+} by 30 μM BAPTA/AM and 1 mM EDTA clearly reduced AKBA- or PMA-induced ROS formation (Figure 2a). As shown in Figure 2b, PMA-induced ROS formation was only slightly affected by wortmannin (50 nM) or LY-294002 (30 μM), inhibitors of phosphatidylinositol 3-kinase (PI 3-K), or by the p38 MAPK inhibitor SB203580 (10 μM). However, U0126 (3 μM) that blocks the activation of p42/44^{MAPK} partially reduced ROS generation and the PKC inhibitor RO-318425 (1 μM) totally abolished the effects of PMA. In contrast, AKBA-induced ROS formation was not at all affected by PKC inhibition (RO-318425), but was clearly reduced by wortmannin or LY-294002, and by U0126, implying an involvement of PI 3-K and p42/44^{MAPK}, respectively (Figure 2b). Also, for AKBA-induced ROS formation, a role for p38 MAPK is not apparent.

11-Keto-boswellic acids cause the release of [^{3}H]AA from PMNL

In PMNL, AA is released from phospholipids upon cell stimulation by the cytosolic PLA_2 ($cPLA_2$). Since $cPLA_2$ is activated by phosphorylation at serine residues by members of the MAPK family and/or elevation of the intracellular Ca^{2+} levels (Gijon & Leslie, 1999), BAs were tested for their ability to elevate the liberation of AA from PMNL. AKBA and KBA considerably enhanced the release of [^{3}H]-labelled AA, whereas

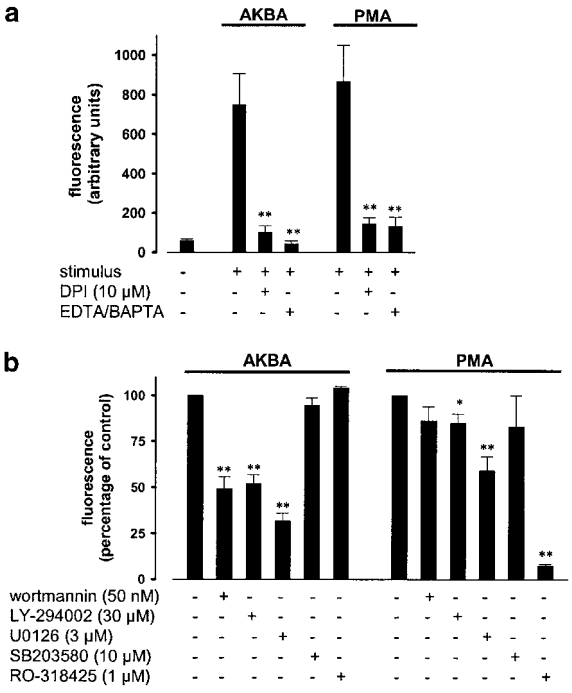


Figure 2 AKBA-induced ROS formation involves NADPH oxidase, PI 3-K, and p42/44^{MAPK}, and requires Ca^{2+}. PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with the indicated compounds for 20 min at RT, prior addition of DCF-DA (1 μg ml^{-1}). After another 2 min, AKBA (30 μM) or PMA (100 nM) were added and the generation of peroxides was measured as described above. (a) Effects of DPI and Ca^{2+} depletion. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units $\pm$s.e., $n=3$. Student's t-test; $^{**}P<0.01$. (b) Effects of pharmacological protein kinase inhibitors. Data were determined 5 min after addition of stimuli and expressed as the percentage of the mean fluorescence $\pm$s.e., $n=4$, of control. Student's t-test; $^{*}P<0.05$, $^{**}P<0.01$. The control values (100%) in the absence of inhibitors for cells stimulated with AKBA were 425 ± 57, and for PMA 675 ± 103 arbitrary fluorescence units.

β-BA and A-β-BA showed only weak effects (Figure 3). Also, under these experimental settings, AKBA and KBA caused even more pronounced responses as compared to 1 μM fMLP. The fMLP- as well as the AKBA-induced AA release was completely abolished when Ca^{2+} was removed by chelation using EDTA and BAPTA/AM (not shown). Experiments using SB203580 (10 μM) and U0126 (3 μM), in order to inhibit p38 MAPK and p42/44^{MAPK} activities, respectively, were performed to estimate the importance of these MAPK for AA liberation. The effect of AKBA was partially suppressed by U0126 (53 ± 4.6%), but was unaffected by SB203580 (not shown), implying that p42/44^{MAPK}, but apparently not p38 MAPK, may contribute to the AKBA-induced AA release.

Effects of 11-keto-boswellic acids on cellular 5-LO product formation in PMNL

In intact cells, 5-LO can be activated upon elevation of the intracellular Ca^{2+} levels and/or phosphorylation by p38 MAPK-regulated MAPKAPKs and p42/44^{MAPK} (Werz *et al.*, 2000; 2002a, b). Although 11-keto BAs are direct inhibitors of 5-LO (Safayhi *et al.*, 1992; 1995), we speculated that, due to their potential to mobilise Ca^{2+} and to activate MAPK, BAs could also stimulate 5-LO in intact cells. As shown in

Figure 4a, when 11-keto-BAs were added to PMNL together with 20 μM AA, AKBA and KBA (30 μM each) caused 3.8- and 2.4-fold increases in 5-LO product formation *versus* control cells that had been stimulated with AA alone, whereas no upregulatory effects were observed for β-BA and A-β-BA. Notably, such upregulatory effects of the 11-keto-BAs were most prominent when Ca^{2+} was chelated by EDTA and BAPTA/AM, whereas, in the presence of Ca^{2+}, AKBA increased 5-LO product formation only by 1.9-fold. Expanding the preincubation period with BAs prior addition of AA (>1 min) strongly reduced the effects of BAs (not shown). The dose–response curve shown in Figure 4b reveals that, at higher concentrations (100 μM), AKBA fails to stimulate 5-LO, possibly due to direct inhibitory enzyme interaction. In order to determine if p38 MAPK and/or $p42/44^{MAPK}$ are required for AKBA-induced 5-LO activation, PMNL were preincubated with 3 μM U0126 and/or 10 μM SB203580 prior stimulation. Upregulation of 5-LO product formation by AKBA (in the absence of Ca^{2+}) was not significantly suppressed by these inhibitors (not shown), suggesting that MAPK are not determinants for AKBA-induced 5-LO activation.

Prolonged exposure of 5-LO to elevated Ca^{2+} or oxidants such as ROS leads to a rapid inactivation of 5-LO (Ford-Hutchinson *et al*., 1994). Hence, it appeared possible, that the potent 5-LO inhibition by AKBA in intact cells after longer preincubation periods (30 min) prior cell stimulation is related to the prominent Ca^{2+} mobilisation and the accompanied

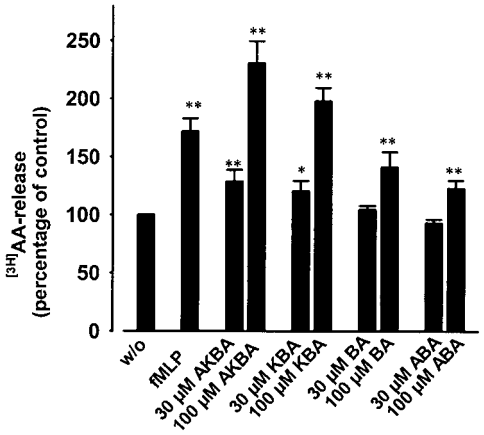


Figure 3 11-Keto-BAs elicit the liberation of AA in PMNL. Freshly isolated PMNL (2×10^6 in 1 ml RPMI 1640 medium) were prelabelled with 0.25 μCi ml^{-1} [^{3}H]AA for 120 min at 37°C and 5% CO_2. After unincorporated [^{3}H]AA was removed, cells (5×10^6 in 1 ml PGC buffer, containing 2 mg ml^{-1} fatty acid-free albumin) were treated with the indicated additives and incubated for 5 min at 37°C. Free (nonesterified) [^{3}H]AA was determined as described in Methods. Results are given as mean ± s.e. ($n=4$). Student's *t*-test; $^*P<0.05$; $^{**}P<0.01$.

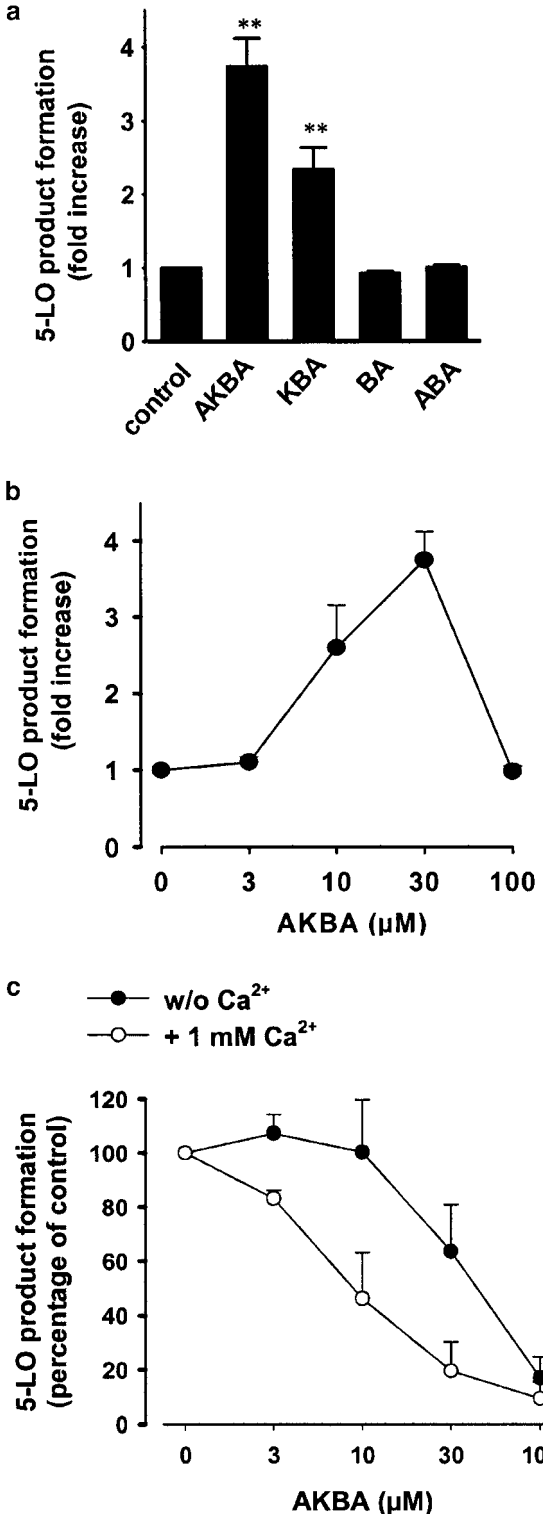


Figure 4 Effects of 11-keto-BAs on the formation of 5-LO metabolites. (a) Freshly isolated PMNL (5×10^6) were resuspended in 1 ml PG buffer containing 1 mM EDTA and preincubated with 30 μM BAPTA/AM for 15 min. Then, cells were stimulated with 20 μM AA alone or together with the indicated BAs (30 μM, each). After 10 min at 37°C, 5-LO products were determined by HPLC. Results are given as mean + s.e., $n=4$–6. Student's *t*-test; $^{**}P<0.01$. (b) Dose–response curve of AKBA. PMNL (5×10^6) were resuspended in 1 ml PG buffer containing 1 mM EDTA and preincubated with 30 μM BAPTA/AM for 15 min. Then, cells were stimulated with 20 μM AA alone or together with the indicated amounts of AKBA. After 10 min at 37°C, 5-LO products were determined. Results are given as mean + s.e., $n=4$–5. (c) Inhibition of 5-LO. PMNL (5×10^6) were resuspended in either 1 ml PGC buffer or PG buffer containing 1 mM EDTA and 30 μM BAPTA/AM. After 15 min at 37°C, AKBA was added, and cells were incubated for another 30 min at 37°C. Then, $CaCl_2$ was adjusted to 1 mM in all incubations and cells were immediately stimulated with 2.5 μM ionophore and 40 μM AA. After 10 min, 5-LO products were determined. Results are given as mean + s.e., $n=3$.

ROS release. PMNL were preincubated for 30 min with AKBA in the presence of Ca^{2+}, which results in Ca^{2+} mobilisation and ROS formation. Alternatively, Ca^{2+} was removed by 1 mM EDTA and 30 μM BAPTA/AM during this preincubation period, conditions where ROS release does not occur. Subsequently, after preincubation, all samples were adjusted to 1 mM Ca^{2+} and cells were immediately stimulated with ionophore and AA to induce 5-LO product formation in intact cells. As can be seen from Figure 4c, the efficacy of AKBA to inhibit 5-LO product formation was strongly impaired when cells were preincubated in the absence of Ca^{2+} (IC_{50} approx. 50 μM), as compared to conditions when Ca^{2+} was present (IC_{50} approx. 8 μM). Thus, potent 5-LO inhibitory effects of AKBA in intact cells by may require Ca^{2+}-mediated processes, such as ROS formation.

Pertussis toxin (PTX) attenuates boswellic acid-induced mobilisation of Ca^{2+} and MAPK activation in PMNL

The proximal signalling pathways involved, as well as the kinetics for Ca^{2+} mobilisation and MAPK activation, resembled those of chemotactic factors, such as fMLP, that act *via* G protein-coupled receptors (GPCR). In order to determine a possible role of G_i or G_0 proteins in the AKBA-induced Ca^{2+} release and MAPK activation, the effects of PTX, an irreversible inhibitor of $G\alpha_{i/0}$ subunits of heterotrimeric G proteins, were assessed in human isolated PMNL. As shown in Figure 5a, PTX (2 μg ml^{-1}) suppressed the Ca^{2+} response induced by 30 μM AKBA in a similar fashion as compared to fMLP stimulation. Similarly, PTX clearly reduced the activation of $p42^{MAPK}$ in PMNL, stimulated with AKBA or KBA, whereas activation of p38 MAPK was only moderately reduced (Figure 5b). In control experiments (Table1 and Figure 5b), PTX also partially decreased the fMLP-induced activation of MAPK and Ca^{2+} mobilisation, whereas MAPK activation (not shown) and Ca^{2+} mobilisation (Table 1) induced by ionomycin (that circumvents G protein signalling) were not suppressed by PTX.

Moreover, PTX (2 μg ml^{-1}) partially inhibited AKBA-induced ROS formation (21.1 $\pm$ 7.6%), but did not at all affect ROS production in PMNL stimulated by PMA. On the other hand, PTX caused only marginal suppression (8.3 $\pm$ 4.1%) of AKBA-induced 5-LO activity in PMNL.

Cell type-dependent induction of MAPK activation, Ca^{2+} mobilisation and ROS generation by BAs

The effects of BAs on the mobilisation of Ca^{2+}, activation of MAPK and formation of ROS were further investigated in various haematopoietic cell lines. For monocytic MM6 cells differentiated with calcitriol and TGFβ, none of the four different BAs (up to 100 μM) induced Ca^{2+} mobilisation or activation of MAPK, and also no significant induction of ROS formation was observed (not shown). In control experiments, PAF, LTB_4 or fMLP caused pronounced elevation of intracellular Ca^{2+}, demonstrating that G protein-coupled Ca^{2+} mobilisation is operative in these cells. Similarly, BAs also failed to activate the B-lymphocytic cell line BL41-E-95-A in this respect (not shown).

In contrast, the effects of BAs in differentiated granulocytic HL60 cells resembled those observed in PMNL from human blood. Thus, AKBA considerably induced mobilisation of

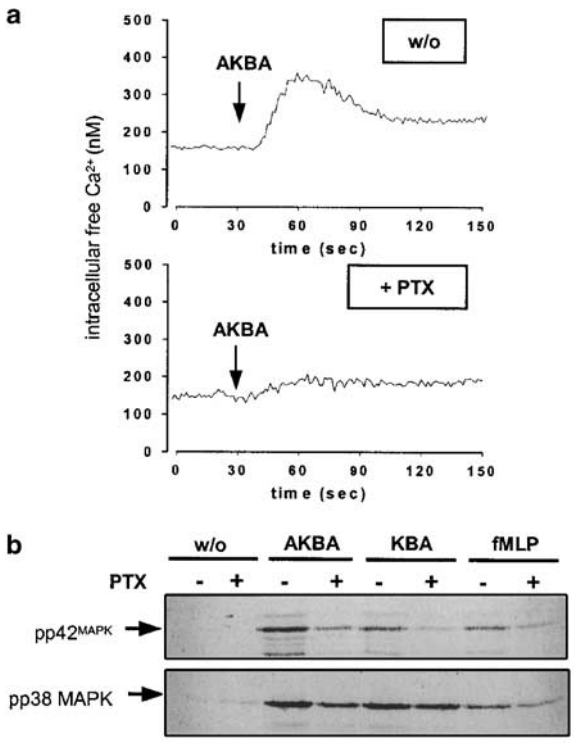


Figure 5 PTX suppresses Ca^{2+} mobilisation and activation of MAPK in PMNL induced by boswellic acids. (a) Ca^{2+} mobilisation. Freshly isolated PMNL (1 ± 10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. Then, cells were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C. After addition of 30 μM AKBA, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of four. (b) MAPK activation. Freshly isolated PMNL (5×10^6 in 100 μl PGC buffer) were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C and then stimulated with 30 μM AKBA, 30 μM KBA, 1 μM fMLP, or left untreated. After 1.5 min at 37°C, incubations were terminated by addition of the same volume of ice-cold SDS-b. Samples were electrophoresed and analysed for dually phosphorylated p38 MAPK or $p44/42^{MAPK}$ by Western blotting.

Table 1 Effects of PTX on Ca^{2+} mobilisation

Stimulus	*Ca^{2+} mobilisation (percentage of control)*
Ionomycin (2.5 μM)	94.1 $\pm$ 8.3
fMLP (100 nM)	39.6 $\pm$ 9.7
AKBA (30 μM)	43.8 $\pm$ 16.8

Freshly isolated PMNL (1×10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. Then, cells were preincubated with or without 2 μg ml^{-1} PTX for 60 min at 37°C. After addition of the indicated stimuli, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. Values (mean $\pm$ s.e., $n = 4$) are given as the percentage of Ca^{2+} mobilisation of PTX-treated cells *versus* control cells that received no PTX.

Ca^{2+}, stimulated activation of p38 MAPK and $p42^{MAPK}$, and upregulated the formation of ROS (Figure 6). Interestingly, KBA was less effective, whereas β-BA and A-β-BA also stimulated HL60 cells for MAPK activation and release of ROS. Nevertheless, β-BA and A-β-BA failed to substantially mobilise Ca^{2+} in differentiated HL60 cells. It should be noted

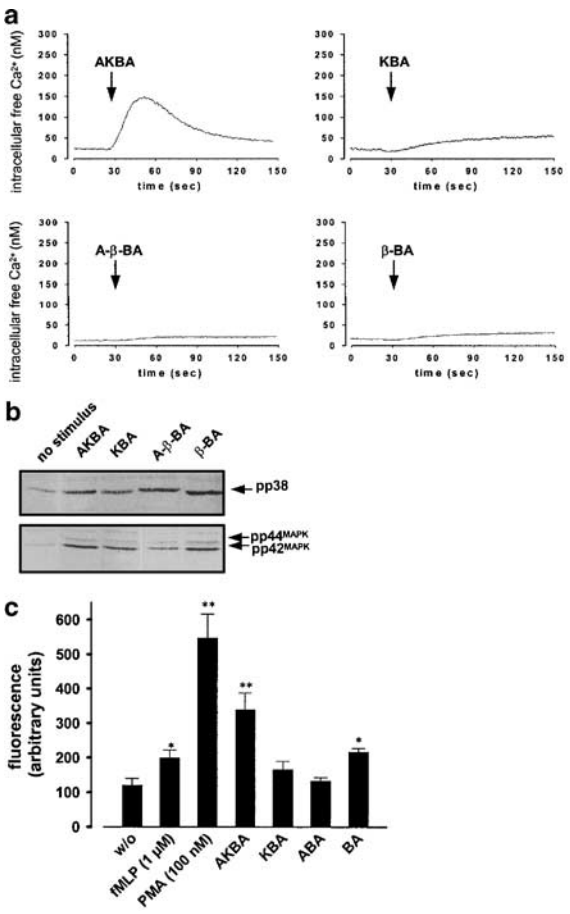


Figure 6 Effects of BAs on Ca^{2+} release, MAPK activation, and ROS production in granulocytic HL60 cells. HL60 cells were differentiated towards granulocytic cells in the presence of 1.5% DMSO, 2 ng ml^{-1} TGFβ, and 500 pM calcitriol for 4 days. (a) Mobilisation of Ca^{2+}. Cells (10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. After addition of BAs (50 μM, each) or 1 μM fMLP, the fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of 4–5, respectively. (b) MAPK activation. Cells ($5 \times 106/100$ μl PGC buffer) were stimulated with 30 μM of BAs, 1 μM fMLP, or left untreated. After 1.5 min at 37°C, incubations were terminated by addition of the same volume of ice-cold SDS-b. Samples were electrophoresed and analysed for dually phosphorylated p38 MAPK or p44/42^{MAPK} by Western blotting. (c) ROS formation. Cells (5×10^7 ml^{-1} PGC buffer) were preincubated with DCF-DA (10 μg ml^{-1}) for 2 min at 37°C prior addition of the indicated stimuli. The generation of peroxides was measured as described. Data determined 5 min after addition of stimuli are expressed as the mean of the fluorescence given in arbitrary units ± s.e., $n = 4$. Student's t-test; $^*P < 0.05$; $^{**}P < 0.01$.

that undifferentiated HL60 cells did neither respond to AKBA nor to the other BAs (not shown), implying that a certain signalling component(s), induced during differentiation, is required to transduce the effects of BAs.

Discussion

We have recently reported that 11-keto-BAs potently stimulate the elevation of intracellular Ca^{2+} levels and activate p38 MAPK as well as p42^{MAPK} (Altmann *et al*., 2002), which are pivotal signalling events that regulate numerous effectors of PMNL. In the present study, we show that 11-keto-BAs in fact elicit functional responses of PMNL or granulocytic HL60 cells such as ROS generation, increased liberation of AA, and its subsequent metabolism by 5-LO. Investigation of the signalling molecules involved and comparison of the kinetics with those of chemotactic ligands for leukocytes, that is, fMLP, PAF or LTB_4, led us to conclude that 11-keto-BAs may transduce their signals in a common way as chemotactic ligands that involve GPCR signalling (see Altmann *et al*. (2002) and references therein). Since elevated levels of AA and ROS are critical inducers of caspase-mediated apoptosis (Hampton *et al*., 1998; Taketo & Sonoshita, 2002), our findings may also provide a molecular basis for the 11-keto-BA-induced caspase activation (Liu *et al*., 2002a, b) and the apoptotic effects observed in various cancer cell lines (Shao *et al*., 1998; Glaser *et al*., 1999; Hoernlein *et al*., 1999; Jing *et al*., 1999; Liu *et al*., 2002a, b).

O_2^- is the precursor of ROS, which are essential for the host defence against microorganisms. The NADPH oxidase of leucocytes is the major source of O_2^- released upon agonist challenge (Chanock *et al*., 1994). Activation of NADPH oxidase requires the presence of Ca^{2+} and multiple phosphorylations of the subunit p47^{phox} by certain PKC isoenzymes or other kinases (Heyworth & Badwey, 1990; Chanock *et al*., 1994; Dewas *et al*., 2000). We demonstrate that, according to their ability to stimulate Ca^{2+} mobilisation and MAPK activation (compare Altmann *et al*., 2002), 11-keto-BAs, but not BAs lacking the 11-keto group, induced a rapid and robust formation of O_2^- and of ROS in PMNL, as determined by lucigenin chemiluminescence and the oxidation of DCF-DA, respectively. Since the NADPH oxidase inhibitor DPI (Hancock & Jones, 1987) abolished ROS formation, ROS derived from the NADPH oxidase system may indeed be responsible. Pharmacological targeting of the proximal signalling pathways revealed that AKBA-induced ROS formation seemingly depends on PI 3-K and on the p42/44^{MAPK} pathway, but does not require p38 MAPK or PKC. In this context, it is interesting that the fMLP-induced phosphorylation of the p47^{phox} component in neutrophils was suppressed by inhibition of p42^{MAPK} (Dewas *et al*., 2000) and PI 3-K (Ding *et al*., 1995), but not when p38 MAPK was blocked by SB203580 (Dewas *et al*., 2000). In contrast, our control experiments utilising PMA as a direct activator of PKC confirm the findings by others, showing that PKC, and to a minor extent also p42^{MAPK}, are required for NADPH oxidase-dependent ROS formation (Cox *et al*., 1985; Heyworth & Badwey, 1990; Dewas *et al*., 2000), whereas a role of PI 3-K or p38 MAPK is not readily apparent. Notably, Ca^{2+} signalling pathways are determinants for ROS formation, since chelation of Ca^{2+} strongly suppressed the signals induced by AKBA and also by PMA. Together, 11-keto BAs may stimulate ROS formation by mobilisation of Ca^{2+} and by activation of p42/44^{MAPK}, apparently involving PI 3-K.

The release of free AA by leukocytes is an important step in the onset of inflammatory reactions and the $cPLA_2$, which is regulated by Ca^{2+} and phosphorylation by MAPK, appears to play a major role for AA liberation in PMNL (Gijon & Leslie, 1999). 11-keto BAs, but not β-BA and A-β-BA, induced the release of considerable amounts of AA, with a similar efficacy as fMLP. The doses of the respective BAs were somewhat higher than those needed for the formation of ROS, which might be due to the fact that $cPLA_2$ favours a sustained Ca^{2+} influx (Qiu *et al*., 1998), which in turn requires higher

concentrations of AKBA or KBA (unpublished observations). In agreement with findings reported for ligands acting *via* GPCR (Qiu *et al*., 1998), AKBA-induced AA liberation also depended on Ca^{2+} and $p42/44^{MAPK}$.

Although BAs have been initially reported as direct-type inhibitors of the 5-LO enzyme, without reducing or iron-chelating properties (Safayhi *et al*., 1992; 1995), we demonstrate that AKBA can paradoxically stimulate cellular 5-LO, when added to PMNL concomitantly with AA. Also, it was reported that low concentrations of *B. serrata* extracts enhanced ionophore-stimulated 5-LO product synthesis in PMNL (Safayhi *et al*., 2000), and 3-oxo-tirucallic acid, that acts as a direct 5-LO inhibitor in cell-free systems, induced and upregulated 5-LO activity in human neutrophils (Boden *et al*., 2001). In intact cells, mobilisation of Ca^{2+}, and also phosphorylation by MAPKAPKs and by $p42/44^{MAPK}$, activates 5-LO for product formation (Werz *et al*., 2000; 2002a, b). Interestingly, AKBA-induced 5-LO activation was most prominent in the absence of Ca^{2+}, and was not sensitive to MAPK inhibitors and hardly sensitive to PTX. Thus, additional unknown factors or pathways induced by AKBA seem operative, which still remain to be determined.

A discrepancy in the efficacy of AKBA is evident for suppression of 5-LO in intact cells ($IC_{50} \approx 2–5\,\mu M$) and cell-free systems ($IC_{50} \approx 15–50\,\mu M$) (Safayhi *et al*., 1995; Werz *et al*., 1997; 1998). Thus, in intact cells, additional factors are operative for the suppression of 5-LO. Since prolonged exposure to elevated Ca^{2+} or oxidants rapidly inactivates 5-LO (Ford-Hutchinson *et al*., 1994), it appeared possible that the potent 5-LO inhibition by AKBA in intact cells is related to the prominent ROS release occurring during preincubation periods (15–30 min) prior cell stimulation. In fact, Ca^{2+} depletion, that prevents ROS formation, impaired the efficacy of AKBA in intact cells (Figure 4c), and the respective IC_{50} value fits well with those obtained in cell-free systems (Werz *et al*., 1998). Intriguingly, the efficacy of AKBA for suppression of 5-LO in MM6 cells ($IC_{50} \approx 15\,\mu M$), that are unable to mobilise Ca^{2+} and to produce ROS in response to AKBA, is significantly reduced as compared to PMNL ($IC_{50} \approx 2–5\,\mu M$).

Upon ligation of their specific GPCR, chemoattractants elicit various functional responses of different leucocytes involving Ca^{2+} mobilisation and activation of MAPK (Herlaar & Brown, 1999; Belcheva & Coscia, 2002). The putative BA receptor, operative in PMNL and mature HL60 cells, seems to be induced during differentiation towards granulocytic cells, since undifferentiated HL60 cells did not respond to BAs with Ca^{2+} mobilisation, MAPK activation or ROS formation. Along these lines, it was found that, in HL60 cells, the G protein-coupled fMLP receptor is also first induced after differentiation (Perez *et al*., 1992). Nevertheless, differentiated monocytic MM6 cells apparently do not possess any BA-inducible signalling pathways or BA receptor(s), although these cells respond to diverse ligands of GPCR (fMLP, LTB_4 or PAF), implying intact G protein signalling pathways in differentiated MM6 cells. Intriguingly, differentiated MM6 cells also failed to mobilise Ca^{2+} in response to 5(S)-HETE or 5-oxo-ETE, which on the other side caused significant Ca^{2+} mobilisation in PMNL (unpublished observations). Accordingly, even the expression of the putative G protein coupled 5-oxo-ETE receptor was found to be cell type-dependent (O'Flaherty *et al*., 2000). Thus, we conclude that the expression of the target (–receptor) of AKBA may be restricted to certain cell types. Finally, it should be noted that, in contrast to PMNL, in differentiated HL60 cells also β-BA and A-β-BA caused cell stimulation (Figure 6a and b), suggesting that, at least in HL60 cells, putative receptor subtypes (not present in PMNL) may exist that accept BAs lacking the 11-keto group.

Our experiments using PTX suggest that a $G_{i/0}$-subunit of a heterotrimeric G protein mediates the effects of 11-keto-BAs in PMNL. Remarkably, the activation of p38 MAPK and of 5-LO was less affected by PTX, implying that, for example, PTX-insensitive G_q subunits, as in the case of the PAF receptor (Shimizu *et al*., 1996), are involved. Also, the PI 3-K inhibitor wortmannin differentially suppressed the activation of p38 MAPK and $p42^{MAPK}$ induced by AKBA (Altmann *et al*., 2002). It is yet unclear whether 11-keto-BAs act *via* a GPCR or alternatively interfere directly with a G protein. Notably, modulation of G proteins by low molecular weight compounds in the absence of a GPCR, both in a positive or a negative way, has been described (see Breitweg-Lehmann *et al*. (2002) and references therein).

The data presented here imply that BAs, at least in concentrations $\geqslant 10\,\mu M$, are potent immunocompetent agents that might be regarded as proinflammatory stimuli. By contrast, cellular and pilot clinical studies indicate the anti-inflammatory effects of *B. serrata* extracts and BAs (Gupta *et al*., 1992; 2001; Gerhardt *et al*., 2001; Krieglstein *et al*., 2001). It is conceivable that, at low concentrations, BAs may have antagonistic activity within certain signalling pathways induced by a second stimulus. In fact, at low concentrations, AKBA ($2–8\,\mu M$) inhibited the activation of $p42/44^{MAPK}$ in meningioma cells stimulated with platelet-derived growth factor (Park *et al*., 2002), and, in our hands, BAs ($0.3–1\,\mu M$) significantly suppressed the PAF-induced Ca^{2+} mobilisation in platelets (unpublished results). Further studies are required to identify the receptor(s) of BAs and the defined mechanisms leading to Ca^{2+} and MAPK signalling, and to reveal if BAs can act as partial agonists at receptors relevant for inflammatory processes. Such knowledge may help to unravel the molecular mechanisms of the anti-inflammatory actions of BAs, and may provide new concepts for the pharmacological intervention with inflammatory diseases.

We thank Astrid Neuß and Sven George for expert technical assistance. This study was supported by grants from the Fonds der Chemischen Industrie, the EU (LEUCHRON, QLRT-2000-01521), and the Deutsche Pharmazeutische Gesellschaft.

References

ALTMANN, A., FISCHER, L., SCHUBERT-ZSILAVECZ, M., STEINHILBER, D. & WERZ, O. (2002). Boswellic acids activate p42(MAPK) and p38 MAPK and stimulate Ca(2+) mobilization. *Biochem. Biophys. Res. Commun.,* **290,** 185–190.

BELCHEVA, M.M. & COSCIA, C.J. (2002). Diversity of G protein-coupled receptor signaling pathways to ERK/MAP kinase. *Neurosignals,* **11,** 34–44.

BODEN, S.E., SCHWEIZER, S., BERTSCHE, T., DUFER, M., DREWS, G. & SAFAYHI, H. (2001). Stimulation of leukotriene synthesis in intact polymorphonuclear cells by the 5-lipoxygenase inhibitor 3-oxo-tirucallic acid. *Mol. Pharmacol.,* **60,** 267–273.

BREITWEG-LEHMANN, E., CZUPALLA, C., STORM, R., KUDLACEK, O., SCHUNACK, W., FREISSMUTH, M. & NURNBERG, B. (2002). Activation and inhibition of G proteins by lipoamines. *Mol. Pharmacol.,* **61,** 628–636.

CHANOCK, S.J., EL BENNA, J., SMITH, R.M. & BABIOR, B.M. (1994). The respiratory burst oxidase. *J. Biol. Chem.,* **269,** 24519–24522.

COX, J.A., JENG, A.Y., SHARKEY, N.A., BLUMBERG, P.M. & TAUBER, A.I. (1985). Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J. Clin. Invest.,* **76,** 1932–1938.

DEWAS, C., FAY, M., GOUGEROT-POCIDALO, M.A. & EL-BENNA, J. (2000). The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47phox phosphorylation in human neutrophils. *J. Immunol.,* **165,** 5238–5244.

DING, J., VLAHOS, C.J., LIU, R., BROWN, R.F. & BADWEY, J.A. (1995). Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J. Biol. Chem.,* **270,** 11684–11691.

FORD-HUTCHINSON, A.W., GRESSER, M. & YOUNG, R.N. (1994). 5-LIPOXYGENASE. *Annu. Rev. Biochem.,* **63,** 383–417.

GERHARDT, H., SEIFERT, F., BUVARI, P., VOGELSANG, H. & REPGES, R. (2001). Therapy of active Crohn disease with *Boswellia serrata* extract H 15. *Z. Gastroenterol.,* **39,** 11–17.

GIJON, M.A. & LESLIE, C.C. (1999). Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J. Leukoc. Biol.,* **65,** 330–336.

GLASER, T., WINTER, S., GROSCURTH, P., SAFAYHI, H., SAILER, E.R., AMMON, H.P., SCHABET, M. & WELLER, M. (1999). Boswellic acids and malignant glioma: induction of apoptosis but no modulation of drug sensitivity. *Br. J. Cancer,* **80,** 756–765.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

GUPTA, I., PARIHAR, A., MALHOTRA, P., GUPTA, S., LUDTKE, R., SAFAYHI, H. & AMMON, H.P. (2001). Effects of gum resin of *Boswellia serrata* in patients with chronic colitis. *Planta Med.,* **67,** 391–395.

GUPTA, O.P., SHARMA, N. & CHAND, D. (1992). A sensitive and relevant model for evaluating anti-inflammatory activity-papaya latex-induced rat paw inflammation. *J. Pharmacol. Toxicol. Methods,* **28,** 15–19.

HAMPTON, M.B., FADEEL, B. & ORRENIUS, S. (1998). Redox regulation of the caspases during apoptosis. *Ann. N.Y. Acad. Sci.,* **854,** 325–328.

HANCOCK, J.T. & JONES, O.T. (1987). The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. *Biochem. J.,* **242,** 103–107.

HERLAAR, E. & BROWN, Z. (1999). p38 MAPK signalling cascades in inflammatory disease. *Mol. Med. Today,* **5,** 439–447.

HEYWORTH, P.G. & BADWEY, J.A. (1990). Protein phosphorylation associated with the stimulation of neutrophils. Modulation of superoxide production by protein kinase C and calcium. *J. Bioenerg. Biomembr.,* **22,** 1–26.

HOERNLEIN, R.F., ORLIKOWSKY, T., ZEHRER, C., NIETHAMMER, D., SAILER, E.R., SIMMET, T., DANNECKER, G.E. & AMMON, H.P. (1999). Acetyl-11-keto-beta-boswellic acid induces apoptosis in HL-60 and CCRF-CEM cells and inhibits topoisomerase I. *J. Pharmacol. Exp. Ther.,* **288,** 613–619.

JING, Y., NAKAJO, S., XIA, L., NAKAYA, K., FANG, Q., WAXMAN, S. & HAN, R. (1999). Boswellic acid acetate induces differentiation and apoptosis in leukemia cell lines. *Leuk. Res.,* **23,** 43–50.

JOHNSON, E.N. & DRUEY, K.M. (2002). Heterotrimeric G protein signaling: role in asthma and allergic inflammation. *J. Allergy Clin. Immunol.,* **109,** 592–602.

KRIEGLSTEIN, C.F., ANTHONI, C., RIJCKEN, E.J., LAUKOTTER, M., SPIEGEL, H.U., BODEN, S.E., SCHWEIZER, S., SAFAYHI, H., SENNINGER, N. & SCHURMANN, G. (2001). Acetyl-11-keto-beta-boswellic acid, a constituent of a herbal medicine from Boswellia serrata resin, attenuates experimental ileitis. *Int. J. Colorectal Dis.,* **16,** 88–95.

LIU, J.J., NILSSON, A., OREDSSON, S., BADMAEV, V. & DUAN, R.D. (2002b). Keto- and acetyl-keto-boswellic acids inhibit proliferation and induce apoptosis in Hep G2 cells *via* a caspase-8 dependent pathway. *Int. J. Mol. Med.,* **10,** 501–505.

LIU, J.J., NILSSON, A., OREDSSON, S., BADMAEV, V., ZHAO, W.Z. & DUAN, R.D. (2002a). Boswellic acids trigger apoptosis *via* a pathway dependent on caspase-8 activation but independent on Fas/Fas ligand interaction in colon cancer HT-29 cells. *Carcinogenesis,* **23,** 2087–2093.

O'FLAHERTY, J.T., TAYLOR, J.S. & KUROKI, M. (2000). The coupling of 5-oxo-eicosanoid receptors to heterotrimeric G proteins. *J. Immunol.,* **164,** 3345–3352.

PARK, Y.S., LEE, J.H., BONDAR, J., HARWALKAR, J.A., SAFAYHI, H. & GOLUBIC, M. (2002). Cytotoxic action of acetyl-11-keto-beta-boswellic acid (AKBA) on meningioma cells. *Planta Med.,* **68,** 397–401.

PEREZ, H.D., KELLY, E. & HOLMES, R. (1992). Regulation of formyl peptide receptor expression and its mRNA levels during differentiation of HL-60 cells. *J. Biol. Chem.,* **267,** 358–363.

QIU, Z.H., GIJON, M.A., DECARVALHO, M.S., SPENCER, D.M. & LESLIE, C.C. (1998). The role of calcium and phosphorylation of cytosolic phospholipase A(2) in regulating arachidonic acid release in macrophages. *J. Biol. Chem.,* **273,** 8203–8211.

ROOS, D., GOLDSTEIN, I.M., KAPLAN, H.B. & WEISSMANN, G. (1976). Dissociation of phagocytosis, metabolic stimulation and lysosomal enzyme release in human leukocytes. *Agents Actions,* **6,** 256–259.

SAFAYHI, H. & SAILER, E.R. (1997). Anti-inflammatory actions of pentacyclic triterpenes. *Planta Med.,* **63,** 487–493.

SAFAYHI, H., BODEN, S.E., SCHWEIZER, S. & AMMON, H.P. (2000). Concentration-dependent potentiating and inhibitory effects of Boswellia extracts on 5-lipoxygenase product formation in stimulated PMNL. *Planta Med.,* **66,** 110–113.

SAFAYHI, H., MACK, T., SABIERAJ, J., ANAZODO, M.I., SUBRAMANIAN, L.R. & AMMON, H.P. (1992). Boswellic acids: novel, specific, non-redox inhibitors of 5-lipoxygenase. *J. Pharmacol. Exp. Ther.,* **261,** 1143–1146.

SAFAYHI, H., RALL, B., SAILER, E.R. & AMMON, H.P. (1997). Inhibition by boswellic acids of human leukocyte elastase. *J. Pharmacol. Exp. Ther.,* **281,** 460–463.

SAFAYHI, H., SAILER, E.R. & AMMON, H.P. (1995). Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-beta-boswellic acid. *Mol. Pharmacol.,* **47,** 1212–1216.

SHAO, Y., HO, C.T., CHIN, C.K., BADMAEV, V., MA, W. & HUANG, M.T. (1998). Inhibitory activity of boswellic acids from *Boswellia serrata* against human leukemia HL-60 cells in culture. *Planta Med.,* **64,** 328–331.

SHIMIZU, T., MORI, M., BITO, H., SAKANAKA, C., TABUCHI, S., AIHARA, M. & KUME, K. (1996). Platelet-activating factor and somatostatin activate mitogen-activated protein kinase (MAP kinase) and arachidonate release. *J. Lipid Mediat. Cell. Signal.,* **14,** 103–108.

SIMCHOWITZ, L. & SPILBERG, I. (1979). Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor. Evidence for the role of calcium. *J. Lab. Clin. Med.,* **93,** 583–593.

TAKETO, M.M. & SONOSHITA, M. (2002). Phospholipase A2 and apoptosis. *Biochim. Biophys. Acta,* **1585,** 72–76.

WERZ, O., BRUNGS, M. & STEINHILBER, D. (1996). Purification of transforming growth factor beta1 from human platelets. *Pharmazie,* **51,** 893–896.

WERZ, O., BUERKERT, E., FISCHER, L., SZELLAS, D., DISHART, D., RÅDMARK, O., SAMUELSSON, B. & AND STEINHILBER, D. (2002b). Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.,* **16,** 1441–1443.

WERZ, O., BUERKERT, E., SAMUELSSON, B., RÅDMARK, O. & AND STEINHILBER, D. (2002a). Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood,* **99,** 1044–1052.

WERZ, O., KLEMM, J., SAMUELSSON, B. & RÅDMARK, O. (2000). 5-Lipoxygenase is phosphorylated by p38 kinase dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 5261–5266.

WERZ, O., SCHNEIDER, N., BRUNGS, M., SAILER, E.R., SAFAYHI, H., AMMON, H.P.T. & STEINHILBER, D. (1997). A test system for leukotriene synthesis inhibitors based on the *in-vitro* differentiation of the human leukemic cell lines HL-60 and Mono Mac 6. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 441–445.

WERZ, O., SZELLAS, D., HENSELER, M. & STEINHILBER, D. (1998). Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.,* **54,** 445–451.

WINKING, M., SARIKAYA, S., RAHMANIAN, A., JODICKE, A. & BOKER, D.K. (2000). Boswellic acids inhibit glioma growth: a new treatment option? *J. Neurooncol.,* **46,** 97–103.

(Received August 28, 2003
Revised October 13, 2003
Accepted October 29, 2003)

VII

Inhibitors of actin polymerisation stimulate arachidonic acid release and 5-lipoxygenase activation by upregulation of Ca^{2+} mobilisation in polymorphonuclear leukocytes involving Src family kinases

Lutz Fischer, Eva Bürkert, Dieter Steinhilber, and Oliver Werz
Institute of Pharmaceutical Chemistry, University of Frankfurt, D-60439 Frankfurt, Germany

Abstract: Here we show, that agents that block actin polymerisation such as latrunculin B (LB), and to a minor extent also cytochalasin D (Cyt D), enhance the release of arachidonic acid (AA) as well as nuclear translocation of 5-lipoxygenase (5-LO) and 5-LO product synthesis in human polymorphonuclear leukocytes (PMNL), challenged with thapsigargin (TG). The concentration-dependent effect of LB (EC_{50} ? 200 nM) declined with prolonged preincubation (> 3 min) prior TG and was barely detectable when PMNL were stimulated with Ca^{2+}-ionophores.

Investigation of the stimulatory mechanisms revealed that LB (or Cyt D) elicits Ca^{2+} mobilisation and potentiates agonist-induced elevation of intracellular Ca^{2+}, regardless of the nature of the agonist. LB caused rapid but only moderate activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulate kinase (ERK)2. The selective Src family kinase inhibitors PP2 and SU6656, blocked LB- or Cyt D-mediated Ca^{2+} mobilisation, and suppressed the upregulatory effects on AA release and 5-LO product synthesis, without affecting AA metabolism evoked by ionophore alone. We conclude that in PMNL, inhibitors of actin polymerisation cause enhancement of intracellular Ca^{2+} levels through Src family kinase signaling, thereby facilitating the release of AA and 5-LO product formation, elicited by agonists that moderately induce Ca^{2+} mobilisation.

Key words: *leukotriene, polymorphonuclear leukocyte, latrunculin, cytochalasin, inflammation, cytoskeleton.*

INTRODUCTION

Massive formation and release of leukotrienes (LTs) occurs mainly in phagocytes upon stimulation, and is initiated by the oxygenation of free arachidonic acid (AA) via 5-lipoxygenase (5-LO) [1]. 5-LO and its products have been implicated in a variety of pathophysiological events and contribute to a number of inflammatory and allergic disorders including asthma, arthritis, psoriasis and cardiovascular diseases such as atherosclerosis, myocardial infarction and stroke [1-3]. Therefore, the understanding of the mechanisms regulating the biosynthesis of 5-LO product synthesis attracts particular attention, but is still incompletely understood.

For 5-LO product synthesis in phagocytes, the liberation of AA by the cytosolic phospholipase (cPL)A_2 is one of the rate-limiting steps. Ca^{2+} and/or phosphorylation at serine residues are of importance for $cPLA_2$ activation, depending on the cell type and the stimulus [4]. In analogy to $cPLA_2$, also 5-LO can be activated by Ca^{2+} and/or phosphorylation [5-7]. $cPLA_2$ and 5-LO translocate from a soluble cellular locale to the nuclear membrane [8, 9], where 5-LO activating protein (FLAP) serves as an AA-binding protein, believed to facilitate AA transfer to 5-LO [10].

It was shown that a threshold level of $[Ca^{2+}]_i$ is required for activation and translocation of $cPLA_2$, and it was proposed that phosphorylation may reduce the requirement for Ca^{2+} [11]. Also for 5-LO activation, Ca^{2+} and phosphorylation may act in conjunction [5, 6, 12, 13]. In fact, Ca^{2+}-ionophores that cause prominent and rapid elevation of $[Ca^{2+}]_i$ induce substantial release of AA and LTs, which is also evident when phosphorylation is blocked [5, 11]. Agents, including thapsigargin (TG), N-formyl-methionyl-leucyl-phenylalanine (fMLP) or zymosan that elicit only moderate and/or transient Ca^{2+} mobilisation are poor inducers of the AA cascade.

Previous studies have proposed a regulatory role of the cytoskeleton for the capacity of leukocytes to form LTs. For example, it was shown that preincubation of polymorphonuclear leukocytes (PMNL) with cytochalasins (Cyt) upregulates LT formation induced by fMLP [14-16]. Moreover, 5-LO is able to bind to actin [17] and interacts with the actin-binding protein coactosin-like protein (CLP) [18, 19]. On the other hand, 5-LO interferes with actin polymerisation and a modulatory role for 5-LO in actin dynamics was suggested [19]. Thus, there are several indications that reorganization of the actin skeleton, may have impact on the regulation of LT biosynthesis.

In the present study the effects of latrunculin B (LB) and Cyt D, inhibitors of actin polymerisation, on 5-LO product release from PMNL were investigated and the underlying mechanism were explored. We found that the LB- or Cyt D-mediated upregulation of 5-LO product synthesis depended on the stimulus and was primarily due to an

increased liberation of AA, but also to an enhanced translocation of 5-LO to the nucleus. Analysis of the signaling and activation pathways of these processes suggest that LB and Cyt D mediate their effects by elevation of $[Ca^{2+}]_{i,}$ through Src family kinase signaling.

MATERIALS AND METHODS

The 5-LO antibody (AK-7, 1551) was kindly provided by Dr. Olof Rådmark, Karolinska Institute Stockholm, Sweden. RPMI 1640 medium was from GibcoBRL, Life Technologies (Rockville, MD). Fetal calf serum, Ca^{2+}-ionophore A23187, ionomycin, AA, TG, fMLP, Sigma (Deisenhofen, Germany); LB, PP2, PP3, SU6656, Calbiochem (San Diego, USA); Cyt D, Fura-2, Alexis (Grünberg, Germany); [^{3}H]AA, GE Healthcare Bio-Sciences (Freiburg, Germany); HPLC solvents, Merck (Darmstadt, Germany).

Cells

PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany) as described [5]. In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation at 4000 ? g/20 min/20°C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes. PMNL (10^7 cells /ml; purity > 96-97%) were finally resuspended in PBS containing 1 mg/ml glucose, and 1 mM $CaCl_2$ (PGC buffer) or 1 mM EDTA (PG buffer) was added, as indicated.

Measurement of intracellular Ca^{2+} levels

Freshly isolated PMNL (10^7/ml PGC buffer)were incubated with 2 µM Fura-2/AM for 30 min at 37°C, washed, resuspended in 1 ml PGC buffer and transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. [20]. F_{max} (maximal fluorescence) was obtained by lysing the cells w0ith 1% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Determination of 5-LO product synthesis

Freshly isolated PMNL (7.5 × 106) were finally resuspended in 1 ml PGC buffer. After preincubation with the indicated compounds at 37°C, 5-LO product formation was started by the addition of the indicated stimuli (DMSO, final concentration = 0.3 % (vol/vol), was used as solvent) with or without exogenous AA as indicated. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol and 30 µl of 1 N HCl, 200 ng prostaglandin B1 and 500 µl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [5]. 5-LO product formation is expressed as ng of 5-LO products per 106 cells which includes LTB4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined.

SDS-PAGE and Western blotting

Freshly isolated PMNL (5 ? 10^6) were resuspended in PGC buffer, final volume was 100 µl. After addition of the indicated stimuli, samples were incubated at 37°C and the reaction was stopped by addition of 100 µl of ice-cold 2 ? sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% ?-mercaptoethanol), vortexed and heated for 6 min at 95°C. Total cell lysates (20 µl) were mixed with 4 µl of glycerol/0.1% bromphenolblue (1:1, vol/vol) and analyzed by SDS-PAGE on a 10% gel. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. After electroblot to nitrocellulose membrane (Amersham Pharmacia), blocking with 5 % non fat dry milk for 1 h at RT, membranes were washed and incubated with primary antibodies (ABs) overnight at 4°C. Phospho-specific antibodies (AB) recognizing ERK1/2 (Thr202/Tyr204) or p38 MAPK (Thr180/Tyr182) were obtained from New England Biolabs. All ABs were used as 1:2,000 dilution. The membranes were washed and incubated with 1:1,000 dilution of alkaline phosphatase conjugated IgGs (Sigma) for 2 h at RT. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer.

Determination of release of [^{3}H]-labeled arachidonic acid from PMNL

Freshly isolated PMNL were resuspended at 2 ? 10^6 in 1 ml RPMI 1640 medium containing 4.8 nM [^{3}H]AA (corresponding to 0.25 µCi/ml, specific activity 200 Ci/mmol) and incubated for 120 min at 37^{o}C in 5 % CO_2 atmosphere. Thereafter, cells were collected by centrifugation, washed once with PBS and twice with PBS containing 2 mg/ml fatty acid free albumin, to remove unincorporated [^{3}H]AA. Labeled PMNL (10^7) were resuspended in 1 ml PGC buffer containing 2 mg/ml fatty acid free

albumin. The reaction was started by addition of the indicated stimuli. After 5 min at 37°C, the samples were placed on ice for 2 min and cells were centrifuged at 400 ? g for 5 min at rt. Aliquots (100 µl) of the supernatants were measured in a beta-counter (Micro Beta Trilux, Perkin Elmer, Foster City, CA) to detect the amounts of [^{3}H]-labeled AA released into the medium.

Subcellular fractionation by detergent lysis

Subcellular localization of 5-LO was investigated as described previously [21]. In brief, freshly isolated PMNL (3×10^7) in 1 ml PGC buffer were incubated at 37°C for 10 min with the indicated stimuli and chilled on ice. Nuclear and non-nuclear fractions were obtained after cell lysis by 0.1% NP-40. Aliquots of these fractions were immediately mixed with the same volume of SDS-b, heated for 6 min at 95°C, and analyzed for 5-LO protein by SDS-PAGE and immunoblotting.

Statistics

The program "GraphPad PRISM 3.0" was used for statistical comparisons. Statistical evaluation of the data was performed using Student´s *t* test for unpaired observations. A *P* value of < 0.05 was considered as significant.

RESULTS

LB and Cyt D elevate TG-induced 5-LO product synthesis from endogenous AA.

Addition of TG (0.01 to 10 µM) to isolated human PMNL resuspended in PGC buffer induced a concentration-dependent induction of 5-LO product formation (EC_{50} ? 2 µM, **fig. 1A**). When extra- and intracellular Ca^{2+} was removed using 1 mM EDTA and 30 µM BAPTA/AM, respectively, no 5-LO product formation was detectable (not shown), implying that Ca^{2+} is absolutely required for TG-induced 5-LO activation. Depending on the concentration of TG, co-addition of 3 µM LB caused an up to 18-fold increase in 5-LO product synthesis (**fig. 1A**). Again, when Ca^{2+} was chelated by EDTA and BAPTA/AM, no 5-LO product synthesis was observed in response to TG plus LB (not shown). The magnitude of the stimulatory effect of LB was most prominent when low concentrations of TG (0.01 to 1 µM) were used for PMNL challenge. In contrast, LB failed to upregulate 5-LO product synthesis at 10 µM TG. Addition of LB to PMNL alone caused no significant 5-LO product synthesis (not shown). Concentration-response experiments revealed an EC_{50} of ? 0.2 µM LB, maximal effects were obtained at 3 µM (**fig. 1B**). Moreover, LB gave the best effect when added simultaneously with TG, whereas the upregulatory effects of LB decreased over time. Thus, preincubation with 3 µM LB for 30 min caused an only two-fold increase of TG-

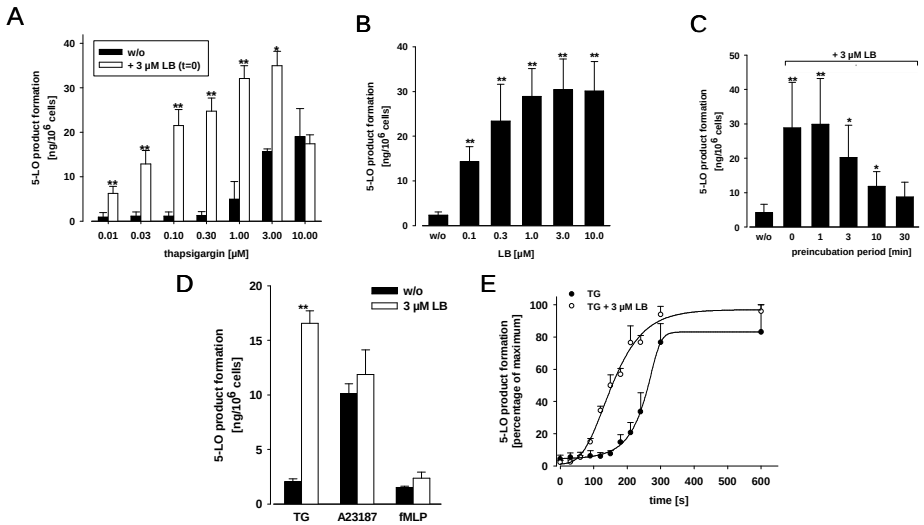


Fig. 1 Latrunculin B upregulates 5-LO product synthesis induced by thapsigargin.
For all experiments freshly isolated PMNL (7.5×10^6 in 1 ml PGC buffer) were used. Results are given as mean + S.E., n = 5-6. Student *t* test; **P*< 0.05; ***P*< 0.01. (**A**) PMNL were incubated with or without 3 µM LB together with the indicated concentrations of TG at 37°C for 10 min and 5-LO products were determined by HPLC as described in the Methods section. (**B**) Cells were incubated with 1 µM TG together with the indicated concentrations of LB at 37°C for 10 min and 5-LO products were determined. (**C**) PMNL were preincubated with 3 µM LB for the indicated periods at 37°C. Then, 1 µM TG was added and after another 10 min, and 5-LO products were determined. (**D**) PMNL were incubated with 1 µM TG, 2.5 µM ionophore, or 1 µM fMLP together with or without 3 µM LB at 37°C for 10 min, and 5-LO products were determined. (**E**) PMNL were incubated with 1 µM TG together with or without 3 µM LB at 37°C. Incubations were stopped by addition of the same volume of methanol after the times indicated and 5-LO products were determined.

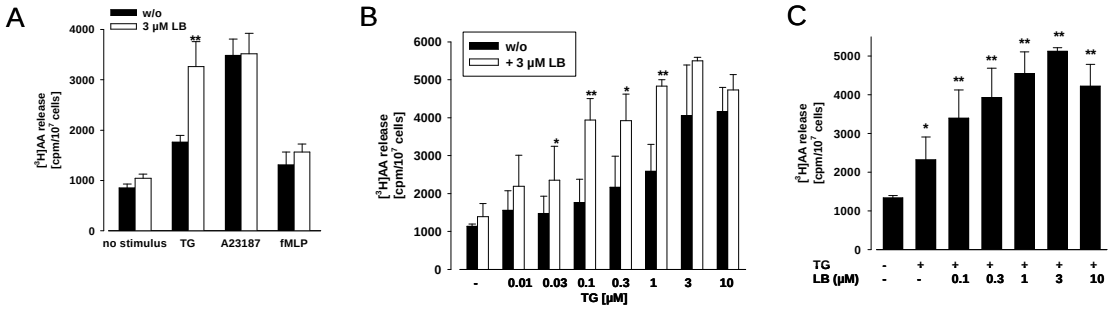


Fig. 2 Latrunculin B upregulates the release of AA induced by thapsigargin.
Freshly isolated PMNL (2 ? 10^6 in 1 ml RPMI 1640 medium) were prelabeled with 0.25 µCi/ml [^{3}H]AA for 120 min at 37°C and 5 % CO_2. Unincorporated [^{3}H]AA was removed, and cells (10^7) were resuspended in 1 ml PGC buffer, containing 2 mg/ml fatty acid-free albumin and incubated as described below. (**A**) Cells were stimulated with 1 µM TG, 2.5 µM ionophore A23187, or 1 µM fMLP together with or without 3 µM LB for 5 min at 37°C and free (nonesterified) [^{3}H]AA was determined as described in the Methods section. (**B**) Cells were stimulated with the indicated concentrations of TG with or without 3 µM LB. After 5 min at 37°C, free [^{3}H]AA was determined. (**C**) Cells were incubated with 1 µM TG together with the indicated concentrations of LB. After 5 min at 37°C, free [^{3}H]AA was determined. Results are given as mean ± S.E. (n = 4). Student *t* test; **P*< 0.05; ***P*< 0.01.

induced 5-LO product synthesis (**fig. 1C**).
Next, LB was coincubated with other stimuli known to induce 5-LO product formation. LB caused only a slight increase of 5-LO product synthesis when ionophore A23187 (2.5 µM, optimized concentration [21]) was used as agonist and upregulated fMLP-induced 5-LO activity only about 1.6-fold (**fig. 1D**). Interestingly, upregulation of TG-induced 5-LO product synthesis by LB was obvious only when endogenous substrate was converted by 5-LO, thus, in the presence of exogenous AA (10 µM), LB caused no significant enhancements (not shown). Moreover, LB not only increased the total amount of 5-LO products formed, it also significantly shortened the lag-phase of 5-LO product synthesis from about 160 to 70 sec (**fig. 1E**). Together, LB selectively upregulates TG-induced 5-LO product formation from endogenous substrate.
In addition to LB, also the actin polymerisation inhibitor Cyt D (10 µM) caused upregulation of 5-LO product synthesis induced by TG and slightly also by fMLP, but not by ionophore. However, the magnitude of these upregulatory effects of Cyt D (? 3.6-fold) on top of TG were less pronounced as compared to LB (? 18-fold, see fig. 1A), which fits to the potency of these compounds to inhibit actin polymerisation [22].

LB elevates TG-induced AA release.

In accordance with 5-LO product formation, LB upregulates AA release from PMNL induced by TG, but only marginally when induced by fMLP, and fails in response to ionophore (**fig. 2A**). Again, the release of AA was less efficiently increased by Cyt D as compared to LB (not shown). In analogy to 5-LO product synthesis, effects of LB on the release of AA were most pronounced at suboptimal concentrations of TG (0.01 to 1 µM), whereas LB failed at TG concentrations >3 µM (**fig. 2B**). The EC_{50} for upregulation of TG-induced AA release was 0.2 µM LB, maximal effects were obtained at 3 µM LB (**fig. 2C**), and prolonged preincubation with LB prior TG reduced the stimulatory effect of LB (not shown).

LB promotes the translocation of 5-LO to the nuclear membrane.

In order to determine, if LB influences 5-LO redistribution, PMNL were challenged with TG or ionophore and subcellular localisation of 5-LO was determined. Stimulation of PMNL with TG (1 µM) caused only moderate translocation of 5-LO to the nucleus, but co-addition of LB caused significant enrichment of 5-LO in this locale (**fig. 3**). LB alone had no effect. In contrast, ionophore (2.5 µM) induced substantial 5-LO translocation which was not further increased by LB (**fig. 3**).

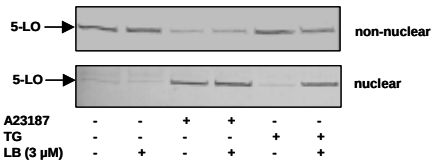


Fig. 3 Latrunculin B enhances 5-LO redistribution to the nucleus. Freshly isolated PMNL (3 × 10^7 in 1 ml PGC buffer) were stimulated with 2.5 µM A23187 or 1 µM TG together with or without 3 µM LB for 5 min at 37°C. 5-LO was detected in nuclear and non-nuclear fractions by immunoblotting after subcellular fractionation. Similar results were obtained in two additional independent experiments.

LB induces Ca^{2+} mobilisation and augments agonist-induced elevation of $[Ca^{2+}]_i$.

Elevation of $[Ca^{2+}]_i$ is a determinant for the release of AA by $cPLA_2$ as well as for 5-LO product synthesis [23]. It appeared possible that LB upregulates AA liberation and 5-LO activation by increasing $[Ca^{2+}]_i$. Ionomycin, a Ca^{2+}-ionophore related to A23187, was used, since A23187 possesses fluorescent properties and is not suitable for measurement of $[Ca^{2+}]_i$ using Fura-2. At 1 µM, TG induced a moderate and rather delayed increase in $[Ca^{2+}]_i$ from 60 +/- 8 to 230 +/- 15 nM) as compared to ionomycin (from 60 +/- 8 to 869 +/-

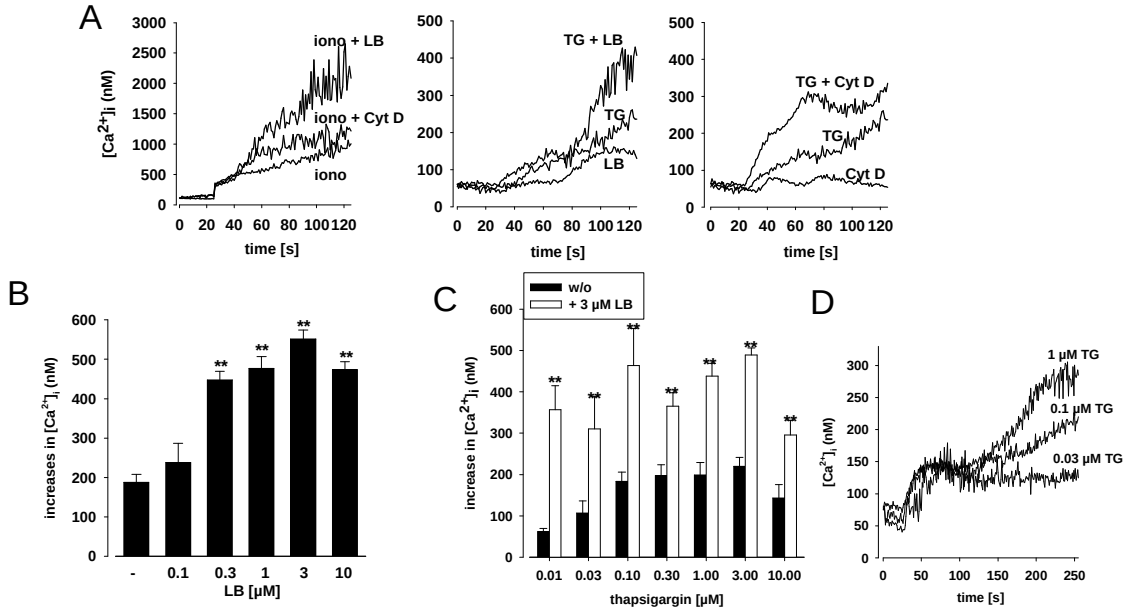


Fig. 4 Latrunculin B and cytochalasin D induce Ca^{2+} mobilisation and upregulate agonist-induced Ca^{2+} mobilisation. Freshly isolated PMNL (1×10^7/ml PGC buffer) were loaded with 2 µM Fura-2 for 30 min at 37°C. After washing, cells (1×10^7) were resuspended in PGC buffer, the fluorescence was measured, and $[Ca^{2+}]_i$ was calculated as described. Values are given as mean + S.E., n = 3-5; curves are representative for 3-5 experiments. (**A**) Cyt D (10 µM), LB (3 µM), TG (1 µM), and ionomycin (1 µM) were added alone or combined as indicated 25 s after the measurement was started. As control, the solvent (DMSO) was added that gave no significant signal. The monitored curves show one typical experiment out of four. (**B**) Cells were stimulated with 1 µM TG together with the indicated concentrations of LB. The maximal $[Ca^{2+}]_i$ after stimulation was determined within 125 s of measurement. (**C**) Cells were stimulated with the indicated concentrations of TG with or without 3 µM LB. The maximal values obtained with TG alone or with TG plus LB were recorded after 220 or 120 sec, respectively. (**D**) TG was added at the indicated concentrations. A second raise of $[Ca^{2+}]_i$ 100 sec after TG was added is observed at TG concentrations = 0.1 µM.

313 nM) within 110 sec of measurement (**fig. 4A**). Interestingly, LB (3 µM) alone elevated the $[Ca^{2+}]_i$ from 60 +/- 6 to 218 +/- 26 nM, with a delay of about 40 sec (**fig. 4A**). Similarly, Cyt D caused elevation of $[Ca^{2+}]_i$, although to a minor extent (82 +/- 34 nM). Co-addition of LB or Cyt D with TG gave additive effects resulting in enhanced $[Ca^{2+}]_i$ (462 +/- 50 and 349 +/- 77 nM, respectively). Such additive signals of Cyt D or LB were also observed with ionomycin, where again, LB was more effective as compared to Cyt D (**fig. 4A**). Concentration-response studies regarding augmentation of TG-induced elevation of $[Ca^{2+}]_i$ determined an EC_{50} for LB at 0.2 µM with maximal effects at 3 µM (**fig. 4B**). The enhancement of $[Ca^{2+}]_i$ induced by TG alone was concentration-dependent (**fig. 4C**). However, in combination with LB a clear concentration-dependent effect was not readily apparent. It should be noted that the values of $[Ca^{2+}]_i$ given in fig. 4C were recorded 220 sec after addition of TG and 120 sec after stimulation with TG plus LB, reflecting $[Ca^{2+}]_i$ at the time points where the velocity of 5-LO product formation is close to maximum (see fig. 1E). Obviously, at low TG concentrations (< 0.1 µM), $[Ca^{2+}]_i$ enters a plateau (**fig. 4D**), which is followed by a second raise at higher concentrations of TG.

The PLC inhibitor U-73122 (but not its inactive analogue U-73343, not shown) blocked LB-induced Ca^{2+} mobilisation as well as the upregulatory effect of LB, but did not reduce TG-induced Ca^{2+} mobilisation (**table 1**). Finally, LB elicited $[Ca^{2+}]_i$ also in the absence of extracellular Ca^{2+} (presence of 1 mM EDTA), which was more rapid but only about 10 to 15 % of the signal obtained in the presence of extracellular Ca^{2+}, and also this effect was abolished by U-73122 (not shown). Therefore, it appears that LB evokes mobilisation of Ca^{2+} from internal stores involving PLC.

Table 1 Effects of U-73122 on elevation of $[Ca^{2+}]_i$. Freshly isolated PMNL (1×10^7/ml PGC buffer) were loaded with 2 µM Fura-2 for 30 min at 37°C. After washing, cells (1×10^7) were resuspended in PGC buffer and preincubated with or without 3 µM U-73122, prior stimulation with LB and TG as indicated. The fluorescence was measured, and intracellular free Ca^{2+} was calculated as described. The maximal $[Ca^{2+}]_i$ after stimulation was determined within 130 s of measurement. Values are given as mean + S.E., n = 3.

stimulus	increase in $[Ca^{2+}]_i$ (nM)	
	w/o	+ 3 µM U-73122
LB	187 ± 26	21 ± 3
TG	207 ± 18	210 ± 10
TG + LB	565 ± 100	198 ± 12

Effect of LB on the activation of MAP kinases.

Release of AA by $cPLA_2$ and 5-LO activation is regulated also by p38 MAPK and ERK1/2 [4-7]. Addition of LB to PMNL rapidly induced activation of ERK2 and p38 MAPK within 15 to 30 sec, and subsequently slightly declined (**fig. 5A**). Maximum activation was determined at 3 µM of

LB (not shown). However, compared to fMLP or ionophore, MAPK activation elicited by LB was only moderate (**fig. 5B**). Moreover, LB did not potentiate activation of ERK2 or p38 MAPK induced by ionophore, fMLP, or TG (**fig. 5B**). Together, MAPK may play only a minor role in transmitting signals by LB.

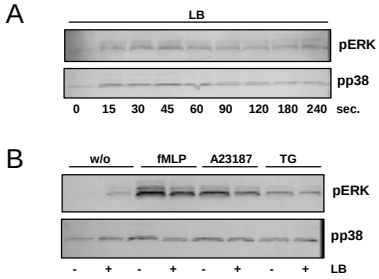


Fig. 5 Effects of latrunculin B on MAPK activation. Freshly isolated PMNL (5×10^6 in 100 µl PGC buffer) were stimulated as indicated and described below. Incubations were terminated by addition of the same volume of ice-cold SDS-b. Samples were electrophoresed and analyzed for dually phosphorylated p38 MAPK or ERK2 by Western blotting. (**A**) Cells were stimulated with 3 µM LB for the indicated times at 37°C. (**B**) Cells were stimulated with 1 µM fMLP, 2.5 µM ionophore A23187, or 1 µM TG, together with or without 3 µM LB at 37°C for 1.5 min. The results shown are representative for 3-4 experiments.

Src family kinases mediate the stimulatory effects of LB on Ca^{2+} mobilisation.

It was reported that LB or Cyt D evoke activation of members of the Src family kinases in certain cell types [24, 25], and Src family kinases in turn are involved in activation of PLC? that mediates mobilisation of Ca^{2+} from internal storage sites [26]. We investigated the involvement of Src family kinases in LB-mediated Ca^{2+} responses using the selective Src family kinase inhibitors PP2 [27], and for control the negative analogue PP3, as well as SU6656 [28]. PP2 acts as a competitive inhibitor of ATP-binding, with IC_{50} values of 4 - 20 nM for crude $p56^{lck}$, $p59^{fynT}$, and Hck *in vitro*, whereas for cellular studies, much higher concentrations of PP2 (IC_{50} = 0.55 µM) are required, probably due to high $[ATP]_i$ compared with low concentrations used in *in vitro* kinase assays [27]. Neither PP2 (and PP3, 2 µM, each) nor SU6656 (5 µM) caused suppression of TG-induced elevation of $[Ca^{2+}]_i$ (**fig. 6**). However, PP2 (but not PP3) or SU6656 substantially reduced Ca^{2+} mobilisation elicited by LB (or Cyt D, not shown). Importantly, also the upregulatory effects of LB on top of TG were reduced by PP2 (but not by PP3) or by SU6656 (**fig. 6**). Thus, selective inhibition of Src family kinases prevents LB effects regarding Ca^{2+} mobilisation.

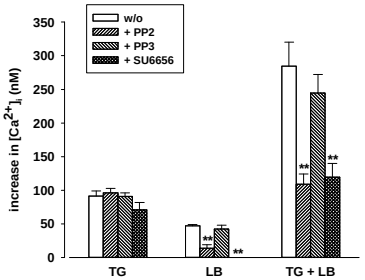


Fig. 6 Effects of Src family kinase inhibitors on Ca^{2+} mobilisation. Freshly isolated PMNL (1×10^7/ml PGC buffer) were loaded with 2 µM Fura-2 for 30 min at 37°C. After washing, cells (1×10^7) were resuspended in PGC buffer and preincubated with PP2, PP3 (2 µM, each), or SU6656 (5 µM) for 15 min at 37°C and then stimulated with TG (1 µM) and LB (3 µM) as indicated. The fluorescence was measured and $[Ca^{2+}]_i$ was calculated as described. The maximal $[Ca^{2+}]_i$ after stimulation was determined within 130 s of measurement. Values are given as mean + S.E., n = 3-5. Student *t* test; **P*< 0.05; ***P*<

Inhibition of Src family kinases prevents the stimulatory effects of LB on AA release, 5-LO product synthesis, and 5-LO translocation.

Next, the involvement of Src family kinases in LB-mediated stimulation of AA release, 5-LO product synthesis, and 5-LO translocation was evaluated. As shown in **fig. 7A**, PP2 and SU6656 (but not PP3) strongly blocked the upregulated 5-LO product synthesis by 3 µM LB in PMNL stimulated with TG. In control experiments, PP2 and SU6656 failed to significantly suppress 5-LO product synthesis induced by ionophore alone (**fig. 7B**), indicating that Src family kinases are not generally involved in 5-LO activation and that the inhibitors do not interfere with 5-LO catalytic activity.
A similar pattern of the effects of Src family kinases inhibitors was found when the release of AA (**fig. 7C** and **D**) and 5-LO translocation (**fig. 8**) were investigated. Thus, PP2 and SU6656 (but not PP3) abolished the stimulatory effects of 3 µM LB on top of TG, but failed to reduce ionophore-evoked responses.

DISCUSSION

Although previous studies have demonstrated that inhibitiors of actin polymerisation augment the capacity of agonist-challenged PMNL to release AA metabolites [14-16], the underlying mechanisms and the signaling molecules involved have not been defined thus far. Here we show that treatment of human PMNL with the actin polymerisation inhibitors LB or Cyt D leads to an elevation of basal and agonist-induced $[Ca^{2+}]_i$, involving Src family kinases. The elevated $[Ca^{2+}]_i$ appears to contribute to an increased availability of free AA and to an enhanced nuclear 5-LO translocation. Both processes together might be attributable for substantial 5-LO product synthesis.
For 5-LO product synthesis, AA must be liberated from phospholipids, implying that activation of the Ca^{2+}-dependent $cPLA_2$ is a determinant [29, 30].

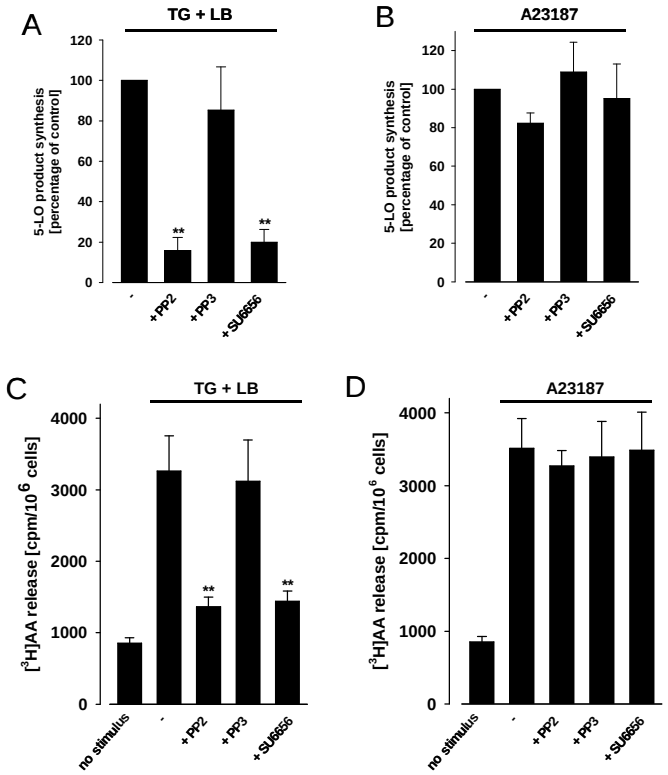


Fig. 7 Effects of Src family kinase inhibitors on AA release and 5-LO product synthesis.
(**A** and **B**) Effects of PP2, PP3, and SU6656 on 5-LO product synthesis. Freshly isolated PMNL (7.5×10^6/ml PGC buffer) were preincubated with PP2, PP3 (2 µM, each), or SU6656 (5 µM) for 15 min at 37°C and then either stimulated with 1 µM TG plus 3 µM LB (left panel), or with 2.5 µM ionophore A23187 (right panel). After 10 min at 37°C, 5-LO products were extracted and determined by HPLC as described. (**C** and **D**) Effects of PP2, PP3, and SU6656 on AA release. Freshly isolated PMNL (2 ? 10^6/ml RPMI 1640 medium) were prelabeled with 0.25 µCi/ml [^{3}H]AA for 120 min at 37°C and 5 % CO_2. Unincorporated [^{3}H]AA was removed and cells (5 ? 10^6) were resuspended in 1 ml PGC buffer, containing 2 mg/ml fatty acid-free albumin. Cells were preincubated with PP2, PP3 (2 µM, each), or SU6656 (5 µM) for 15 min at 37°C and then stimulated with 1 µM TG plus 3 µM LB (left panel), or with 2.5 µM ionophore A23187 (right panel). After 5 min at 37°C, free [^{3}H]AA was determined. Results are given as mean ± S.E., n = 4. Student *t* test; $^{**}P < 0.01$.

Agonists that induce the release of AA by $cPLA_2$ and the subsequent conversion via 5-LO encompass a variety of agents, which are all capable to raise $[Ca^{2+}]_i$. These stimuli include ionophores, TG, G protein-coupled receptor (GPCR) agonists such as fMLP, cytokines like IL-8, and phagocytic particles like zymosan or urate crystals [23]. The extent of LT synthesis correlates to the ability to elevate $[Ca^{2+}]_i$. Thus, Ca^{2+}-ionophores cause strong AA release and LT formation, whereas naturally-occurring ligands, which moderately raise $[Ca^{2+}]_i$, are much less efficient [31-34]. In our study, ionophore elevated $[Ca^{2+}]_i$ to about 800 nM and caused a much stronger AA liberation and 5-LO product synthesis as compared to TG that increased $[Ca^{2+}]_i$ to only 230 nM. Accordingly, LB augmented AA release and metabolism only in combination with TG, but not together with ionophore, although $[Ca^{2+}]_i$ was upregulated by LB regardless of the stimulus. Previous studies have addressed the Ca^{2+} requirement of $cPLA_2$ and 5-LO in PMNL, and it was found that $cPLA_2$ requires a threshold level of approx. 350 to 400 nM $[Ca^{2+}]_i$ [31], whereas 5-LO showed a linear dependence on Ca^{2+} and saturates below these concentrations [31]. Obviously, co-stimulation of TG plus LB, raising $[Ca^{2+}]_i$ to approx. 460 nM, is required for efficient AA release (and subsequent 5-LO product synthesis), since the $[Ca^{2+}]_i$ levels obtained with TG alone do not reach the threshold level (350-400 nM $[Ca^{2+}]_i$) needed for $cPLA_2$ activation. The $[Ca^{2+}]_i$, levels after ionophore stimulation (approx. 800 nM) are high enough for $cPLA_2$ activation and augmentation by LB (to > 1000 nM) causes no significant further enhancements. Moreover, the duration of the lag-phase in 5-LO product synthesis (fig. 1E) correlates to the period of time required to reach the threshold levels of $[Ca^{2+}]_i$ for AA release and 5-LO product synthesis. Thus, a correlation between the ability of these stimuli to elevate $[Ca^{2+}]_i$ and to recruit $cPLA_2$ and 5-LO is apparent. However, since high concentrations of TG (?1 µM) cause substantial formation of 5-LO products (fig. 1A) without inducing a further increase of $[Ca^{2+}]_i$ as compared to TG at 0.1 or 0.3 µM (fig. 4C), other unknown effectors than Ca^{2+} may amplify AA release and conversion by 5-LO. Nevertheless, Ca^{2+} seems to be absolutely required for TG-induced 5-LO product formation, since TG (0.01 up to 10 µM) was not effective in the absence of intra and extracellular Ca^{2+}.

Besides Ca^{2+}, phosphorylation of serine residues by members of the MAPK family regulates activation of $cPLA_2$ [4] but also of 5-LO [6, 7, 21], in particular when $[Ca^{2+}]_i$ is low, whereas enzyme phosphorylation is less important when Ca^{2+} mobilisation is substantial [5, 11]. Since LB caused only moderate enhancement of MAPK activation but at the same time strongly elevated $[Ca^{2+}]_i$, we conclude that phosphorylation plays a minor role in transmitting the upregulatory effects of LB or Cyt D to $cPLA_2$ and 5-LO.

In the presence of exogenous AA, LB was unable to upregulate TG-induced 5-LO product synthesis, again implying that LB elevates 5-LO product synthesis mainly by affecting $cPLA_2$. Also others found that moderate elevation of $[Ca^{2+}]_i$ by natural "weak" agonists is sufficient to induce substantial formation of 5-LO products from exogenous substrate [31, 32]. Hence, an insufficient AA release at suboptimal $[Ca^{2+}]_i$ levels is mainly attributable for low 5-LO product formation, implying that $cPLA_2$ rather than 5-LO is the rate-limiting enzyme. Nevertheless, LB had a clear stimulatory effect on 5-LO, inasmuch as it promoted translocation to the nucleus.

LB is a potent monomer-sequestering and F-actin-depolymerizing drug that *in vivo* disrupts microfilament organization and inhibits microfilament-mediated processes [22]. LB is about 10- to 100-fold more potent than Cyts in several experimental cell-based models and the mechanisms of how the drugs disrupt actin microfilaments differ [22]. Thus, Cyts act primarily by binding to the fast-growing or barbed end of the actin filament [35], whereas LB binds to monomeric actin, thereby preventing actin polymerisation [36]. All the effects of LB in our study were concentration-dependent with a consistent EC_{50} ? 0.2 µM and maximal efficacy at concentrations around 3 µM, which fits to the effective concentrations of LB reported to suppress actin polymerisation [22, 36].

Dynamic reorganization of the actin skeleton is central to a variety of PMNL functions, including locomotion, adhesion spreading and phagocytosis (for review, see [37]). Most stimuli activating the AA pathway also evoke actin polymerisation with similar kinetics, implying that these processes may influence each other. In fact, both $cPLA_2$ as well as 5-LO bind to certain cytoskeletal proteins. $cPLA_2$ was found to interact with vimentin [38], and 5-LO can bind alpha-actinin, actin [17] and CLP [18, 19]. Regarding the modulation of the AA pathway, positive as well as negative regulatory effects of actin polymerisation were reported. Thus, in fMLP-stimulated PMNL, actin polymerisation suppresses AA metabolism, visualized by a Cyt-evoked increase in LTB_4 formation [14-16]. Also, suppression of the basal F-actin content and of stimulus-induced actin assembly by botulinum toxin C2 increased 5-LO product formation in response to soluble chemoattractants, paralleled by a loss of motile and phagocytotic functions [39]. In accordance with our findings, Cyts increased the $[Ca^{2+}]_i$ in human PMNL after stimulation with fMLP [40]. Similarly, in LB-treated RBL-2H3 mast cells exposed to antigen, an increased extend in the $[Ca^{2+}]_i$ was measured, accompanied by enhanced AA release [41].

On the other hand, actin polymerisation may positively influence the AA pathway. Thus, Cyts inhibited the release and metabolism of AA in thrombin- or collagen-stimulated platelets [42, 43]. Notably, in human platelets, inhibition of actin polymerisation by Cyt D or latrunculin A inhibited the TG-evoked Ca^{2+} entry [44]. Also in complement-stimulated glomerular epithelial cells [45] or zymosan-challenged macrophage-like P388D1 cells [46], Cyt D or LB reduced $cPLA_2$ activity and AA release. Together, the effect of actin polymerisation on the AA pathway seemingly depends on the cell type, the nature of the stimulus, and the transmitting signaling pathways, but appears to be tightly connected to the $[Ca^{2+}]_i$.

Analysis of the pathways mediating the enhancement of $[Ca^{2+}]_i$ by LB suggests a role of PLC isoenzymes and Src family kinases. U-73122 (that inhibits PLC) is capable to inhibit receptor-coupled Ca^{2+} mobilisation from internal stores [47]. U-73122 blocked the LB-evoked rise in $[Ca^{2+}]_i$, suggesting that LB leads to release of Ca^{2+} from internal stores through the PLC pathway. Among the PLC isoenzymes, the PLC?1 and -2 isotypes are expressed in hematopoietic cells [48] and are activated in response to chemoattractants by tyrosine phosphorylation involving nonreceptor protein tyrosine kinases (PTKs) such as Src family kinases [49, 50]. The selective Src family kinase inhibitors PP2 [27] and SU6656 [28] selectively blocked the effects of LB on Ca^{2+} mobilisation, AA release, 5-LO product synthesis, and 5-LO translocation, indicating the involvement of Src family kinases. It should be noted that the structurally related PP3 used as negative control for PP2 had no such effects, and PP2 or SU6656 did not block PMNL responses evoked by TG or ionophore in the absence of LB, excluding inhibitory effects on the enzymatic machinery of AA metabolism. Among the Src family kinase members, Fgr, Hck, and Lyn are expressed in PMNL [51], and using double knockout hck-/- fgr-/- PMNL or PP1-treated cells a role of these kinases in Cyt B-induced degranulation was evident [52]. Also, there are indications that actin polymerisation signals through Src family kinases. For example, the nonreceptor PTKs Fer and Fyn are activated by LB-induced actin depolymerisation in CHO cells [24], and actin disassembly stimulates the nonreceptor PTK c-Abl in fibroblasts [53, 54] and c-Src in smooth muscle cells [25].

In conclusion our data indicate that inhibitors of actin polymerization have a prominent influence on the release of AA and the subsequent conversion to proinflammatory 5-LO products by modulating the

$[Ca^{2+}]_i$ through Src family kinase/PLC signaling. Since PMNL migration and adhesion to sites of inflammation is connected to an extensive cytoskeleton remodeling [37], the committed polymerisation of actin may be regarded as a regulatory process, limiting Ca^{2+}-dependent PMNL functions in response to natural occurring inflammatory stimuli, down regulation of the biosynthesis of proinflammatory 5-LO products might be one example.

ACKNOWLEDGMENTS

This study was supported by the EU (LEUCHRON, QLRT-2000-01521) and the Deutsche Forschungsgemeinschaft (GRK 137/3). We thank Dr. Olof Rådmark for critical reading of the manuscript and valuable comments, and Sven George for expert technical assistance.

REFERENCES

1. Funk, C.D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871-1875.
2. Helgadottir, A., Manolescu, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorsteinsdottir, U., Samani, N.J., Gudmundsson, G., Grant, S.F., Thorgeirsson, G., Sveinbjornsdottir, S., Valdimarsson, E.M., Matthiasson, S.E., Johannsson, H., Gudmundsdottir, O., Gurney, M.E., Sainz, J., Thorhallsdottir, M., Andresdottir, M., Frigge, M.L., Topol, E.J., Kong, A., Gudnason, V., Hakonarson, H., Gulcher, J.R., Stefansson, K. (2004) The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 36, 233-239.
3. Dwyer, J.H., Allayee, H., Dwyer, K.M., Fan, J., Wu, H., Mar, R., Lusis, A.J., Mehrabian, M. (2004) Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N Engl J Med* 350, 29-37.
4. Gijon, M.A., Leslie, C.C. (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J Leukoc Biol* 65, 330-336.
5. Werz, O., Bürkert, E., Samuelsson, B., Rådmark, O., Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* 99, 1044-1052.
6. Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., Steinhilber, D. (2002) Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *Faseb J* 16, 1441-1443.
7. Werz, O., Klemm, J., Samuelsson, B., Rådmark, O. (2000) 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proc Natl Acad Sci U S A* 97, 5261-5266.
8. Peters-Golden, M., McNish, R.W. (1993) Redistribution of 5-lipoxygenase and cytosolic phospholipase-A2 to the nuclear fraction upon macrophage activation. *Biochem. Biophys. Res. Commun.* 196, 147-153.
9. Pouliot, M., Mc Donald, P.P., Krump, E., Mancini, J.A., Mc Coll, S.R., Weech, P.K., Borgeat, P. (1996) Colocalization of cytosolic phospholipase A(2), 5-lipoxygenase, and 5-lipoxygenase-activating protein at the nuclear membrane of A23187-stimulated human neutrophils. *Eur J Biochem* 238, 250-258.
10. Mancini, J.A., Abramovitz, M., Cox, M.E., Wong, E., Charleson, S., Perrier, H., Wang, Z.Y., Prasit, P., Vickers, P.J. (1993) 5-Lipoxygenase-Activating Protein Is an Arachidonate Binding Protein. *FEBS Lett* 318, 277-281.
11. Qiu, Z.H., Gijon, M.A., de Carvalho, M.S., Spencer, D.M., Leslie, C.C. (1998) The role of calcium and phosphorylation of cytosolic phospholipase A2 in regulating arachidonic acid release in macrophages. *J Biol Chem* 273, 8203-8211.
12. Bürkert, E., Szellas, D., Rådmark, O., Steinhilber, D., Werz, O. (2003) Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. *J Leukoc Biol* 73, 191-200.
13. Werz, O., Szellas, D., Steinhilber, D., and Rådmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPKAP kinase-2. *J Biol Chem* 277, 14793-14800.
14. Palmer, R.M., Salmon, J.A. (1983) Release of leukotriene B4 from human neutrophils and its relationship to degranulation induced by N-formyl-methionyl-leucyl-phenylalanine, serum-treated zymosan and the ionophore A23187. *Immunology* 50, 65-73.
15. Ham, E.A., Soderman, D.D., Zanetti, M.E., Dougherty, H.W., McCauley, E., Kuehl, F.A., Jr. (1983) Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *Proc Natl Acad Sci U S A* 80, 4349-4353.
16. Salari, H., Braquet, P., Naccache, P., Borgeat, P. (1985) Characterization of effect of N-formyl-methionyl-leucyl-phenylalanine on leukotriene synthesis in human polymorphonuclear leukocytes. *Inflammation* 9, 127-138.
17. Lepley, R.A., Fitzpatrick, F.A. (1994) 5-lipoxygenase contains a functional Src homology 3- binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins. *J. Biol. Chem.* 269, 24163-24168.
18. Provost, P., Samuelsson, B., Rådmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. USA* 96, 1881-1885.
19. Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B., and Radmark, O. (2001) 5-Lipoxygenase interacts with Coactosin-Like Protein. *J. Biol. Chem.* 276, 16520-16527.
20. Grynkiewicz, G., Poenie, M., Tsien, R.Y. (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260, 3440-3450.
21. Werz, O., Klemm, J., Samuelsson, B., and Radmark, O. (2001) Phorbol ester upregulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human PMNL. *Blood* 97, 2487-2495.
22. Spector, I., Shochet, N.R., Blasberger, D., Kashman, Y. (1989) Latrunculins--novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. Cell Motil Cytoskeleton 13, 127-144.
23. Werz, O. (2002) 5-Lipoxygenase: Cellular biology and molecular pharmacology. *Current Drug Targets - Inflammation and Allergy* 1, 23-44.
24. Fan, L., Di Ciano-Oliveira, C., Weed, S.A., Craig, A.W., Greer, P.A., Rotstein, O.D., Kapus, A. (2004) Actin depolymerization-induced tyrosine phosphorylation of cortactin: the role of Fer kinase. *Biochem J* 380, 581-591.
25. Samarakoon, R., Higgins, P.J. (2003) Pp60c-src mediates ERK activation/nuclear localization and PAI-1 gene expression in response to cellular deformation. *J Cell Physiol* 195, 411-420.
26. Rhee, S.G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* 70, 281-312.

27. Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 271, 695-701.
28. Blake, R.A., Broome, M.A., Liu, X., Wu, J., Gishizky, M., Sun, L., Courtneidge, S.A. (2000) SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 20, 9018-9027.
29. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., Shimizu, T. (1997) Role of cytosolic phospholipase A(2) in allergic response and parturition. *Nature* 390, 618-622.
30. Leslie, C.C. (1997) Properties and regulation of cytosolic phospholipase A2. *J Biol Chem* 272, 16709-16712.
31. Schatz-Munding, M., Hatzelmann, A., Ullrich, V. (1991) The involvement of extracellular calcium in the formation of 5-lipoxygenase metabolites by human polymorphonuclear leukocytes. *Eur. J. Biochem.* 197, 487-493.
32. McDonald, P.P., McColl, S., Naccache, P.H., Borgeat, P. (1991) Studies on the activation of human neutrophil 5-lipoxygenase by natural agonists and Ca^{2+} -ionophore A23187. *Biochem. J.* 280, 379-385.
33. Clancy, R.M., Dahinden, C.A., Hugli, T.E. (1983) Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by N-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. *Proc Natl Acad Sci U S A* 80, 7200-7204.
34. Wong, A., Cook, M.N., Foley, J.J., Sarau, H.M., Marshall, P., Hwang, S.M. (1991) Influx of Extracellular Calcium Is Required for the Membrane Translocation of 5-Lipoxygenase and Leukotriene Synthesis. *Biochemistry* 30, 9346-9354.
35. Cooper, J.A. (1987) Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 105, 1473-1478.
36. Coue, M., Brenner, S.L., Spector, I., Korn, E.D. (1987) Inhibition of actin polymerization by latrunculin A. *FEBS Lett* 213, 316-318.
37. Fenteany, G., Glogauer, M. (2004) Cytoskeletal remodeling in leukocyte function. *Curr Opin Hematol* 11, 15-24.
38. Nakatani, Y., Tanioka, T., Sunaga, S., Murakami, M., Kudo, I. (2000) Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A(2)alpha. *J Biol Chem* 275, 1161-1168.
39. Grimminger, F., Sibelius, U., Aktories, K., Suttorp, N., Seeger, W. (1991) Inhibition of cytoskeletal rearrangement by botulinum C2 toxin amplifies ligand-evoked lipid mediator generation in human neutrophils. *Mol Pharmacol* 40, 563-571.
40. Nowak, D., Bialasiewicz, P., Antczak, A., Krol, M., Piasecka, G. (1995) Effect of cytochalasin B on intracellular free calcium concentration in human polymorphonuclear leukocytes after repeated stimulation with n-formyl-methionyl-leucyl-phenylalanine. Int J Clin Lab Res 25, 116-120.
41. Frigeri, L., Apgar, J.R. (1999) The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. *J Immunol* 162, 2243-2250.
42. Siess, W., Lapetina, E.G., Cuatrecasas, P. (1982) Cytochalasins inhibit arachidonic acid metabolism in thrombin-stimulated platelets. *Proc Natl Acad Sci U S A* 79, 7709-7713.
43. Nakano, T., Hanasaki, K., Arita, H. (1989) Possible involvement of cytoskeleton in collagen-stimulated activation of phospholipases in human platelets. *J Biol Chem* 264, 5400-5406.
44. Rosado, J.A., Jenner, S., Sage, S.O. (2000) A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J Biol Chem* 275, 7527-7533.
45. Cybulsky, A.V., Takano, T., Papillon, J., Khadir, A., Bijian, K., Le Berre, L. (2004) The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2. *Am J Physiol Renal Physiol* 286, 466-476.
46. Akiba, S., Ohno, S., Chiba, M., Kume, K., Hayama, M., Sato, T. (2002) Protein kinase Calpha-dependent increase in Ca2+-independent phospholipase A2 in membranes and arachidonic acid liberation in zymosan-stimulated macrophage-like P388D1 cells. *Biochem Pharmacol* 63, 1969-1977.
47. Bleasdale, J.E., Bundy, G.L., Bunting, S., Fitzpatrick, F.A., Huff, R.M., Sun, F.F., Pike, J.E. (1989) Inhibition of phospholipase C dependent processes by U-73, 122. *Adv Prostaglandin Thromboxane Leukot Res* 19, 590-593.
48. Noh, D.Y., Shin, S.H., Rhee, S.G. (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta* 1242, 99-113.
49. Ptasznik, A., Traynor-Kaplan, A., Bokoch, G.M. (1995) G protein-coupled chemoattractant receptors regulateLyn transduction pathways. *Curr Opin Cell Biol*7,176-182
50. Ptasznik, A., Traynor-Kaplan, A., Bokoch, G.M. (1995) G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase. Shc adapter protein signaling complexes. *J Biol Chem* 270, 19969-19973.
51. Perskvist, N., Zheng, L., Stendahl, O. (2000) Activation of human neutrophils by Mycobacterium tuberculosis H37Ra involves phospholipase C gamma 2, Shc adapter protein, and p38 mitogen-activated protein kinase. *J Immunol* 164, 959-965.
52. Erpel, T., Courtneidge, S.A. (1995) Src family protein tyrosine kinases and cellular signal.
53. Mocsai, A., Ligeti, E., Lowell, C.A., Berton, G. (1999) Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J Immunol* 162, 1120-1126.
54. Woodring, P.J., Hunter, T., Wang, J.Y. (2001) Inhibition of c-Abl tyrosine kinase activity by filamentous actin. *J Biol Chem* 276, 27104-27110.

XI. Curriculum vitae

PERSÖNLICHES

Geburtsdaten: geboren am 14. September 1974 in Oberhausen/Rheinland

Staatsangehörigkeit: deutsch

SCHUL- und HOCHSCHULAUSBILDUNG

Mai 2001 - Januar 2005	Biochemische Promotion unter der Leitung von PD Dr. Oliver Werz am Institut für Pharmazeutische Chemie der Johann Wolfgang Goethe-Universität Frankfurt
Januar 2002	Approbation als Apotheker
Dezember 2001	Pharmazeutische Prüfung (3. Abschnitt)
Mai 2001 - Oktober 2001	2. Hälfte des praktischen Jahres am Institut für Pharmazeutische Chemie der Johann Wolfgang Goethe-Universität Frankfurt
November 2000 - April 2001	1. Hälfte des praktischen Jahres in der Titus-Apotheke in Frankfurt
Oktober 1995 - Oktober 2000	Studium der Pharmazie an der Johann Wolfgang Goethe-Universität Frankfurt
Mai 1994	Allgemeine Hochschulreife
1985 - 1994	Besuch des Heinrich Heine - Gymnasiums, Oberhausen
1981 - 1985	Besuch der Erich Kästner - Grundschule, Oberhausen

AUSLANDSAUFENTHALT

September 2003 - Oktober 2003	Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Schweden

WEHRDIENST

Juli 1994 - Juni 1995	6./ Wachbataillon BMVg in Siegburg

BERUFSERFAHRUNG und PRAKTIKA

Seit April 2003	Teilnahme an Seminaren im Rahmen der Weiterbildung zum Fachapotheker für Pharmazeutische Analytik
Januar 2002 – Januar 2005	Vertretung des Apothekenleiters Titus-Apotheke, Frankfurt
Januar 2002 – Januar 2005	Leitung von Workshops sowie Vorträge im Rahmen des Europäischen Graduiertenkollegs „Roles of Eicosanoids in Biology and Medicine“
Mai 2001 – Januar 2005	Betreuung der Pharmaziestudenten des 8. Semesters im Rahmen des Arbeitskreispraktikums
Mai 2001 – Januar 2005	Einarbeitung von technischen Angestellten sowie wissenschaftlichen Mitarbeitern des Institut für Pharmazeutische Chemie der Johann Wolfgang Goethe-Universität Frankfurt
August 1996	Famulatur in der Hindenburg-Apotheke, Oberhausen
Juli 1996	Famulatur in der Möllebeck-Apotheke, Hünxe

Akademische Lehrer

Meine akademischen Lehrer neben PD Dr. Oliver Werz und Prof. Dr. Dieter Steinhilber waren:

Prof. Dr. H. Blume

Prof. Dr. Th. Dingermann

Prof. Dr. J. Dressman

Prof. Dr. E. Ehlers

Prof. Dr. H. Hoffmann (†)

Prof. Dr. J. Kreuter

Prof. Dr. G. Lambrecht

Prof. Dr. H. Linde (†)

Prof. Dr. W. E. Müller

Prof. Dr. C.R Noe

Prof. Dr. G. Schmalzing

Prof. Dr. A. Zimmer

XII. Publication list

Fischer L, Steinhilber D, Werz O. Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610. *Br. J. Pharmacol*. 2004;142:861-868. Epub 2004 Jun 2014.

Fischer L, Szellas D, Rådmark O, Steinhilber D, Werz O. Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *FASEB J.*. 2003;17:949-951.

Fischer L, Bürkert E, Werz O. Latrunculin B, an inhibitor of actin polymerization, upregulates arachidonic acid release and 5-lipoxygenase product formation: involvement of tyrosine kinase signalling and Ca^{2+}. *Manuscript*

Werz O, Bürkert E, **Fischer L**, Szellas D, Dishart D, Samuelsson B, Rådmark O, Steinhilber D. 5-Lipoxygenase activation by MAPKAPK-2 and ERKs. *Adv Exp Med Biol*. 2003;525:129-132.

Werz, O, Bürkert E, **Fischer L**, Szellas D, Dishart D, Samuelsson B, Rådmark O, Steinhilber D. Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *FASEB J.*. 2002;16:1441-1443.

Altmann A, Pöckel D, **Fischer L**, Schubert-Zsilavecz M, Steinhilber D, Werz O. Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leukocytes. *Br. J. Pharmacol*. 2004;141:223-232. Epub 2003 Dec 2022.

Altmann A, **Fischer L**, Schubert-Zsilavecz M, Steinhilber D, Werz O. Boswellic acids activate p42(MAPK) and p38 MAPK and stimulate Ca^{2+} mobilization. *Biochem. Biophys. Res. Commun*. 2002; 290:185-190.

Arnold C, Albert D, **Fischer L**, Hörnig M, Rådmark O, Steinhilber D, Werz O. (2005) 1-Oleoyl-2-acetylglycerol stimulates 5-lipoxygenase activity via a putative (phospho)lipid-binding site within the N-terminal C2-like domain. *Manuscript*

Wissenschaftliche Mitarbeit bei der Erstellung der 9. Auflage des pharmazeutischen Wörterbuchs „Hunnius“, Herausgegeben im de Gruyter-Verlag.

Präsentationen bei Kongressen

Fischer L, Szellas D, Rådmark O, Steinhilber D und Werz O. Inhibition of 5-Lipoxygenase: Novel aspect for the development of nonredox-type inhibitors.
Poster im Rahmen des ZAFES Kick Off Symposiums „Lipid Signaling“, Frankfurt, Oktober 2004.

Fischer L, Steinhilber D und Werz O. Pharmakologisches Profil des 5-Lipoxygenase Inhibitors CJ-13,610.
Vortrag im Rahmen der „Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft“. Freudenstadt-Lauterbad, März 2004.

Fischer L, Szellas D, Rådmark O, Steinhilber D und Werz O. Phosphorylation and stimulus-dependent inhibition of cellular 5-lipoxygenase by nonredox-type inhibitors.
Poster für die „Jahrestagung der Deutschen Pharmazeutischen Gesellschaft“. Berlin, Oktober 2002.

Fischer L, Szellas D, Rådmark O, Steinhilber D und Werz O. Phosphorylation and stimulus-dependent inhibition of cellular 5-lipoxygenase by nonredox-type inhibitors.
Poster für die „12th International Conference on Prostaglandins, Leukotrienes and other Bioactive Lipid Research“. Istanbul, Türkei, August 2002.

Fischer L, Szellas D, Rådmark O, Steinhilber D und Werz O. Efficacy of nonredox-type 5-LO inhibitors depends on the enzyme`s phosphorylation status.
Vortrag und Poster im Rahmen des „European Graduate Student Meeting“. Frankfurt, Februar 2000

XIII. Acknowledgments

Die vorliegende Arbeit entstand am Institut für Pharmazeutische Chemie der Universität Frankfurt in Zusammenarbeit mit dem „Institutionen för medicinsk biokemi och biofysik“, Karolinska Institutet, Stockholm.
Die Arbeit stand unter der Leitung von PD Dr. Oliver Werz.
Das Projekt wurde finanziert über den Fonds der Chemischen Industrie, die EU (LEUCHRON, QLRT-2000.01521) und die Deutsche Pharmazeutische Gesellschaft.

Mein herzlichster Dank gilt...

- … PD Dr. Oliver Werz für seine hervorragende und vorbildliche wissenschaftliche Betreuung, motivierende und „tiefsinnige“ Gespräche auch in schwierigeren Zeiten und manche Kletterstunde, inklusive „Doppeldoktor“.
- … Prof. Dr. Dieter Steinhilber für die Aufnahme in seinen Arbeitskreis, die Bereitstellung des Themas, die idealen Arbeitsbedingungen, die intensive Förderung dieser Arbeit, die Aufnahme in das Europäische Graduiertenkolleg „Roles of Eicosanoids in Biology and Medicine“ und die damit verbundenen Aufenthalte in Stockholm sowie den Besuch von mehreren Kongressen.
- … Prof. Dr. Olof Rådmark for his hospitality during my stay in his lab, for excellent scientific advices and discussions as well as for his precise revisions of my manuscripts. Many thanks, Olle.
- … Daniel Pöckel für neue hilfreiche wissenschaftlich/biochemische Aspekte und Kniffe im Labor, das Korrektur lesen dieser Arbeit, den Versuch der Etablierung von DoKo-Runden, das gemeinsame Fahren der schwarzen 35, „J'en ai marre!“ und den regelmäßigen Donnerstagskick.
- … Nicole Schnur für das gemeinsame Durchleben von Höhen und Tiefen während des Studiums und der Promotion, sowie manch konstruktive Kritik.
- … Dr. Nadine Meindl, Dr. Sabine Seuter, Dr. Anja Altmann, Dr. Uwe Janßen-Timmen für einige molekularbiologische Tipps und die Aushilfe mit Platten und Puffern, die Einweisung in das weite Feld der Boswelliasäuren, ein paar klaren Worten und die sehr gute Zusammenarbeit.

… Dagmar Szellas für die gewissenhafte und exzellente Einarbeitung in die Geheimnisse des Labors, Zellkultur, Western-Blotting, 5-LO Assays, Proteinreinigung, Transfizieren von HeLa-Zellen sowie den einen oder anderen Puffer

… dem AK Steinhilber für die gute Zusammenarbeit und vielen gemeinsamen Unternehmungen.

… Brigitte Welter, Astrid Brüggehof, Astrid Neuß und Sven George für die Beschaffung von jeder Menge Literatur, die Bestellung von Chemikalien, diversen Maxi-Preps und die Mithilfe und Entlastung im Labor.

… den Mitgliedern des EU Graduiertenkolleg „Roles of Eicosanoids in Biology and Medicine“ für jede Menge Workshops, diverse Reisen und Summer Schools.

… den Angestellten des Instituts für Pharmazeutische Chemie, für die oft unbemerkte Arbeit im Hintergrund, ohne die das Leben am Institut viel schwerer wäre.

… den Mitarbeitern des Zentrallabors des Markus-Krankenhauses, Frankfurt/Main, für die Belieferung mit Blutkonserven, ohne die es diese Arbeit so nicht gäbe.

… meinen Freunden aus Oberhausen und Frankfurt für den Rückhalt im Leben außerhalb des Labors.

Mein besonderer Dank gilt Dr. Eva Bürkert für die schier unendliche Geduld im (Iso-)Labor und die gewonnene tiefe Freundschaft und
insbesondere meinen Eltern und meiner Schwester Anne für die unermüdliche Unterstützung, das entgegengebrachte Vertrauen und die gegebene Liebe.