Pharmacological actions and targets of boswellic acids in human leukocytes and platelets

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1 Abbreviations

AA	arachidonic acid
AC	adenylyl cyclase
A(β)BA	3-*O*-acetyl-(β-)BA
(A)KBA	(3-*O*-acetyl-)11-keto-BA
A(D/T)P	adenosine (bis/tris)phosphate
ATL	aspirin-triggered lipoxin
(β-)BA	(β-)boswellic acid
BC-4	mixture of α-BA and β-ABA (1:1) extracted from *B. carterii*
B. spec	*Boswellia species*
CaLB	Ca^{2+}/lipid binding domain
CaMK	Ca^{2+}/calmodulin-dependent kinase
(c/s/i)PLA_2	(cytosolic/secretory/Ca^{2+}-independent) phospholipase A_2
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CSF	colony-stimulating factor
COX	cyclooxygenase
CYP	cytochrome P450
cys-LT	cysteinyl leukotriene
DAG	diacylglycerol
DCF-DA	dichlorofluorescein diacetate
EET	epoxyeicosatrienoic acid
ER/SR	endoplasmatic/sarcoplasmatic reticulum
ERK	extracellular signal-regulated kinase
ETP	endogenous thrombin potential
FLAP	5-lipoxygenase-activating protein
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
G(D/T)P	guanosine (bis/tris)phosphate
GEF	guanine exchange factor
GM-CSF	granulocyte/macrophage colony stimulating factor
GP	glycoprotein
GPx	glutathione peroxidase
GPCR	G protein-coupled receptor
Grb-2	growth factor receptor-bound protein 2
GSH	glutathione (reduced state)
(12-)HHT	(12-)hydroxyheptadecatrienoic acid
HL-60	human leukaemia cell line
HLE	human leukocyte elastase
H(P)ETE	hydro(pero)xy-eicosatetraenoic acid
(13-)HPODE	(13-)hydroperoxyoctadecadienoic acid
IFN	interferone
IKK	inhibitor of NF-κB (IκB)-kinase

IL	interleukin
IP_3	inositol trisphosphate
JNK	c-Jun *N*-terminal kinases
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LO	lipoxygenase
LT	leukotriene
LTA_4H	LTA_4 hydrolase
LTC_4S	LTC_4 synthase
LX	lipoxin
Mac-1	macrophage antigen-1
MAPEG	membrane associated proteins in eicosanoid and GSH metabolism
MAPK(KK)	mitogen-activated protein kinase (kinase kinase)
MAPKAPK	MAPK-activating protein kinase
MEK	MAPK and ERK kinase
MMP	matrix metalloproteinase
MNK	MAPK-integrating kinase
MK-2/3	MAPKAPK-2/3
NF-κB	nuclear factor κB
NO	nitric oxide
PAF(-AH)	platelet-activating factor (acetylhydrolase)
PAR	protease activated receptor
PDGF	platelet-derived growth factor
PG	prostaglandin
PGI_2	prostacyclin
PH	pleckstrin homology
PIP_2	phosphatidylinositol-4,5-bisphosphate
PI-3 K	phosphoinositide-3 kinase
PL	phospholipase
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane Ca^{2+}-ATPase
PMNL	polymorphonuclear leukocytes
PK(A/C)	protein kinase A/C
PSGL	P-selectin glycoprotein ligand
PT	pentacyclic triterpene
PTx	pertussis toxin
PtdCho/Ins	phosphatidyl choline/inositol
p-tyr	phospho-tyrosine
p12-LO	platelet-type 12-lipoxygenase
RA	rheumatoid arthritis
RGS	regulators of G protein signalling
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase

SERCA	SR/ER Ca^{2+}-ATPase
SH(2/3)	src homology (2/3) domain
SOCE	store-operated Ca^{2+} entry
TA	tirucallic acid
TF	tissue factor
TGF	transforming growth factor
TLR	Toll-like receptor
TM	transmembrane
TNF	tumour necrosis factor
TRAP	thrombin receptor-activated peptide
Tx	thromboxane
vWf	von Willebrand factor

2 Introduction

2.1. Inflammation

Inflammation is the physiological reaction of the immune defence system to a variety of noxae, including internal stimuli (tissue injury, lesions) and external stimuli (pathogen infection, bacterial toxins). On the one hand, inflammation consists of an exudative component, resulting in blood vessel dilatation and enhanced capillary permeability around the site of irritation or infection. As a consequence, redness, heat, swelling (edema) and pain are caused. On the other hand, the second (cellular) component of inflammation involves leukocytes, capable to enter the tissue through permeabilised capillary walls. At the first stage, phagocytes attempt to eliminate the infectious stimuli, but if the inflammatory stimulus persists, cytokines (interleukin-1 (IL-1), tumor necrosis factor (TNF)) are released that activate the endothelium in order to upregulate the expression of adhesion receptors (VCAM, ICAM, selectins). Other inflammatory cells such as additional granulocytes, monocytes, T and B cells are recruited to the inflamed tissue. Typically, neutrophilic granulocytes, also termed polymorphonuclear leukocytes (PMNL), are found in acute states of inflammation, where they are responsible for phagocytosis and chemokine/second messenger release so as to promote and finally terminate the inflammatory process. At the infectious site, they can undergo a respiratory burst to attack intruders, and secrete proteases to finally cleanse damaged tissue. In wound repair, initially blood platelets are triggered by tissue collagen to release mediators, express glycoproteins and aggregate, resulting in the formation of a fibrin/fibronectin-based clot, that attracts other cells like PMNL. In subsequent processes, collagen deposition leads to the re-formation of the extracellular matrix (ECM), and angiogenesis is induced (for reviews, see [1, 2]).

In chronic or persisting inflammation, however, the short-lived neutrophils are gradually substituted by monocytes from the peripheral blood. At the infected or inflamed tissue, monocytes enter the tissue and mature into macrophages, which eliminate apoptotic PMNLs after a few days [2, 3]. Macrophages are powerful producers of reactive oxygen species (ROS), supposed as a defence mechanism but also causing destruction to the surrounding tissue if the cells remain too long at the infectious site [4]. Thus, in many diseases with chronic inflammation (arthritis, rheumatism), it is beneficial to downregulate the activity of leukocytes by pharmacological agents in order to prevent progressive tissue degradation.

2.1.1. Inducers/Mediators of inflammation

In case of exogenous infections, the tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) may be secreted by bacteria [5]. fMLP is a potent chemotactic for neutrophils, where it binds to a specific G protein-coupled receptor (GPCR) [6, 7], causing neutrophil activation via intracellular mobilisation of Ca^{2+} and activation of protein kinases such as the mitogen-activated protein (MAP) kinase cascade [8, 9] but also of cytosolic phospholipase (cPL)A_2 [10].

Cytosolic phospholipase (cPL)A_2 predominantly liberates AA which either functions as mediator itself, inducing PMNL adhesion to the endothelium, ROS formation, chemotaxis and MAP kinase activation [11-13], or serves as a precursor for bioactive lipid mediators (platelet-activating factor, PAF) and eicosanoids. These include prostaglandins (PGD_2, PGE_2), prostacyclin (PGI_2), and thromboxane (TxA), derived from cyclooxygenase (COX) and PGE synthases, as well as hydroxyl-eicosatetraenoic acids (HETEs), leukotriene (LT)B_4, cysteinyl (Cys)-LTs, and lipoxins, synthesised by lipoxygenases. PAF, derived from lyso-PAF, attracts and stimulates granulocytes and monocytes/macrophages, enhances vascular permeability and aids to maintain an inflammatory state [14]. PAF also participates in platelet activation by binding to its corresponding G_i- and/or G_q-coupled receptor [15]. Per se, PAF is a moderate stimulus for neutrophils and a strong stimulus for eosinophils, that for instance causes substantial superoxide anion ($^{\cdot}O_2^-$) formation and generally shares many biological effects with fMLP [16]. COX products usually activate specific GPCRs and have diverse physiological effects mainly related to pain sensation, vascular homeostasis, and inflammation. Lipoxygenase (LO) products are discussed in a later section. Taken together, eicosanoids act as local hormones with short half-life. In contrast, cytokines share many features with “classical” hormones. They are polypeptides, synthesised and released by many inflammatory and other cells, encompassing interleukins (ILs), interferones (IFNs), colony stimulating factors (CSF), tumor necrosis factors (TNFs), transforming growth factor β (TGF-β), and chemotactic cytokines (chemokines) [17]. Cytokines bind to membrane-embedded cytokine receptors that induce cytosolic responses but also modulate transcription factors via members of the MAP kinase family, the c-Jun *N*-terminal kinases (JNKs). They have proinflammatory and immunostimulant properties, modulate the haematopoietic system and control proliferation, differentiation and cell survival [18]. Chemokines are the largest group of cytokines (>40 human members), and act via specific GPCRs to mediate leukocyte activation [19, 20]. Accordingly, leukocyte- or fibroblast-derived IL-8 is the most important chemokine for neutrophil activation, inducing a respiratory burst [21].

2.2. Physiology of haematopoietic cells

2.2.1. Polymorphonuclear leukocytes (PMNL)

Mature PMNL evolve in about 14 days from myeloblasts via myelocytes. They contain numerous ribosomes, mitochondria, and granules, as well as a spectrum of receptors linked to the innate immune defence [22]. Regularly, PMNLs roll along walls of postcapillary venules or various membranes and tissues for signs of chemoattractants. For proper recruitment to sites of infection, PMNLs require priming by a variety of agents such as cytokines, chemokines (IL-8), growth factors, lipid mediators, adhesion factors, or bacterial stimuli (fMLP). Many of these agents act on Toll-like receptors (TLRs) of neutrophils, inducing mobilisation of secretory vesicles but not yet full activation. That is, in turn, achieved by direct interaction with pathogen-derived structures (peptidoglycans, lipopolysaccharides) via TLRs, CD14, and other cell-surface receptors, resulting in prolonged cell survival, phagocytosis, respiratory burst and degranulation [23]. In addition, complement-opsonised microbes are easily recognised by PMNL complement receptors, further triggering PMNL activation.

Neutrophil degranulation occurs in conjunction with phagocytosis, as granules either exocytose or fuse with phagosomes. Granules contain various anti-bacterial proteins such as α-defensins, cathepsins, proteinase-3, elastase and lysozyme, supposed to permeabilise and degrade microbial structures [22]. The regulatory mechanisms that govern degranulation are not yet fully elucidated but are believed to comprise, amongst other things, intracellular Ca^{2+} mobilisation, Src family kinase and p38 MAPK activation [23, 24]. As an unwanted side effect, protease release also causes destruction of healthy tissue, a process that may contribute to some chronic disease states.

The neutrophil respiratory burst has important bactericidal effects. It consists of reactive $^{\cdot}O_2^-$ produced primarily by the membrane-bound NADPH oxidase complex [25]. Other sources are the mitochondrial respiratory chain, xanthine oxidase, COX and LOs (yielding lipid hydroperoxides as side products) and the cytochrome p450 system. The NADPH oxidase complex consists of components that normally reside in the cytosol ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, Rac2) as well as in membrane compartments ($gp91^{phox}$/$p22^{phox}$, Rap1A) that associate at the membrane upon neutrophil activation. Phosphorylation of the cytosolic components by protein kinase (PK)C but also by ERKs and p38 MAPK are major inducers of membrane translocation [26]. The active enzyme transfers electrons from NADPH to intraphagosomal molecular oxygen, yielding $^{\cdot}O_2^-$ that has destructive properties within phagosomes. The short-lived superoxide anion is rapidly dismutated to H_2O_2 and derivatives like hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$), which act

efficiently on bacteria [27]. Moreover, these oxidants activate matrix metalloproteinases (MMPs) that promote tissue breakdown [28]. Within granulocytes, catalases and glutathione peroxidases (GPx) provide intracellular protection against oxidants. In many pathophysiological settings such as tumor progression, neurodegeneration, atherosclerosis, but also Diabetes mellitus and aging, an imbalance between generated reactive oxygen species (ROS) and antioxidative effectors exists, causing 'oxidative stress' that includes the destruction of intact tissue by often chronically activated inflammatory cells [29]. Here again, intervention with pharmacological agents can reasonably cause a slowdown of disease progression. Notably, patients suffering from a genetic disorder called chronic granulomatous disease express defective subunits of the NADPH oxidase, unable to generate sufficient amounts of H_2O_2 and other reactive oxygen radicals. These patients sustain recurrent infections and have a remarkably reduced lifespan, thereby underlining the crucial importance of ROS generation in host defence [30].

Finally, the resolution of an infection is accompanied by PMNL apoptosis. Aged PMNL typically undergo spontaneous apoptosis after few days [31], but this can be delayed by cytokine stimulation (tumour necrosis factor (TNF)-α, lipopolysaccharide (LPS), toxins) to allow proper pathogen clearance. In contrast, bacterial ingestion by PMNL accelerates apoptosis, followed by encirclement and degradation by neighbouring macrophages [32]. Thus, neutrophil apoptosis is an essential process in the termination of an infection and health maintenance.

2.2.2. Monocytes and macrophages

Monocytes are myeloid cells that circulate in the blood for 1 to 2 days before they extravasate into tissues for terminal maturation to 'tissue' macrophages. This process occurs regularly, therefore resident tissue macrophages represent an important component of the vigilant unspecific host defence in the body. At sites of infection or irritation, they accumulate in large numbers [33]. Extensive macrophage extravasation is an early event in the onset of inflammation, various diseases, and wound healing. As stated above, the initial infiltration occurs through neutrophils with a maximum after 1 to 2 days, followed by monocyte recruitment that peaks not until 2 to 5 days after the wounding. In damaged tissue, macrophages are responsible for debris clearance and attraction of other cell types by release of cytokines and chemokines. The late phase of an inflammation is characterised by macrophages acting in concert with lymphocytes and epithelial cells by virtue of mutual cytokine stimulation (IL-12, IFN-γ, TGF-β) to induce tissue repair [34]. Furthermore, macrophages release 'secretory leukocyte protease inhibitor' (SLPI) which

can be regarded as a marker indicating the switch from tissue damage to tissue repair. SPLI has anti-inflammatory and wound-healing effects, and together with the action of fibroblasts, lymphocytes and endothelial cells, subsequent processes like tissue remodelling and angiogenesis are induced [34, 35]. However, macrophages are also implicated in tumour growth, since they are recruited by tumour-derived chemoattractants and associate with tumours. They may even constitute a large portion of the tumour mass [36]. By secretion of various factors, these cells participate in tumour proliferation, survival, metastasis and angiogenesis [37]. Intriguingly, in many aspects (cytokine equipment, receptor expression, or motility) macrophages resemble tumour cells [33], and not surprisingly, more than 15 percent of cancers worldwide evolve from chronic inflammations initiated by infections [20].

With their capacitiy to secrete proangiogenic cytokines and enzymes, macrophages exert beneficial action in wound healing but at the same time cause unwanted effects in rheumatoid arthritis (RA). RA consists of a chronic inflammation of the synovium in joints, bearing infiltrated leukocytes and hyper-proliferative fibroblasts [38]. Persistent presence of macrophages is linked to tissue damage by formation of ROS, but also to the generation of new blood vessels that facilitates migration of leukocytes and fibroblasts. Angiogenesis is a prominent feature of RA which is promoted by macrophages [33]. Lastly, macrophages play an important role in atherosclerosis, a process due to dysfunction of endothelial cells. Monocytes infiltrate into arterial intima, differentiate into macrophages and ingest large quantities of oxidised low-density lipoprotein (LDL), thus becoming “foam” cells at the arterial wall [39]. In disease progression, the arterial wall thickens and the diameter decreases, causing hypoxic conditions and promoting vessel obstruction [33]. Altogether, there are several disease types where pharmacological regulation of macrophage activity appears favourable regarding disease progression [37].

2.2.3. Platelets

Platelets play a central role during vascular wound healing. Injured endothelium allows blood components to interact with the subendothelial matrix, leading to multiple interactions between endothelium, neutrophils, monocytes, and platelets. Initial platelet tethering under shear conditions is governed by interaction of its two main adhesion receptors, platelet glycoprotein (GP) Ibα and GPVI, to von Willebrand factor (vWf) and collagen, respectively [40]. Monocytes and PMNL are recruited via P-selectin expression on collagen-bound platelets and endothelial cells. Monocyte interaction with platelets and endothelium relies on the binding of monocyte P-selectin GP ligand-1 (PSGL-1) to P-

selectin [41], and is further strengthened by activation of macrophage antigen-1 (Mac-1) by its corresponding ligand on adherent platelets (MCP-1, monocytic chemotactic protein-1) [42]. Meanwhile, the coagulation process is initiated by exposure of extravascular tissue factor (TF) to blood components, but also by TF on microvesicles released by stimulated monocytes at injured vessel walls [43]. TF-bearing vesicles concentrate at these sites in close proximity to adhered platelets, inducing the coagulation cascade. The cascade is spatially directed to the platelet surface by means of the prothrombinase complex (factor Xa) which cleaves plasma prothrombin into thrombin, thus generating a pivotal coagulation stimulus [44]. Interestingly, this prothrombinase complex can also be associated on monocytes, where neutrophil-derived cathepsin G activates factor X to factor Xa bound to the monocyte integrin Mac-1 ($\alpha_M\beta_2$). Thrombin is a strong pro-aggregatory stimulus, but its effect can still be enhanced by cathepsin G and elastase released from PMNL that cleave the α_{IIb} subunit of platelet integrin $\alpha_{IIb}\beta_3$, enabling it to bind fibrinogen and hence induce aggregation [45]. More platelets are attracted by binding of GPIb-IX-V complex to vWf released from endothelial cells which respond in this way upon exposure to ROS generated by activated adherent leukocytes [46]. GPIb-IX-V receptor occupation induces diverse intracellular signalling events by integration of multiple stimuli. Activation of the second major adhesion receptor GPVI, predominantly by collagen, induces activation of the platelet integrins $\alpha_2\beta_1$ and $\alpha_{II}b\beta_3$ (GPIIb-IIIa) by virtue of tyrosine kinase activation, thus allowing firm adhesion to collagen and promoting aggregation [47].

Thrombin is a protease that acts on protease-activated receptors (PARs), present on platelets but also on PMNL and monocytes, where it acts as a chemotactic factor. In platelets, however, activation of PARs induces G protein signalling and massive intracellular Ca^{2+} mobilisation that reinforces $\alpha_{II}b\beta_3$-dependent aggregation. Collagen and thrombin agonism on platelets further induces an auto-stimulatory loop involving ADP. The nucleotide is stored in dense vesicles and released upon binding of strong agonists, immediately stimulating platelet purinergic $P2Y_1$ and $P2Y_{12}$ receptors that strengthen platelet activation [47]. Concomitantly, α-granules containing soluble vWf and P-selectin are secreted. Traditionally, platelet shape change was considered as a prerequisite for aggregation [48], comprising the rearrangement of cytoskeletal elements in order to facilitate granule secretion, integrin externalisation and cell-cell interactions. However, current evidence suggests that shape change rather occurs after than before aggregation itself [49].

Besides execution of the coagulation cascade, local inflammation is promoted by various released factors of the intertwined cells, such as neutrophil release of chemokines and AA. AA and derivatives serve as substrate in transcellular metabolism for COX and 12-LO enzymes to generate pro-inflammatory and aggregation-conveying eicosanoids [44]. The intimate physical association between activated platelets and leukocytes via P-selectin binding to PSGL-1 [50] and/or GPIbα association with Mac-1 [51] thus suggests an important contribution of platelets to the progression of an inflammatory response at least in terms of vessel injuries, pointing out platelets as a noteworthy target for the development of anti-inflammatory drugs.

2.3. Typical signalling pathways

2.3.1. Cell surface receptors

Cell surface receptors transduce signals from external, mostly not membrane-permeant, agonists into intracellular signalling events. Four classes of receptors have been defined, (1) ligand-gated ion channels, (2) transmembrane (TM) receptors with intrinsic enzymatic activity, (3) TM receptors with associated tyrosine kinase activity, and (4) G protein-coupled receptors (GPCRs). The first group is mainly found in neuronal tissues for the purpose of rapid synaptic signal transmission. Nonetheless, the IP_3 receptor, a ligand (IP_3)-gated ion (Ca^{2+}) channel, is a prominent representative of this group with high impact in Ca^{2+} signalling in all tissues. Growth factors are important ligands of the second group of receptors; these receptors often possess an intrinsic tyrosine kinase activity on the intracellular domain and are called receptor tyrosine kinases (RTKs). Also the third category of receptors may bind growth factors, but at the cytosolic part, soluble tyrosine kinases have to be recruited for further signal transduction. GPCRs, the fourth group, are intracellularly linked to heterotrimeric G proteins that can bind guanine nucleotides (GDP, GTP). GPCR ligands are mostly peptides or polypeptides, and GPCRs constitute nowadays the main drug targets.

Cell proliferation or differentiation is regulated by growth factors and cytokines via RTKs. RTKs are receptors with one TM helix. Upon binding, RTKs dimerise and autophosphorylate vice versa on tyrosine residues, but they may also phosphorylate other associated proteins [52]. Phosphorylated tyrosine (p-tyr) residues provide a binding matrix for proteins bearing ‘SH2’ domains. SH2 domains are considerably widespread among intracellular proteins, allowing a multitude of signalling cascades to be initiated. Examples are the members of the Ras-Raf activation complex, consisting of the growth factor receptor-bound protein 2 (Grb-2) and associated cytosolic adaptor proteins (Sos, Shc) that

promote activation of the membrane-anchored small G protein Ras and subsequently of the serine/threonine kinase c-Raf. Ras and c-Raf have diverse targets; one central effector, the MAP kinase ERK, is introduced below (section 2.3.3.). As can be appreciated, RTKs function as a site of conjunction for several effector proteins that need to associate for further signal transduction. A second effect of the formation of this 'activation complex' is the amplification of small initial signals to robust cellular responses at the end of the cascade. RTKs are also essential for the activation of PLCγ, since upon association of its two SH2 domains to p-tyr residues on RTKs, PLCγ gains access to its substrate, phosphatidylinositol-4,5-bisphosphate (PIP_2), and can initialise a cascade leading to Ca^{2+} mobilisation and PKC activation [53](see below, 2.3.2.).

GPCRs consist of seven TM helices and possess an intracellular guanine exchange factor (GEF) activity. After receptor occupation by specific ligands, conformational changes allow GDP-bound (inactive) heterotrimeric G proteins to bind to the cytosolic portion of the receptor, which catalyses the exchange of GDP to GTP. GTP binding destabilises the heterotrimer, and the α subunit dissociates from the βγ complex in order to activate or inhibit effector proteins. Intrinsic GTPase activity of the α subunit finally terminates its activity and facilitates re-association of the trimer in its inactive form. Currently four major groups of Gα proteins have been defined, named according to their most prominent members $G\alpha_s$ (stimulating adenylyl cyclase (AC)), $G\alpha_i$ (inhibiting AC), $G\alpha_q$ (activating e.g. PLCβ isoforms), and $G\alpha_{12/13}$ (targeting e.g. cytoskeletal proteins, Rho GEFs). Also the Gβγ complex modulates the activity of effector proteins, for instance $PLC\beta_2$ or phosphoinositide-3 kinase (PI-3 K) [54, 55]. Examples for specific GPCRs on leukocytes are the fMLP and PAF receptors that are linked to $G\alpha_i$ and $G\alpha_i$/ $G\alpha_q$ proteins [56]. A hallmark of platelet activation is the activation of PAR receptors by thrombin. PARs are unique GPCRs, because they are activated by proteolysis of their N-terminal portion, thereby unmasking the endogenous ligand of the receptor. This special mechanism requires subsequent internalisation and degradation, thus, PAR cleavage is a critical step in irreversible platelet activation [55]. The expression pattern of GPCRs varies depending on the cell type, but due to the enormous intracellular amplification of GPCR signals, even limited numbers of expressed GPCRs and low agonist concentrations enable cells to generate a prominent functional response.

2.3.2. The role of Ca^{2+}

Ca^{2+} is a cofactor for many proteins and at the same time second messenger within intracellular signalling events. The crucial role of Ca^{2+} relies on the tight regulation of its

intracellular availability; the concentration between cytosol (10-100 nM) and extracellular space (1.5-2 mM) differs by factor 10^4 to 10^5 [57]. By opening of Ca^{2+} channels on intracellular stores or in the plasma membrane, the cytosolic $[Ca^{2+}]_i$ can instantly be elevated to 500-1000 nM, thus representing a powerful means for the cell to regulate intracellular components and physiological effects [18]. Agonists that induce Ca^{2+} influx in cells such as leukocytes and platelets usually act via GPCRs, since these receptors are mostly employed in rapid signalling. Typically, stimuli like fMLP, PAF, or LTB_4 cause activation of G proteins that recruit PLC isoforms. The following steps are collectively referred to as "store-operated Ca^{2+} entry" (SOCE) [58]. First, PLC hydrolyses PIP_2, yielding membrane-embedded diacylglycerol (DAG) and soluble inositol trisphosphate (IP_3). Second, IP_3 activates specific IP_3 receptors (ligand-gated Ca^{2+} channels) on the endoplasmic reticulum (ER), promoting efflux of stored Ca^{2+} into the cytosol, generating a moderate intracellular Ca^{2+} elevation. Third, the depletion of intracellular Ca^{2+} stores is immediately coupled to the opening of plasma membrane Ca^{2+} channels by a yet unresolved mechanism that may include "conformational coupling" of the participating compartments [59]. As a result, massive Ca^{2+} influx superimposes the intracellular fraction to a substantial elevation that in most cases is rapidly counteracted by activated Ca^{2+} pumps, since sustained elevations of cytosolic Ca^{2+} levels are cytotoxic. Therefore Ca^{2+}-ATPases (located in the plasma membrane, termed PMCA, and in the ER/SR, termed SERCA) rapidly pump Ca^{2+} back into intracellular storage sites or the external lumen [60]. Besides central roles for Ca^{2+} in muscle contraction and the execution of apoptosis, Ca^{2+} has ubiquitous functions as cofactor for various proteins. It is involved in vesicle exocytosis by binding to cytoskeletal elements, cell-matrix interactions, and membrane association of proteins bearing so-called C2 domains [61]. C2 and C2-like domains are found in several enzymes related to intracellular protein kinase cascades and lipid signalling, such as $cPLA_2$, PKC, PLC, and LOs [62, 63]. Binding of Ca^{2+} to C2 domains causes conformational changes, thereby increasing the affinity for membrane phospholipids. As a consequence, the enzymes (like 5-LO, 12-LO, $cPLA_2$, PKC, PLC, and PI-3 K) translocate to membrane compartments where they encounter substrate, binding partners, or additional modifications [63-65].

2.3.3. MAP kinase cascades

MAPKs are central regulators of a vast array of physiological processes in all cell types of multicellular organisms. They are regulated by a evolutionary conserved mechanism of sequential phosphorylations in a three-component cascade (figure 1). Moreover, three

major MAPK pathways have been characterised, culminating in three families of 'terminal' MAPK termed ERKs, JNKs, and p38 MAPK [66].

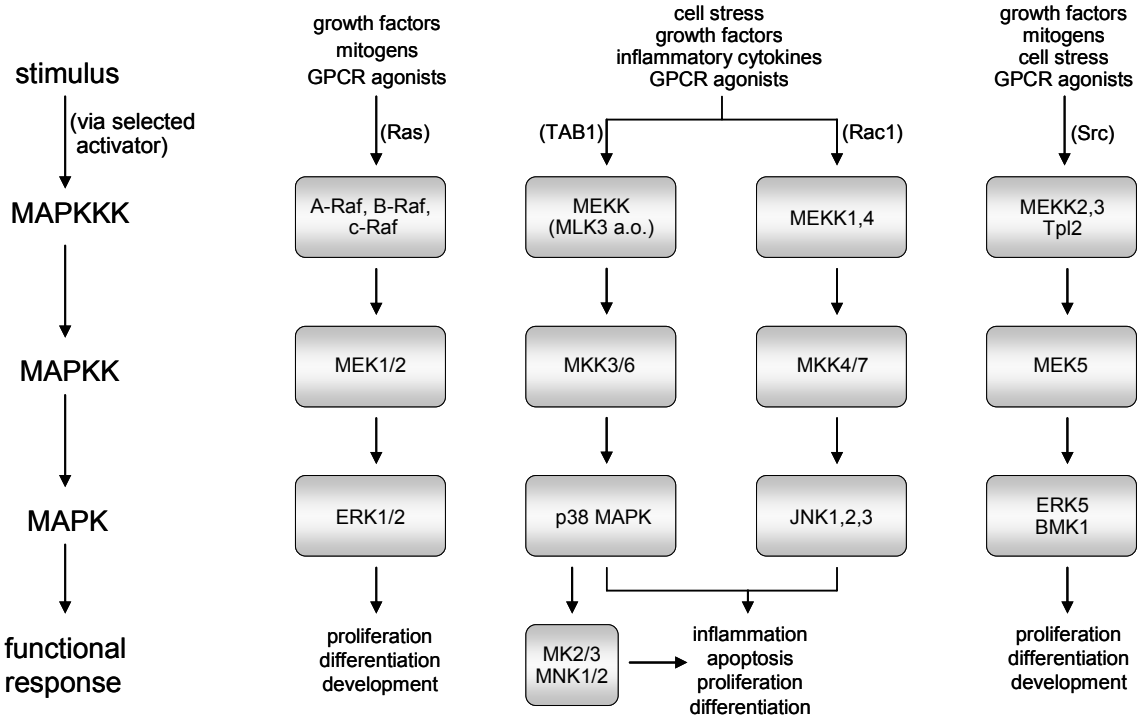

Figure 1: MAPK phosphorylation cascades (a.o., and others)

Humans express two major isoforms of ERKs, previously designated p42/p44 MAPKs. They are activated by various stimuli, such as growth factors, cytokines, GPCR ligands, or mitogens, that converge at the stage of Ras, a small membrane-associated G protein. Activated Ras·GTP recruits Raf, the first member of the tripartite MAPK cascade, as well as other effector proteins like PI-3 kinase (PI-3 K), to the plasma membrane. Raf, which exists in three isoforms (A-Raf, B-Raf, (c-)Raf-1), is a serine/threonine (Ser/Thr) protein kinase, that phosphorylates the downstream kinase MEK (MAPK and ERK kinase) at two serines in its 'activation loop'. Besides, roles for Raf-1 as an inhibitor of apoptosis and activator of the Rho pathway (controlling cytoskeletal remodelling) have recently been suggested [67, 68]. Activated MEK has only one known substrate, that is the ERK members. Interestingly, MEK is a dual-specificity protein kinase that exists in two isoforms in mammals, introducing two phosphate groups at a threonine and a tyrosine residue in the phosphorylation motif TEY. ERKs control cell division, stimulate proliferation, with implications for tumour growth, regulate motility, and influence metabolism. Important targets for ERKs related to eicosanoid generation are $cPLA_2$ and 5-LO [69]. As multifaceted MAPK, activated ERKs are found in the nucleus, cytosol, membrane and cytoskeletal compartments [66, 70]. The analysis of ERK1/2

phosphorylation at Thr185/Tyr187 in cellular samples generally indicates the activation state of the MAPK, reflecting the responsiveness of the cell to the selected stimulus.

JNKs were originally designated “stress-activated protein kinases” (SAPKs, together with p38 MAPK) and are now being recognised as important transcriptional regulators. They form part of the AP-1 transcription complex, thereby controlling many cytokine genes, involving the regulation of apoptosis. In turn, JNKs are activated upon exposure to environmental stress and growth factors in an extremely complex manner by at least 13 enzymes at the stage of MAPKKKs [71].

p38 MAPK also belongs to the SAPKs, because its activation occurs in response to cellular stress factors like radiation, UV light, genotoxic stress, or osmotic shock. Some stimuli like inflammatory cytokines act via GPCRs or RTKs, indicating a role for p38 MAPK in the onset of an inflammatory response, but also in diseases like asthma and autoimmunity [66]. Notably, MAPKAP kinase-2/3 has been identified as target for p38 MAPK. This enzyme is important for the activation of 5-LO (see below), thus providing a link between inflammation/cell stress and the generation of bioactive eicosanoids from free AA [72]. To date, there are four major p38 MAPK isoforms known (α, β, γ, δ). p38α is most widely expressed and represents the primary isoform in all inflammatory cells [73]. In addition to the established MAPKKK-MAPKK-MAPK cascade, p38α is activated by the protein TAB1 at the top stage. Intriguingly, TAB1 is not a protein kinase but rather a scaffold protein without catalytic activity, thus representing an alternative mechanism of activating the MAPK cascade [66].

Termination of MAPK activity is effectuated by a battery of specific phosphatases. By cleaving off the phosphate(s), these enzymes render members of the MAPK cascade inactive again. Specific regulation of phosphatases permits to define the duration and potency of MAPK activity and is therefore a key regulator of MAPK cascades in general [74].

Regarding neutrophil physiology, inflammatory mediators have merely selective effects on the activation of certain members of the MAPK families. For instance, TNF-α specifically activates p38α and δ, whereas GM-CSF, fMLP, or IL-8 primarily activate ERK1/2. However, it has to be noted that MAPK pathways are a complex network, allowing individual members to cross-modulate adjacent routes and vice versa. Thus, determination of the phosphorylation state of single MAPK members solely indicates its potential activity, but does not necessarily elucidate the upstream signalling cascade that led to its activation.

2.4. The arachidonic acid cascade

Arachidonic acid (*all-cis*-5,8,11,14-eicosatetraenoic acid; 20:4/n-6; AA) is a poly-unsaturated, essential (for mammals) fatty acid that is abundantly present in cellular membranes, where it exists in esterified form incorporated into phospholipids (e.g. phosphatidylcholine, PtdCho). AA can be released from phospholipids by the action of phospholipases (PL), foremost $cPLA_2$. Short-lived free AA itself constitutes a second messenger with impact on signalling pathways in the cell, but also serves as a precursor for the generation of eicosanoids by cyclooxygenases (COX), lipoxygenases (LO), and cytochrome p450 enzymes (CYPs). A scheme is presented at the end of chapter 2.4. (figure 4). Alternatively, AA can be re-incorporated into phospholipids, diffuse to the extracellular space, or be degraded enzymatically [75] (figure 2). Since AA represents the precursor for a number of pro-inflammatory metabolites, its release is often induced by primary inflammatory mediators.

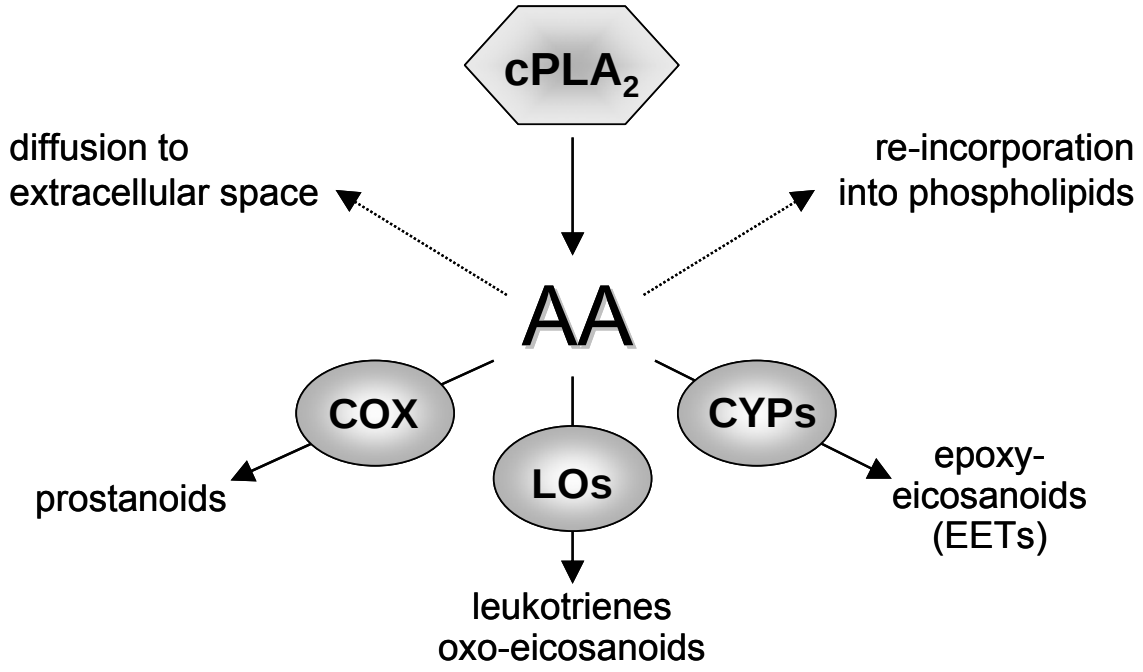

Figure 2: Possible fates of arachidonic acid (AA)

2.4.1. Cytosolic phospholipase A_2

Phospholipases A_2 (PLA_2) comprise a large family of at least 12 members [76], subdivided into four subfamilies of cytosolic (c), secretory (s), and Ca^{2+}-independent (i) PLA_2s as well as PAF acetylhydrolases (PAF-AHs). The most important subgroup, group IV $cPLA_2$, contains four $cPLA_2$ paralogs that are expressed in mammals; designated $cPLA_2$-α, -β, -γ, and -δ. With a molecular weight of 85 kDa, $cPLA_2$-α is the best

characterised isoform in humans and regarded to be primarily responsible for the liberation of substantial amounts of AA in the cell. Accordingly, $cPLA_2$-α-deficient mice fail to produce leukotrienes, prostaglandins, and PAF [77]. Chemically, $cPLA_2$ hydrolyses glycerophospholipids at the sn2-position, yielding free AA and lysophospholipids. The latter can be further converted to PAF, provided the sn-1 position contains an alkyl ether linkage.

$cPLA_2$s differ from the other subfamilies in their pronounced sensitivity for Ca^{2+}, which binds to the Ca^{2+}/lipid binding (CaLB) domain of the enzyme. $sPLA_2$s, which are much smaller (14 kDa), require millimolar concentrations of Ca^{2+} for catalytic activity, play roles in chronic inflammation, and are involved in AA release, lipid digestion, and receptor activation [65]. $iPLA_2$s have molecular masses from 85 to 88 kDa but are structurally different from $cPLA_2$s. They might play a role in lipid remodelling [78]. PAF-AHs are also Ca^{2+}-independent and exist in intracellular and serum isoforms, with different molecular weights. Of all PLA_2 subfamilies, only group IV $cPLA_2$s possess specificity to sn-2 linked AA, with a preference for PtdCho and Ptd-inositol (PtdIns) as substrates [79].

Posttranslational regulation of $cPLA_2$s includes Ca^{2+} binding via its CaLB-domain and phosphorylation events. Importantly, Ca^{2+} binding promotes translocation of the enzyme from soluble compartments to nuclear, ER, or plasmalemmal membranes, where $cPLA_2$ can access and metabolise its substrate [79]. Membrane binding is further facilitated by $cPLA_2$ association with PIP_2 [80]. Phosphorylation, in turn, occurs at serines 505, 515 and/or 727 by ERK2, Ca^{2+}/calmodulin-dependent kinase II (CaMK-II), and the p38-downstream MAPK-integrating kinase (MNK-1), respectively, activates the enzyme, and induces membrane translocation [81, 82]. However, phosphorylation by these kinases is partly cell type-specific (for instance, ERKs do not activate $cPLA_2$ in platelets), and also other kinases (PKC) have been suggested as modulators of $cPLA_2$ activity [79].

Neither mode of activation is obligate, and the degree of $cPLA_2$ activation depends on the stimulus applied. A wide range of extracellular agonists activate $cPLA_2$, including growth factors, cytokines, interferones, integrin engagement or F_c receptor crosslinking, but also non-receptor stimuli such as oxidative stress (at sites of inflammation), shear stress, or UV light [79]. Since induction of $cPLA_2$ yields primarily pro-inflammatory eicosanoids, pharmacological interference with $cPLA_2$ activity (or its activation pathways) may limit the extent of an inflammatory response.

2.4.2. Lipoxygenases

Lipoxygenases (LOs) are a family of enzymes which catalyse the oxygenation of AA and other poly-unsaturated fatty acids. Each lipoxygenase forms a distinct hydroperoxyeicosatetraenoic acid (HPETE) that is subsequently reduced to the corresponding alcohol (HETE). LOs are found in all higher eukaryotic organisms (not in yeast). Their specific designation (5-, 8-, 12-, 15-LO) reflects the position at which molecular oxygen is introduced into the C-20 carbon chain of AA. More than 18 different sequences of mammalian LOs are known; of which six important enzymes are expressed in humans (figure 3) [83, 84]. These are 5(S)-LO, reticulocyte-type 12-/15(S)-LO-I, 15(S)-LO-II, platelet-type (p12(S)-LO), and epidermis-type (e12(R)-LO) [84, 85]. Rat leukocyte-type 12(S)-LO does not exist in humans, the 12-/15-LO-I is the closest human homologue. Mammalian LOs have molecular weights in the range of 72 to 78 kDa and bear N-terminal β-barrel domains that resemble C2 domains in structure and function, thus termed 'C2-like domains' [61, 63]. The C-terminal portion contains the catalytic centre, with a non-heme iron coordinated by three histidines, one asparagine, one isoleucine, and a flexible water molecule in the active site of 5-LO [86]. Today, only the crystal structure of one mammalian LO is available, namely rabbit reticulocyte 15-LO [87]. On the basis of that structure, a model for the tertiary structure of human 5-LO was calculated [88].

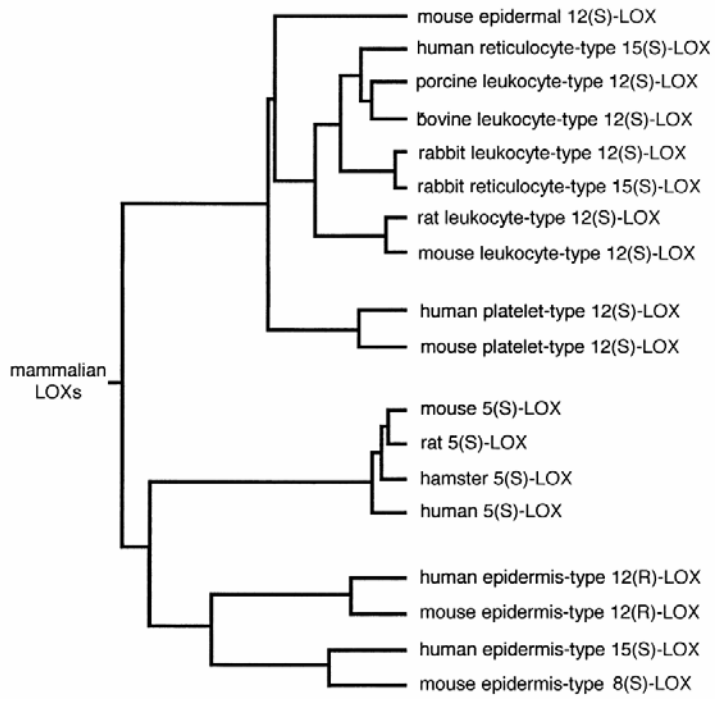

Figure 3: Phylogenetic tree of mammalian LOs (taken from [83], dated 1999).

2.4.2.1. 5-Lipoxygenase

Catalysis

5-LO was first described in rabbit PMNL in the late 1970s, and details of the 5-LO pathway rapidly began to emerge [89, 90]. Two sequential reactions are catalysed by 5-LO, that include first an oxygenase reaction and second the conversion of a formed peroxide into an epoxide (LTA_4 synthase reaction, figure 4). In the first reaction, molecular oxygen is incorporated into AA in a stereospecific manner, by means of a non-heme iron switching between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. Importantly, for the initiation of the reaction, the iron has to be present in the ferric state, a condition that is regulated by the cellular redox tone, e.g. by lipid hydroperoxides. The product of the oxygenation reaction contains a peroxide moiety and is termed 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5(S)-HPETE). It is immediately converted into the instable epoxide LTA_4 by the LTA_4 synthase reaction. This step, too, requires redox cycling of the iron, finally recovering it in the ferric form under release of a water molecule [86]. Continuous activity of 5-LO eventually results in irreversible inactivation (after minutes), also called suicide inactivation, and is characterised by the inability to recover the reduced state of the iron [91]. At the stage of 5-HPETE, parts of the formed products escape subsequent conversion and are reduced (e.g. by GPx) to give 5-hydroxy-eicosatetraenoic acid (5-HETE), that also possesses biological functions and serves as a precursor for 5-oxo-ETE, a highly potent granulocyte chemoattractant [92].

Gene / protein expression

The 5-LO gene is located on chromosome 10 in humans, its expression yields a protein of 672 amino acids in length. It has an unusual promoter structure lacking typical TATA or CCAAT transcriptional initiation elements but is rich in GC sequences. Accordingly, several tandem GC boxes were identified, constituting potential response elements for the transcription factors Sp-1 and Egr-1 that have shown to be required for basal 5-LO transcription [93-95]. Besides, multiple response elements for other transcription factors exist. 5-LO is expressed in cells of myeloid origin, but not in red blood cells, platelets, or endothelial cells. Its expression occurs mainly in parallel with that of 5-LO-activating protein (FLAP, see below). Some other tissues, especially parts of the central nervous system (CNS), also express 5-LO [96].

Crucial factors inducing 5-LO expression are TGF-β and calcitriol (vitamine D3) that upregulate 5-LO mRNA synthesis and protein levels [97, 98]. Furthermore, they modulate 5-LO enzymatic activity in intact cells. In human PMNL and monocytes, GM-CSF is an additional activator of 5-LO and FLAP protein expression [96]. In cells that do not

synthesise 5-LO protein, its transcription is inhibited by histone acetylation and DNA methylation of the 5-LO gene [99, 100].

Regulation of 5-LO activity: cofactors and activation pathways

Early investigations revealed that 5-LO can be activated by Ca^{2+}, ATP, and phospholipids [101]. Without Ca^{2+}, the enzyme is virtually inactive, and 1-2 μM of Ca^{2+} are required for half-maximal activity in vitro [102]. However, in the cell, only 150-350 nM of Ca^{2+} are sufficient to activate 5-LO [103]. Binding of Ca^{2+} to the C2-like domain facilitates selective association with PtdCho, thus directing 5-LO towards membranes, and confers resistance to an endogenous inhibitor, glutathione peroxidase (GPx)-1 [96]. Whereas Ca^{2+} binds in a molar ratio of 2:1 to 5-LO, ATP is bound in equimolar ratio. Presence of ATP enhances 5-LO activity in vitro by factor 6, and is already maximal at a concentration of 0.1 mM, about 10- to 15-fold less than the intracellular concentration [91, 104].

In most leukocytes, 5-LO resides in the cytosol in resting cells and translocates to the nuclear membrane upon activation [105]. In some cell types (alveolar macrophages, mouse mast cells), however, 5-LO is constitutively present within the nucleus [106]. Many proinflammatory stimuli mobilise intracellular Ca^{2+}, as discussed above in the $cPLA_2$ section, hence they also facilitate membrane translocation of 5-LO. Cell stimulation by stress often results in p38 MAPK activation, which is an activator of MAPK-activated protein (MAPKAP) kinases (MK)-2 and -3. Alternative pathways may result in ERK activation. Both enzymes, MK-2/-3 and ERK2, can phosphorylate 5-LO at Ser271 and Ser663, respectively, thereby promoting membrane translocation and induction of full activity [107, 108]. Intriguingly, the presence of free AA strongly enhances phosphorylation of 5-LO [109], suggesting a second regulatory mechanism to selectively activate 5-LO under inflammatory conditions. In contrast, activation of PKA counteracts 5-LO activation and prevents translocation, due to phosphorylation of 5-LO at Ser523 [110].

Once 5-LO associates with the membrane, it moves in close proximity of $cPLA_2$, providing the 5-LO substrate (AA), and to the accessory protein FLAP. FLAP is an 18 kDa membrane-bound protein of the MAPEG superfamily (see section 2.4.3.) and in most tissues it is co-expressed with 5-LO. Its exact function is still elusive, but it was shown that FLAP stimulates 5-LO activity resulting in LTA_4 synthesis [96]. Thus, co-localisation with FLAP and $cPLA_2$ strongly stimulate 5-LO catalysis, therefore the induction of membrane translocation must be regarded as key regulatory step of 5-LO activation. Yet, some additional proteins have been found to bind 5-LO, including Grb-2, TRAP-1, CLP,

RNaseIII, and actin/α-actinin, but the functional relevance of these proteins regarding 5-LO activation will have to be determined [96].

2.4.2.2. 12-Lipoxygenase

Three types of 12-LO are known today, although the individual tissue expression patterns vary between species or are not yet fully elucidated. The leukocyte-type 12(S)-LO has been detected in some glands of the mouse (pineal, pituitary, adrenal, and pancreas) and tissues related to the immune defence, lung and kidney. It is also present in macrophages, leukocytes, and reticulocytes (immature red blood cells). In humans, there is only a 12-/15-LO isoform expressed in reticulocytes and eosinophils, capable to synthesise 12- and 15-HPETE [83, 111]. Epidermal 12(S)-LO is specifically expressed in differentiated keratinocytes of mouse epidermis and in the root of hair follicles, but in humans only as a pseudogene in skin and hair follicles [112]. Platelet-type 12(S)-LO (p12-LO) is primarily present in platelets and epidermis, independent of species [113, 114]. Interestingly, leukocyte-type 12(S)-LO is the primary LO in rat brain, expressed by mature oligodendrocytes and astrocytes [115, 116]. The three 12(S)-LO isozymes show about 60% sequence identity amongst each other and 40% identity in comparison to 5-LO. The most closely related LO is 15-LO with 71-86% identity, which is underlined by the fact that all human 12-LOs and 15-LOs are arranged in a gene cluster on chromosome 17 [111]. In contrast to the 5-LO promoter, the p12-LO *cis*-acting regulatory elements contain a TATA box, as well as three GC boxes for potential Sp-1 binding. In addition, NF-κB and glucocorticoid-responsive elements were detected in a further upstream region of the gene. Accordingly, NF-κB heterodimers were demonstrated to negatively regulate the expression of the human p12-LO gene [117], but otherwise functional implications of these regulatory sites are unknown. On the other hand, several inducers of 12(S)-LO gene expression have been recognised, such as TGF-α and phorbol 12-myristate 13-acetate (PMA) in human epidermis, PDGF in porcine smooth muscle, bradykinin and PAF in rat skin, and angiotensin II in mesangial cells [118, 119].

From a catalytic point of view, 12-LOs function basically in the same way as 5-LO in the first step, except that the introduction of oxygen occurs at position C-12 of the main substrate, AA. The three isoforms differ regarding their product profile. p12-LO primarily generates 12(S)-HPETE from AA, whereas leukocyte-type 12-LO generates considerable amounts of 15-HPETE as well, depending on the species in a ratio between 3:1 and 11:1 (12-HPETE : 15-HPETE). Consistently, by mutation of only two amino acids, porcine 12-LO could be converted into a 15-LO [120]. 12-HPETE is either reduced to the

corresponding alcohol (12-HETE), or may be further enzymatically transformed to diHETEs and hepoxilins. As in 5-LO, 12(S)-LO presumably consists of a N-terminal β-barrel domain (bearing the Ca^{2+} binding site) and a C-terminal portion containing a hydrophobic binding pocket. Three crucial histidines (361, 366, 541) and the C-terminal isoleucine have been identified as essential residues for coordination of the iron in the porcine leukocyte isoform [120].

AA is not the only substrate for 12-LOs. Especially (porcine/murine) leukocyte-type 12(S)-LO also accepts various C-18 and C-20 fatty acids but can also act on esterified substrates, such as phospholipids, cholesterol esters, and LDL [111, 121-123]. For example, lineleic acid (C18:2) is transformed into 13-hydroperoxyoctadecadienoic acid (13-HPODE), a bioactive lipid hydroperoxide [124]. In contrast, the tolerance for other substrates than AA exhibited by p12-LO is rather low. In analogy to 5-LO, leukocyte-type 12(S)-LO is rapidly suicide inactivated (within minutes), whereas no self-inactivation was reported for p12-LO [83, 111].

The subcellular localisation of 12(S)-LOs differs depending on the activation state of the cells. Porcine leukocyte-type 12(S)-LO as well as rat and human p12-LO were found to reside in the cytosol of PMNL and platelets, respectively, and activation of human p12-LO goes along with translocation to the plasma membrane, when a strong stimulus like thrombin is applied [111]. Moreover, binding of Ca^{2+} apparently promotes membrane association of rat p12-LO [64]. At membranes, p12-LO was reported to associate with $cPLA_2$ and $sPLA_2$ upon collagen stimulation via the GPVI receptor, hence supporting the view of membrane compartments as site where catalysis of p12-LO occurs [125].

Other regulatory factors for 12(S)-LO have not been established so far. Evidence is lacking for an involvement of FLAP and ATP in promoting catalysis, and also the role of phosphorylation is unclear. Recently, activation of 12-LO in oligodendrocytes was suggested to be the consequence of ERK activation, but no evidence for direct phosphorylation of 12-LO was given [126].

The physiological consequences of 12-LO activation are described below in the 12-HETE section (2.5.2.). Besides enhanced formation of 12-LO products, altered 12-LO expression gives rise to some clinical implications (for review, see [111]). For example, p12-LO deficiency enhances the risk of hemorrhages rather than thrombosis, accompanied by bleeding complications. Mice lacking p12-LO showed increased platelet sensitivity to ADP, indicating that an anti-aggregatory product is formed by p12-LO. Generally, 12(S)-LO seems to be associated with blood vessel disorders and inflammation. Furthermore, the leukocyte-type 12-LO gene is important for the development of atherosclerosis in mice,

probably due to increased LDL oxidation by 12-LO [127]. A role for p12-LO was also determined in human rheumatoid arthritis [111]. Regarding cancer progression, p12-LO mRNA was found to be elevated in prostate cancer tissues, implying that p12-LO promotes tumour growth, angiogenesis, and metastasis [128, 129]. Likewise, breast cancer patients show elevated levels of leukocyte-type 12(S)-LO in tissue sections as compared to healthy control persons [130].

2.4.2.3. 15-Lipoxygenase

Two isoforms of arachidonate 15-LOs are known at present, designated according to their tissue origin reticulocyte-type (type I)-15LO and epidermis-type (type II)-15-LO. 15-LO-II has only 40 % amino acid identity with type I and occurs in prostate, skin, and cornea [131]. Since especially the type I enzyme shares many properties with human leukocyte-type 12-LO, these enzymes are considered as orthologs, hence uniformly referred to as 12/15-LOs [127, 132]. For that reason, 15-LOs shall be discussed here only briefly.

The main products of 15-LO-I are 15(S)-HETE (80 %) and 12(S)-HETE (20 %), when AA is used as substrate. Catalytic activity lasts only for 1-2 minutes until turnover-related inactivation of the enzyme. In contrast, 15-LO-II converts AA purely to 15(S)-HETE and is catalytically active for more than one hour [131, 132]. Importantly, the concomitant presence of 12- and 15-LOs as well as 5-LO in eosinophils, monocytes/macrophages, and other tissues allows the immediate generation of bioactive diHETEs and lipoxins (lipoxygenase interacting products, containing a trihydroxytetraene structure). DiHETEs are considered to possess pro-inflammatory, neutrophil chemoattractant properties [133], whereas lipoxins (LXA_4, LXB_4) exert anti-inflammatory effects via specific GPCRs. Lipoxins are involved in the late phase of a local inflammation, promoting tissue repair and debris clearance by macrophages, meanwhile inhibiting neutrophil chemotaxis [134, 135]. Accordingly, 15-LO expression is enhanced by T helper cell-derived IL-4 and IL-13, and overexpressed 15-LO in monocyte/macrophages mediate protective effects in early atherosclerosis by preventing lipid deposition in vessel walls [111]. 15-LO-II apparently functions as negative regulator of cell proliferation, and is downregulated during malignant transformation of prostate cells [131]. 15(S)-HETE, in turn, was shown to have inhibitory effects on p12-LO and neutrophil 5-LO, indicating interrelations between LOs and their products [136]. Transcriptional regulation of 15-LOs is comparable to 12-LO, and as post-translational regulatory effectors the peroxide tone, nitric oxide, Ca^{2+}-dependent membrane binding, and suicide inactivation have been described, whereas again no role was evident for a phosphorylation/dephosphorylation cycle [137].

2.4.3. LTA_4 hydrolase and LTC_4 synthase

LTA_4 hydrolase (LTA_4H) converts LTA_4 to LTB_4, a potent lipid mediator with chemotactic and chemokinetic properties towards leukocytes [138]. LTA_4H, a protein of 69 kDa (610 amino acids), is an epoxide hydrolase with a second enzymatic function, an anion-dependent aminopeptidase activity. Its crystal structure was published some years ago [139]. The enzyme can be inhibited by covalent binding of its substrate and by divalent cations. It is widely distributed among mammalian tissues and hematopoietic cells [140, 141]. The occurrence of LTA_4H does not generally coincide with 5-LO expression, hence giving rise to putative transcellular metabolism [142]. In mature cells, LTA_4H normally resides in the cytosol, but depending on the differentiation state also nuclear localisation was observed [143].

LTC_4 synthase (LTC_4S) is the other enzyme that metabolises LTA_4 [144]. By conjugation with glutathione (GSH), the cysteinyl-leukotriene (Cys-LT) LTC_4 is formed, which can be further converted to LTD_4 and LTE_4 by extracellular enzymes (γ-glutamyl-transpeptidase, followed by a dipeptidase) [145-147]. Cys-LTs are potent bronchoconstrictors and pro-inflammatory mediators, mainly generated by activated eosinophils, basophils, mast cells, macrophages, and platelets, whereas neutrophils preferentially convert LTA_4 into LTB_4 [148]. LTC_4S is a structural homologue to FLAP, both belonging to the MAPEG (membrane associated proteins in eicosanoid and GSH metabolism) superfamily. Other members are the microsomal glutathione-S-transferases (MGST) 1-3 and PGE_2 synthase [148, 149]. LTC_4S exists as an 18 kDa protein in homodimers, and Mg^{2+} enhances the catalytic activity [144].

2.4.4. Cyclooxygenases

Cyclooxygenases (COX) enzymes comprise two isoforms; COX-1 that is mainly connected to physiological functions and COX-2, which is mainly involved in pathological processes. In addition, a splice variant of COX-1 was described in dogs, termed COX-3. The two genes are located on chromosomes 9 and 1, respectively [150], and encode for proteins of rather similar features regarding molecular mass (67 and 72 kDa, respectively), intracellular localisation (nuclear or ER membranes [151]), and enzymatic function. COX-1 is expressed in many tissues, but COX-2 expression is apparently restricted to cells related to inflammation, such as macrophages, fibroblasts, or endothelial cells [151]. Platelets were long assumed to contain COX-1 only, since *de novo* (inducible) expression in these denucleated cells is not possible. Recent evidence however suggests, that in the precursor cells, megakaryocytes, COX-2 mRNA is formed, allowing

the synthesis of functional COX-2 enzymes in platelets [152, 153]. A causal relation to aspirin resistance in some patients is currently under debate [150].

Concerning catalytic activity, COX bears a cyclooxygenase active site, where the hydroperoxy endoperoxide prostaglandin (PG)G_2 is formed from AA, and a heme with peroxidase activity that converts PGG_2 to PGH_2. Besides PGH_2, COX also generates 12-hydroxyheptadecatrienoic acid (12-HHT) from AA. Under inflammatory conditions, PGH_2 synthesis exceeds 12-HHT, but presence of reducing agents (GSH, 1 mM) shifts the ratio towards 12-HHT generation [154]. PGH_2 serves as precursor for various prostanoids, including thromboxane (Tx)A_2, PGD_2, E_2, $F_{2\alpha}$, and prostacyclin (PGI_2). Prostanoids are pivotal mediators of fever, pain, and inflammation, and also play roles in the regulation of the gastrointestinal tract, reproduction, and the cardiovascular system [155]. Remarkably, prostanoids do not possess uniform properties. For instance, prostacyclin and TxA_2 are mutual antagonists, the latter acting as pro-thrombotic and pro-aggregatory vasoconstrictor, while PGI_2 mediates opposite effects [150]. Due to their central role in the progression of many diseases, COX-targeting non-steroidal anti-inflammatory drugs (NSAIDs, e.g. aspirin) are long since established on the drug market.

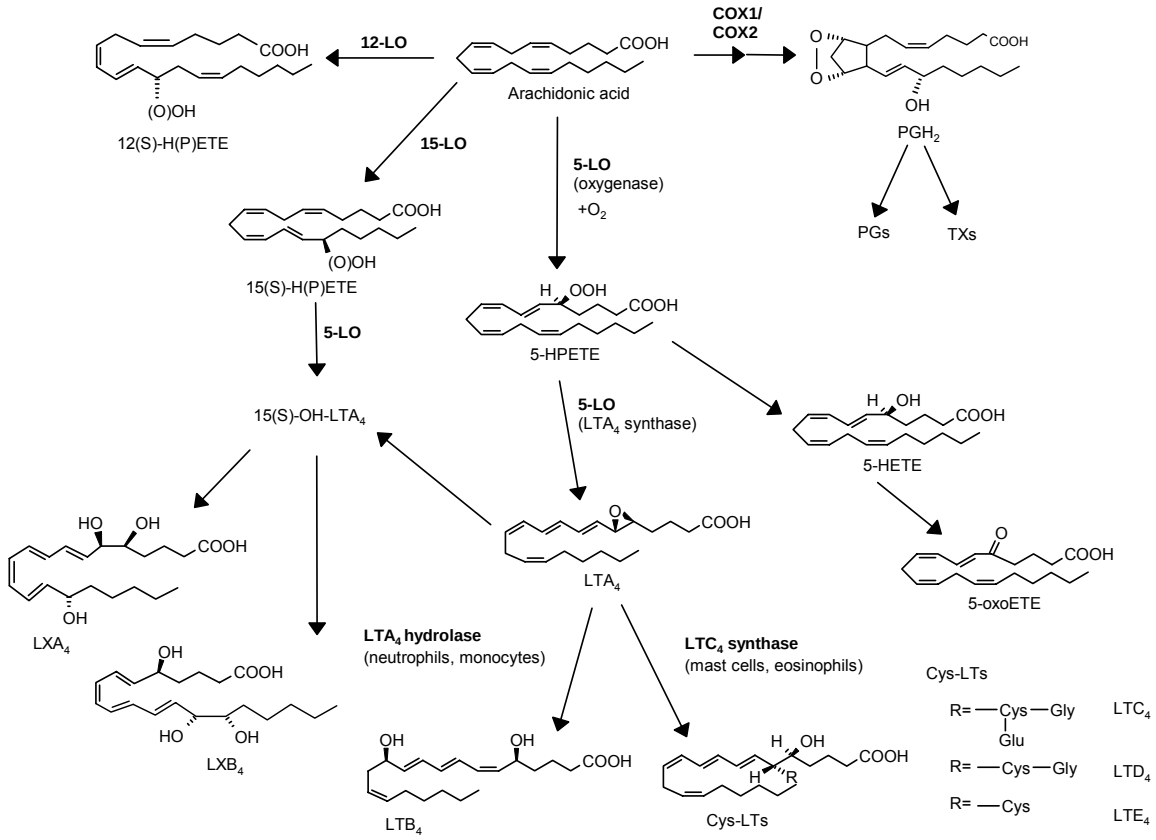

Figure 4: Derivatisation cascade of arachidonic acid

2.5. Biological functions of eicosanoids

2.5.1. Leukotrienes

Leukotrienes are short-lived 'local hormones' with potent pro-inflammatory properties. Upon intracellular formation at nuclear membranes of leukocytes (neutrophils, eosinophils, mast cells, macrophages), they diffuse into the extracellular space and activate specific GPCRs on target cells or are further metabolised. LTB_4, the primary LT derived from neutrophils, acts on BLT-1 and -2 receptors, which are expressed in several cell types including leukocytes (mainly BLT-1), smooth muscle cells, and endothelial cells ([156] and overview in table 1). The affinity of LTB_4 to BLT-1 is about 20-fold higher than for the more ubiquitously expressed BLT-2. Furthermore, BLT-1 binds LTB_4 more specifically as compared to BLT-2 that also accepts 12(S)-HPETE or 15(S)-HETE as ligands. Thus, BLT-1 is regarded as the primary, most sensitive LTB_4 receptor that governs LTB_4 effects in inflamed tissues [157, 158]. Receptor activation results in enhanced activation of granulocytes, chemotaxis, rolling and adhesion to vessel walls, as well as stimulation of ROS release and lysosomal enzyme secretion [159]. Ca^{2+} appears to be a key mediator in BLT signalling, as LTB_4 induces intracellular Ca^{2+} mobilisation, and neutrophils from BLT1-knock-out mice have impaired Ca^{2+} signalling and chemotaxis [160, 161]. LTB_4 is further assumed to promote the development of atherosclerosis and cell survival [162].

CysLTs are synthesised during anaphylactic reactions, are potent bronchoconstrictors by causing smooth muscle contraction, enhance endothelial permeability in post-capillary venules, and promote eosinophil recruitment. Thus, involvement of CysLTs in inflammation is evident. As stated above (section 2.4.3.), only some cell types (eosinophils, mast cells, etc.) are able to generate and release CysLTs. Transcellular metabolism is an important source for precursors of CysLTs [156]. CysLTs specifically activate two known GPCRs, of which CysLT1 receptor is expressed in leukocytes, mast cells, endothelium, lung, and nasal mucosa [163], and CysLT2 receptor is found in immunity-relevant tissues (liver, spleen, lymph nodes), as well as in heart, adrenal gland, and peripheral blood leukocytes [164]. Both CysLT receptors are activated by all CysLTs in different rank order of potency. Based on the potent pro-inflammatory activities of CysLTs, the pharmaceutical industry has sought to develop blockers of LT synthesis and LT receptors. Remarkably, only CysLT1 receptor antagonists have successfully entered the markets (zafirlukast, montelukast, pranlukast) [156].

2.5.2. 5- and 12-H(P)ETE

H(P)ETEs are hydro(pero)xy derivatives of AA with biological activities. Compared to LTs, their effects are less well characterised, although there is substantial formation by LOs upon activation of the AA cascade. 5(S)-HETE is a growth-inducing agent, has chemokine properties, and acts via a putative GPCR in leukocytes [165], thereby inducing ERK and Akt activation [166]. The 5-HETE receptor, which in contrast to BLT and CysLT receptors has not been cloned yet, is suggested to be involved in eosinophil recruitment to sites of allergic reactivity, mitosis, and nerve impulse transmission. Moreover, a role of 5-HETE in tumour progression can be appreciated, since it is constitutively produced in prostate cancer cells, and cells undergo apoptosis when the synthesis of 5(S)-HETE is blocked [167, 168].

5(S)-HETE can be converted by specific dehydrogenases in human PMNL, monocytes, and lymphocytes into 5-oxo-ETE, a compound that has been investigated in more detail. Intriguingly, 5-oxo-ETE is the most active chemoattractant for human eosinophils, promoting the transmigration through basement membranes. It is further involved in the recruitment of inflammatory cells in allergy and asthma. On the cellular level in neutrophils, 5-oxo-ETE causes Ca^{2+} mobilisation, degranulation, actin remodelling, and adherence by induced surface expression of integrins [169]. These effects are presumably transduced through a specific 5-oxo-ETE GPCR [170, 171].

12(S)-HETE is a bioactive mediator promoting cell proliferation in cancer cells [172], cell motility [173], and angiogenesis [174]. It acts as a renal vasoconstrictor, and 12(S)-HETE levels are increased in diabetes and hypertension, whereas vasodilatory properties were reported in coronary and cerebral vasculatures [175]. Exposure of 12-HPETE to platelets results in inhibition of main platelet functions, probably mediated by enhanced cGMP levels and suppressed Ca^{2+} mobilisation [176]. A receptor for 12-HETE as part of a larger receptor complex was identified in lung carcinoma cells, and a nuclear binding site for 12-HETE has been suggested as well ([177] and table 1). On the protein level, 12-HETE activates a signalling network including PLC, PLD, PKCα, ERKs and Src family kinases [178, 179].

2.5.3. 15-HETE, oxo-ETEs, lipoxins, hepoxilins, and EETs

Controversial effects have been reported for 15-HETE. Although it is the major eicosanoid in some inflammatory diseases (proctocolitis) and serves as a quantitative marker for the accompanying inflammatory process in cancer patients [137, 180], several anti-inflammatory implications of 15-HETE have been demonstrated. For instance, it inhibits

superoxide formation, exocytosis, and migration in stimulated human PMNL, and was found to block the synthesis and biological effects of LTB_4. Additionally, it was reported to induce apoptosis [131]. However, in some settings, masking of 15-HETE effects by other 15-LO products could not be excluded, such as 12-/15-diHETEs, 5-oxo-15-HETE, the trihydroxy derivatives lipoxin (LX)A_4 and LXB_4, and hepoxilins [137]. Consistently, lipoxins are currently regarded as lipid mediators with primary anti-inflammatory properties [181]. Their synthesis requires intercellular 'collaboration' of different LOs (5-/12-/15-LO) as well as COX for the aspirin-triggered lipoxins (ATLs). A lipoxin-specific GPCR (designated ALX) in PMNL and macrophages has been identified and cloned [182]. Downstream effectors of ALX are modulated in cell type-specific fashion. Functionally, lipoxin actions terminate neutrophil infiltration and initiate the resolution of inflammatory processes via macrophage activation [183]. Hepoxilins, in contrast, are PMNL-activating hydroxyepoxide derivatives of 12-HPETE, with hepoxilin A3 (HxA3) being the most prominent representative [184, 185]. Platelets can form HxA3 and thereby cause neutrophil chemotaxis and promote migration across intestinal epithelia. This effect may be mediated by mobilisation of intracellular Ca^{2+} in PMNL [186].

Finally, epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) epoxygenase-derived products from AA with diverse biological functions. Primary epoxidation products of AA are 5,6-, 8,9-, 11,12-, and 14,15-EET. EETs are particularly involved in the regulation of normal and pathophysiological vascular function, predominantly exhibiting protective (anti-inflammatory) effects. These actions are mediated in part by NF-κB inhibition [187] but also via membrane receptor activation [188]. On the other hand, EETs stimulate endothelial cell proliferation and selectively (5,6-EET, 8,9-EET) cause angiogenesis [189]. Thus, central physiological and pathological functions in immunity and the cardiovascular system are governed by an amazingly complex network of AA derivatives, set off by LOs, COX, and CYPs.

An overview of eicosanoid effects and receptors is given in table 1.

agent	biological effects	receptor	reference
LTB_4	pro-inflammatory functions (neutrophil activation, chemotaxis) promotes atherosclerosis and survival of tumour cells	BLT1, -2	[159, 162]
$LTC_4/D_4/E_4$	smooth muscle contraction (bronchi) ↑ vascular permeability ↑ eosinophil recruitment	$CysLT_{1, -2}$	[148]
5(S)-HETE	growth induction eosinophil recruitment tumour promotion	putative 5-HETE receptor	[165, 167, 168]
5-oxo-ETE	eosinophil chemoattractant pro-inflammatory (chemoattractant)	putative 5-oxo-ETE receptor	[170, 171]
12(S)-HETE	cell proliferation, motility angiogenesis vasoconstriction/dilatation	putative receptor complex	[172-175]
15(S)-HETE	inflammation marker in cancer anti-inflammatory functions (leukocyte ROS formation ↓, migration ↓)	unknown	[131, 137, 180]
lipoxins	anti-inflammatory functions (neutrophil infiltration ↓) macrophage activation	ALX	[181, 182]
hepoxilins	neutrophil activation (chemoattraction, migration ↑)	unknown	[185, 186]
EETs	regulation of vasculature (anti-inflammatory, protective effects) endothelial cell proliferation angiogenesis	putative membrane and intracellular receptors	[188-190]

Table 1: Selection of functional effects exerted by eicosanoids

2.6. Boswellic acids

2.6.1. Historical overview and sources

The lipophilic fraction of the gum resin from *Boswellia (B.) serrata*, termed 'frankincense', has been traditionally applied in Indian ayurvedic medicine for the topical treatment of joint diseases and various inflammatory disorders, in particular chronic illnesses. Initial scientific studies demonstrated analgesic and psychopharmacological effects of *B. serrata* gum resin in rats [191, 192], supporting a potential therapeutic value. Dried extracts of the gum resin (olibanum or 'Salai guggal'), named Sallaki, were first marketed in India, and are nowadays approved in a part of Switzerland (sold as H15

Gufic). Besides *B. serrata* (mainly growing in India), other important sources for BAs exist, including *B. sacra* (Arabian peninsula), *B. carterii*, and *B. frereana* (both Eastern Africa)[193].

The pentacyclic triterpenes (PTs) boswellic acids (BAs) were identified as the major ingredients of the lipophilic fraction of olibanum (figure 5), as listed in table 2. BAs exist in an α (geminal methyl groups at C-20) or β (vicinal methyl groups at C-19/C-20) configuration. Various pharmacological studies indicate that the β-configurated derivatives possess superior efficacy over the respective α-isomers. Further structural variation is given by a carbonyl moiety at C-11, yielding the 11-keto-BAs, or an acetyl moiety at the C-3 oxygen yielding 3-*O*-acetyl-BAs. Both, 3-*O*-acetyl and 11-keto groups are today believed to constitute crucial pharmacophores of the molecule. α-Amyrin and ursolic acid, which are closely related BA analogues existing in significant quantities, lack several specific residues (e.g. the C4-COOH-group) of BAs (figure 5).

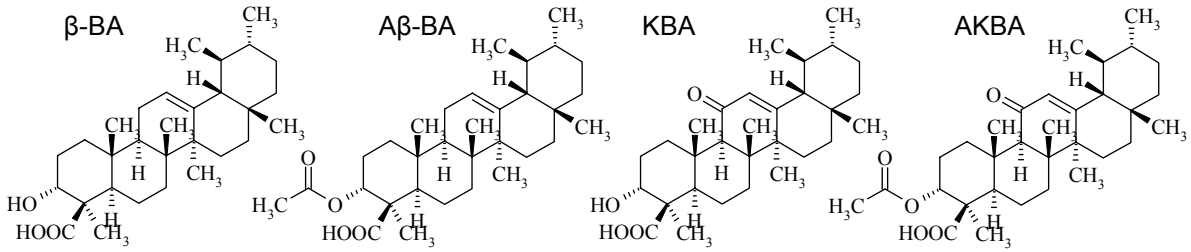

Figure 5: Structures of the four major β–configurated BAs.

ingredient	content in %
AKBA	3.7 %
Aβ-BA	10.5 %
KBA	6.1 %
β-BA	18.2 %
α-BA	13.2 %
Aα-BA	3.3 %

Table 2: Composition of the pentacyclic triterpenes in the dry extract of the gum resin from *B. serrata* (according to [194])

2.6.2. BAs in disease treatment

To date, a number of animal studies and clinical trials with human subjects have been carried out. Isolated BAs or preparations of *B. spec* containing diverse BAs revealed pharmacological properties with potential therapeutic value for the treatment of inflammation (rheumatoid arthritis, osteoarthritis, autoimmune encephalomyelitis, ileitis, colitis, hepatitis), pain, cancer, hyperlipidemia and hypercholesterolemia, and allergy (anaphylaxis) in experimental animal models [195-202].

A prerequisite for a desired pharmacological effect in the human body of an administered bioactive compound is sufficient bioavailability. In one study, the plasma concentrations of BAs after oral administration of *B. serrata* extracts (4 × 786 mg extract/day for 10 days) in a brain tumour patient were analysed. The levels of BAs in plasma were 10.1, 2.4, 0.34, and 0.1 μM for β-BA, Aβ-BA, KBA and AKBA, respectively [203]. Focusing on the pharmacokinetics, food intake profoundly affects the kinetic profile of BA plasma levels, as high-fat meal strongly increases the plasma concentrations of various BAs as compared to the fasted state [194].

In fact, clinical studies on humans confirmed the therapeutic potential for the intervention with several disease types. Administration of *B. spec* extracts to patients with osteoarthritis showed beneficial effects, as expressed by reduced pain sensation and disability score, as well as increased knee flexion [204, 205]. No significant effects were however found in a trial of rheumatoid arthritis [206]. In a study of colitis ulcerosa patients, a *B. serrata* preparation was compared to sulfasalazine treatment and observed to be equally potent in the improvement of disease parameters (stool properties, blood parameters, histopathology) [207]. A similar approach was pursued to evaluate *B. serrata* effects in Crohn's Disease, here the extract (H15) was compared to mesalazine. The *B. serrata*-treated patients displayed a remarkable improvement in the Crohn's Disease Activity Index score, and the extract was clearly superior to mesalazine [208]. Only one study addressed the efficacy of *B. spec* extracts in bronchial asthma, a major leukotriene-related disease. Again, a large fraction (70 %) of the gum resin extract-treated patients experienced an amelioration of the disease, indicating effectiveness of *B. serrata* treatment [209]. One of the most promising targets for *B. serrata* treatment yet appears to be cancer, particularly central nervous system (CNS) tumours. Two studies were carried out, one with 19 young people suffering from intracranial tumours, and another with 12 glioblastoma and leukoencephalopathy patients. H15 was used as drug and applied for several months. The results show that in the first trial, no anti-proliferative effects of palliative H15 treatment were observed, nevertheless an improvement in health parameters

and neurological symptoms in some patients was apparent [210]. Also in the second study, anti-neoplastic effects were absent, but tumour-associated progressive edema were reduced in a significant fraction of patients. Besides, all leukoencephalopathy patients improved clinically, indicating beneficial effects of H15 treatment in this disease form [211]. Accordingly, H15 is today approved for the clinical indication 'peritumoral edema', but only in a part of Switzerland. To conclude, the number of clinical studies is still far from sufficient to reasonably evaluate the potential therapeutic benefits of BA treatment. Good tolerance and some encouraging results should give reason to conduct further trials with BA preparations.

2.6.3. BAs in immunity and inflammation

2.6.3.1. Modulation of the immune response

Various ingredients of the extracts of *B. spec.* have been isolated, purified, and subjected to cellular and molecular studies, in order to identify the pharmacological principles and mechanisms of action of the gum resin. First reports indicated that *B. spec* extracts antagonise the host defence system by impairment of leukocyte infiltration and the complement system [212-214]. Other mechanisms that may contribute to the modulation of the immune response include anti-allergic/anaphylactic effects (inhibition of mast cell degranulation, suppression of macrophage NO production) and alteration of T helper (TH) cell signalling (TH1 cytokine inhibition, TH2 cytokine potentiation) [201, 202, 215].

2.6.3.2. Anti-inflammatory effects

In order to explain the anti-inflammatory actions of BAs stated in several animal models (see above, 2.6.2.), 5-LO, the key enzyme in LT biosynthesis (section 2.4.2.), was suggested as a target for BAs (see below) [216, 217]. LTs have long been recognised as potent mediators of inflammation and allergy. Thus, the concept that suppression of LT formation by BAs is the underlying mechanism of the anti-inflammatory actions of BAs appeared reasonable. It was first observed that *B. serrata* extracts inhibited the generation of LTB_4 in rat neutrophils [216]. Inhibition of LT biosynthesis by isolated BAs was later confirmed by other studies, and 3-*O*-acetyl-11-keto-BA (AKBA) was identified as the most efficient derivative with IC_{50} values in the range of 1.5-8 μM, depending on the experimental settings (e.g. animal/human, cell type, stimulus etc.) [197, 217-222]. Even though direct inhibition of 5-LO by AKBA was demonstrated [219], significant higher concentrations were required to directly suppress 5-LO activity *in vitro* as compared to intact cells, suggesting that potent inhibition of LT formation might be due to interference

with cellular events required for activation of 5-LO (see below, section 2.6.4.). Moreover, for cellular 5-LO activity, up-regulatory effects of BAs were reported, since low concentrations of *B. serrata* extracts potentiated 5-LO product synthesis in stimulated PMNL [218]. Implications of isolated AKBA or KBA applied to PMNL, platelets, and monocytes were addressed in this thesis.

Representing a major pro-inflammatory pathway, NF-κB signalling was found to be suppressed by 3-*O*-acetyl-BAs in human peripheral monocytes. This blockade caused inhibition of LPS-stimulated TNFα expression, apparently by direct interference with 'inhibitor of NF-κB-kinases' (IKKs) [223]. NF-κB downregulation might also explain altered effector levels observed in a humane genome screen using TNFα-stimulated human microvascular cells [224]. The results of this screen substantiate anti-inflammatory effects of BAs. Thus, the expression of pro-inflammatory effectors (matrix metalloproteinases (MMPs), reported as targets before [225] and adhesion receptors (VCAM-1, ICAM-1)) was inhibited. Consistently, a recent report using a semi-synthetic form of AKBA revealed the inhibition of P-selectin upregulation and leukocyte-platelet adherence in induced colitis in mice [199]. Taken together, BAs exert contrasting effects on various cell types implicated in immunity. Discrepancies also remain between the observed anti-inflammatory actions of either crude *B. spec.* extracts or purified BAs that also evoke a number of stimulatory and activating effects. Therefore, a straightforward explanation capable to relate cell-type specific effects of BAs to physiological observations made with preparations of *B. spec.* can not yet be provided.

2.6.4. Molecular targets

2.6.4.1. 5-Lipoxygenase

In 1991, AKBA was claimed as a novel specific nonredox-type inhibitor of 5-LO, the IC_{50} value was determined at 1.5 μM [217]. Other BAs were weaker inhibitors (IC_{50} up to 8 μM). These conclusions were however drawn from experiments assessing 5-LO inhibition in intact rat PMNL, but no data were provided using crude human 5-LO enzyme in cell-free assays. Nonetheless, a direct molecular interaction of AKBA with (rat) 5-LO was shown later ($IC_{50} \approx 8$ μM in rat PMNL cytosolic fractions, and 16 μM on affinity-purified 5-LO from human PMNL) [219]. Deviating from these findings, in our group the IC_{50} of AKBA was determined around 50 μM, using the cytosolic fractions of differentiated (human) HL-60 and monocytic Mono Mac 6 cells [221, 222]. The discrepancies in the potency of AKBA may be due to different cell types and distinct species (rat, human) as well as varying experimental conditions.

Binding of AKBA was suggested to occur at a selective site for PTs that is different from the AA-binding site of 5-LO [219]. Non-active BAs (β-BA) as well as related PTs (amyrine) antagonised AKBA-mediated 5-LO inhibition, and it was concluded that the PT ring system is crucial for binding to the selective effector site, whereas functional groups (i.e. the 11-keto and C4-carboxylic moiety) are essential for molecular inhibition of 5-LO [220].

In a smart approach using a photoaffinity analogue of AKBA, modified at the 3-*O*-acetyl position, the existence of a second, regulatory binding site for PTs and AA on 5-LO (distinct from the primary substrate binding site) was verified. Binding of AKBA and AA was shown to be competitive and Ca^{2+}-dependent [226]. Since the C2-like domain of 5-LO binds Ca^{2+} [227], and this domain also interacts with other hydrophobic molecules such as phospholipids [228] or glycerides [63], a common binding site for hydrophobic molecules such as BAs can be appreciated. Interference of BAs with other AA-metabolising enzymes (12-LO, COX) has once been ruled out [217], but been revised in this thesis, and is presented below.

protein/ enzyme	source/ setting	most potent BA	(a)ctivating/ (i)nhibiting	EC_{50}/IC_{50}	reference
5-LO	rat/human PMNL	AKBA	i	16/50 µM	[219, 221]
HLE	human	AKBA	i	15 µM	[229]
CYP 2C8/2C9/3A4	human, recombinant	β-BA>KBA >AKBA	i	5-10 µM	[230]
topoisomerase I	human	AαBA>AβBA >AKBA	i	3-30 µM	[231]
topoisomerase IIa	human	AαBA>AβBA >AKBA	i	1-30 µM	[231]
IKKα/β	human, recombinant	AαBA, AKBA	i	1-10 µM	[223]
IKKα/β	PC-3 cells	AKBA> AβBA	i	3-10 µM	[232]

Table 3: Molecular targets of BAs

2.6.4.2. Other molecular targets

Within the last decade, several targets capable to interact with BAs have been suggested (table 3). Besides 5-LO, human leukocyte elastase (HLE) was identified as target for BAs [229, 233]. Purified HLE is inhibited in a competitive manner in the low micromolar

range (IC_{50} = 4-6 µM) by ursolic acid and derivatives thereof, which are structurally related to BAs [233]. Other serine proteases (pancreatic elastase, α-chymotrypsin, trypsin) were also inhibited, but less efficiently [233]. HLE inhibition by ursolic acid (IC_{50} = 0.9-2.4 µM) was later confirmed and expanded to BAs, that blocked HLE with IC_{50} values of about 15 µM (AKBA) [229]. Since HLE release is increased upon PMNL activation in a variety of inflammatory and hypersensitivity-based diseases, inhibition of HLE by BAs may indeed contribute to the antiphlogistic properties of BAs.

Recently, *B. spec.* extracts as well as purified BAs were accounted as potent and non-selective inhibitors of the drug-metabolising CYP enzyme family [230]. In a setup using a mixture of recombinant CYP enzymes, β-BA, KBA and AKBA inhibited CYP 2C8/2C9 and 3A4 activity in the range of 5 to 10 µM. Although the physiological significance of CYP inhibition by BAs is still unknown, one should nonetheless consider an influence on the pharmacokinetics of other drugs administered in conjunction with *B. spec.* extracts.

In contrast to the classical approach of target identification, that was directed from phenomenological descriptions via identification of signalling networks involved toward elucidation of single target molecules, powerful analytical methods nowadays allow a reverse approach. This includes identification of targets e.g. by genome screening [224] or selective protein fishing (presented in this work) without initial knowledge about the physiological and functional relevance of the obtained interaction. Obviously, verification of found molecular target should follow *in vitro* and *in vivo*.

Note that more molecular targets of BAs (topoisomerases, IKK) are discussed in a later section (2.6.6.).

2.6.5. Modulation of intracellular signalling networks

BAs interfere with multiple cellular signalling routes within as well as between cells. Key players of intracellular signalling networks are protein kinases (PKs) such as PKA, PKB/Akt, PKC and MAP kinases (MEK/ERK, p38, JNKs). Most of the PKs are activated by phosphorylation events, terminated by dephosphorylation via corresponding phosphatases. There are so far no descriptions of BA effects on phosphatases, but there is substantial evidence for a modulation of diverse kinase activities.

The first detailed investigation on cellular activation in this context was performed with two tetracyclic triterpenes extracted from *B. serrata* resin, namely 3-oxo-tirucallic acid (3-oxo-TA) and 3-acetoxy-TA [234]. Both compounds (10 µM) enhanced LT formation in stimulated PMNL in a Ca^{2+}- and MEK/ERK-dependent fashion, accompanied by

increased nuclear 5-LO translocation. However, at higher concentrations, inhibitory properties became apparent.

Interestingly, also AKBA induced p38 MAPK and ERK1/2 activation in PMNL, and in parallel, AKBA caused a marked elevation of $[Ca^{2+}]_i$ [235]. Since both Ca^{2+} and MAPKs are necessary for liberation of AA [65], we investigated in this work whether AKBA induced the release of AA from PMNL, presumably connected to formation of pro-inflammatory LTs (see paper I). This investigation was expanded to human platelets, and strong effects of BAs were found (see papers II and III). In that regard, AKBA antagonised TNFα-induced phosphorylation of Akt and p38 MAPK in human myeloid KBM-7 cells without interference of ERK activation [236]. In contrast, AKBA (IC_{50} = 15 μM) even prevented ERK1/2 phosphorylation and cell motility in PDGF-stimulated meningioma cells [237]. Since the Ras-ERK route transmits survival signals in tumorigenic cells [238], ERK inhibition could (at least partly) explain the antiproliferative effects of AKBA observed in that study [237]. Putative antagonistic effects of AKBA on MAPK activation and Ca^{2+} signalling in monocytic cells were further investigated in this thesis (see paper V).

In summary, BA-mediated modulation of signalling cascades follows no unique pattern and obviously strongly depends on the cell type and the experimental conditions. Accordingly, further elucidation of the complex network of BA effects on the level of cellular signalling cascades is reasonably required.

2.6.6. Effects of BAs on cell proliferation, differentiation, and death

Shortly after the first descriptions of BA-mediated immunosuppression, anti-proliferative and cytotoxic effects of *B. spec.* preparations and BAs were reported. Studies revealed growth inhibition of human leukemic HL-60 cells (IC_{50} for AKBA ≈ 30 μM) [239, 240], accompanied by inhibition of DNA, RNA and protein synthesis (IC_{50} AKBA from 0.5 to 7.1 μM) [241, 242]. Likewise, *B. serrata* extracts arrested growth of certain tumours [200, 242].

Inhibited proliferation often goes hand in hand with enhanced differentiation. Accordingly, *B. carterii* extracts induced differentiation of HL-60 cells [239], and BC-4, a 1:1 mixture of α- and β-ABA, promoted differentiation of HL-60, U937, ML-1, and B16F10 mouse melanoma cells [225, 243].

Cell differentiation was often accompanied by the incidence of cytotoxicity, and when examined in more detail, revealed signs and attributes of apoptotic cell death. Indeed, BAs, foremost AKBA, induced apoptosis in several glioblastoma cell lines with effective

concentrations at 20 μM [244]. Apoptosis due to BAs was further reported in HL-60 cells [240, 243, 245, 246], several other human leukaemia and brain tumour cell lines [245], and various cancer cell lines of different tissues [225, 232, 247].

It was once suggested that the occurrence of apoptosis in otherwise highly proliferative cells might be a consequence of topoisomerase inhibition [248, 249]. In relation to that, *B. carterii* ingredients were reported to inhibit murine topoisomerase II already in 1991 [250], and purified AKBA (but not amyrin) potently inhibited topoisomerase I from calf thymus and induced apoptosis in topoisomerase I-expressing HL-60 cells at concentrations of ≥30 μM [240]. Direct binding of acetyl-BAs to purified human topoisomerases I and IIa was shown later with comparabale IC_{50} values found for AβBA (IC_{50} = 10-30 μM) and AKBA (IC_{50} = 30-50 μM) [231]. The inhibitory mechanism relies on competition of BAs with DNA for topoisomerase binding. Interestingly, AαBA was the most potent analogue (IC_{50} = 1-3 μM), indicating different structural requirements for target binding as compared to 5-LO (section 2.6.4.). Thus, 5-LO inhibition as major cause for apoptosis induction was clearly ruled out [231]. Since also another report documented inhibition of murine topoisomerase II by BC-4 from *B. carterii* [225], it is conclusive that topoisomerase inhibition may contribute to BA-evoked apoptosis.

Tackling the mechanisms of BA-induced apoptosis induction, the initiator caspase-8 was identified as a central mediator [246, 247, 251]. Caspase-8, in turn, activates the 'executer' caspase 3. Although caspase-8 is typically activated in response to ligation/activation of upstream death receptors such as Fas, the apoptotic effect of BAs is mediated via a pathway independent of Fas/FasL interaction [236, 247]. Controversial results were obtained regarding the induction of apoptosis mediators. Pro-apoptotic (Bax) and anti-apoptotic (Bcl-2, Bcl-X_L) protein levels were unchanged in human myeloid leukaemia cell lines treated with BC-4 [246], whereas in PC3 cells, Bcl-2, Bcl-X_L, and the cell cycle protein cyclin D1 were downregulated after AKBA and ABA (10 μM, each) treatment, in conjunction with loss of mitochondrial cytochrome c [232]. Interestingly, non-transformed human fibroblasts were resistant toward apoptosis in parallel experiments, implying selective cytotoxicity of AKBA in transformed cancer cells [232].

As a molecular mechanism of BA-mediated apoptosis, the same group suggested direct inhibition of constitutive IKK activity by AKBA, leading to suppression of the pro-survival transcription factor NF-κB [232]. In a recent study using human myeloid KBM-5 cells, a blockade of the anti-apoptotic kinase "Akt" was instead suggested as responsible mechanism resulting in reduced NF-κB signalling and low expression levels of Bcl-2 and

Bcl-X_L as well as the Fas receptor. Direct inhibition of IKK activity was ruled out in that study [236].

Collectively, the recruitment of pro-apoptotic mediators and downstream effector caspases by BAs is unequivocal, and typical apoptotic markers such as DNA laddering and poly (ADP-ribose) polymerase (PARP) cleavage support apoptotic cell death signalling [232, 236, 246]. However, the primary target protein(s) initialising apoptotic signals are still not clearly defined.

2.7. Aim of this work

Frankincense preparations containing bioactive BAs have proven to be effective in the treatment of chronic inflammatory diseases for centuries in the Indian ayurvedic medicine. In the course of an upcoming awareness for natural (plant-derived) compounds as safe and well-tolerable drugs during the last decades in our society, the scientific community is increasingly turning the focus to the elucidation of the mechanism by which these remedies act on the molecular and cellular level. This interest is substantiated by recent clinical trials demonstrating beneficial effects of *B. spec* preparations in several disease forms such as inflammatory bowel diseases and some cancer forms (see sections above).

However, the utilised extracts consist of a plethora of different compounds. Although BAs represent the largest fraction and are considered as primary pharmacological principles, application of this mixture in cellular experiments in order to identify molecular targets is not feasible, due to the heterogeneity of the extract and the diversity of ingredients. Thus, investigations on the cellular level should start with the purified compounds, namely BAs. That has been done since about 15 years, and has yielded a number of relevant target proteins, including 5-LO [252]. AKBA was discerned as the most potent BA. Subsequently, modulation of LT formation, which are key mediators of inflammation with additional implications in carcinogenesis, was described by a series of studies. Also other targets, related to cell proliferation and apoptosis, were identified [223, 236]. None of these findings, though, can presently provide a satisfying explanation for the anti-inflammatory effects of the extracts observed in clinical settings and the traditional folk medicine. Hence, this thesis was designed to further elucidate the actions of BAs on cellular physiology and intracellular signalling networks. Haematopoietic cells (leukocytes, platelets) were used as a model system, as they are primary governors of inflammatory processes and wounding.

Investigations on the cellular and molecular effects of BAs were started in this group by Oliver Werz [222], and a first thesis work focusing on the effects of BAs on neutrophils

was performed by Anja Altmann. Based on these initial findings [235], studies were continued and expanded toward other related cell types (monocytic cells, platelets). The results are presented in this thesis.

First, we examined a putative correlation of intracellular Ca^{2+} mobilisation and ERK1/2 activation by 11-keto-BAs [235] to the generation of ROS, release of AA and formation of LTs (paper I). Second, the effects of BAs on Ca^{2+} mobilisation, MAPK activation, and functional responses in human platelets were investigated (paper II). The work with human platelets further revealed interactions of BAs with platelet-type 12-LO, and the implications on the formation of p12-LO products were described (paper III). In this publication, an artificial construct of immobilised BAs was utilised to identify putative target molecules. The synthesis of this construct was documented in paper IV. Lastly, turning focus towards monocytes, which are the pivotal leukocytes in chronic inflammation, the actions of BAs on cellular physiology as compared to PMNL were investigated and surprising effects were attained (published in paper V).

3 Methods

Method	Paper
Isolation of human PMNL	I, II
Isolation of human platelets	II, III
Isolation of human monocytes	V
Cell culture of human suspension cells	I, V
5-/12-LO activity assay	I, III
COX product evaluation assay	III
AA release assay	I–III
SDS-gel electrophoresis and Western Blot	I, II, V
Spectrofluorimetric Ca^{2+} imaging	I, II, V
Immobilisation of BAs	IV
Measurement of cellular peroxide formation	I
Determination of superoxide formation	I
Cell viability assessment	II
PLC activity assay	V
Selective protein precipitation (target fishing)	III
Platelet shape change and aggregation	II
Thrombin generation assay (ETP)	II
FACS analysis of adhesion receptor expression	II
Primary cells:	
PMNL, freshly isolated from human leukocyte concentrates (buffy-coats)	I
Human platelets, freshly isolated from buffy-coats or whole blood	II, III
Monocytes, isolated from buffy-coats	V
Cell lines:	
HL-60 cells (human acute myeloid leukaemia cells)	I
MonoMac 6 cells (monocytes/macrophages)	I, V

4 Results and Discussion

Paper I

Altmann, A.; Poeckel, 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 leucocytes. *Br J Pharmacol* **2004,** 141, (2), 223-232.

Paper II

Poeckel, D.; Tausch, L.; Altmann, A.; Feisst, C.; Klinkhardt, U.; Graff, J.; Harder, S.; Werz, O., Induction of central signalling pathways and select functional effects in human platelets by β-boswellic acid. *Br J Pharmacol* **2005,** 146, (4), 514-524.

Paper III

Poeckel, D.; Tausch, L.; Kather, N.; Jauch, J.; Werz, O., Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase. *Mol Pharmacol* **2006,** 70, (3), 1071-1078.

Paper IV

Kather, N.; Tausch, L.; Poeckel, D.; Werz, O.; Herdtweck, E.; Jauch, J., Immobilisation of Boswellic acids at EAH Sepharose™ for "target fishing". *Tetrahedron* **2006**, submitted.

Paper V

Poeckel, D.; Tausch, L.; George, S.; Jauch, J.; Werz, O., 3-O-Acetyl-11-keto-boswellic acid decreases basal intracellular Ca^{2+} levels and inhibits agonist-induced Ca^{2+} mobilisation and MAP kinase activation in human monocytic cells. *J Pharmacol Exp Ther* **2006,** 316, (1), 224-232.

4.1. **Paper I:** Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes

Background and aims:

BAs, the active principles from Frankincense, have proved their beneficial action in the traditional treatment of various inflammatory diseases. Recent clinical studies supported an anti-inflammatory and anti-cancerogenic potential of BAs or *B. spec* extracts. However, little is known about the molecular mechanisms and cellular effects of BA action. Initial studies postulated 5-LO inhibition as pivotal mode of action, conferring anti-inflammatory effects via suppression of LT formation [217]. Due to the high concentrations of AKBA (50 μM) required to directly inhibit purified 5-LO [221, 222], other molecular effects of AKBA or of related BAs probably contribute to the observed pharmacological influence of *B. spec* preparations. In a first study devoted to enlighten *in vitro* BA actions on isolated inflammatory cells, the related tetracyclic triterpene 3-oxo-TA promoted ERK activation and moderate Ca^{2+} mobilisation in human PMNL [234]. In our group, AKBA was shown to cause substantial Ca^{2+} elevation in human PMNL, comparable to the chemotactic agonist fMLP, as well as phosphorylation of the MAPK members ERK2 and p38 [235]. These stimulatory effects were surprising, suggesting apparent pro-inflammatory action of AKBA. In order to elucidate whether these agonistic effects have relevant implications for cellular physiology, we determined functional responses including generation of ROS, liberation of AA, and biosynthesis of LTs in PMNL that were exposed to AKBA.

Results:

PMNL belong to the group of phagocytes that can respond to exogenous stimuli with an oxidative burst. Here, the release of ROS by PMNL upon stimulation with AKBA was evaluated by two separate methods. First, the peroxide-sensitive dye DCF-DA (dichlorofluorescein diacetate) was applied in a spectrofluorimetric assay to detect all forms of ROS. Second, lucigenin was used to specifically detect superoxide anions, the product of NADPH oxidase. We found that the 11-keto-BAs (AKBA, KBA; 30 μM each) caused robust formation of ROS and superoxide anions, even exceeding the potency of Ca^{2+} ionophore A23187 and of fMLP. Only the potent activator of the PKC-NADPH oxidase pathway, PMA (0.1 μM), was slightly superior to AKBA (30 μM). The prominent ROS formation could be blocked by application of the NADPH oxidase inhibitor DPI (diphenyleneiodonium chloride), indicating involvement of this enzyme, and Ca^{2+}

depletion by the chelators BAPTA/AM and EDTA, showing Ca^{2+}-dependency of AKBA action. Furthermore, inhibitors of PI-3 K (wortmannin, LY-294002) and ERK (U0126) imply participation of these kinases, whereas no role was evident for PKC (RO-318425).

Ca^{2+} and MAPK activation are determinants for $cPLA_2$ activation, leading to AA liberation from endogenous phospholipids. In an AA release assay using radiolabeled AA (incorporated into cellular membranes), we recorded an increase in free AA by PMNL after exposure to BAs. The rank order of potency was AKBA>KBA>β-BA>Aβ-BA. High concentrations of BAs (≥ 30 μM) were necessary to provoke measurable AA release. Inhibitor studies point toward an involvement of Ca^{2+} and ERKs in the signalling route leading to $cPLA_2$ activation. Enhanced AA release could lead to formation of LTs. Thus, 5-LO products of BA-stimulated PMNL were analysed by HPLC. BAs (foremost AKBA, 30 μM), added simultaneously with AA (20 μM), increased 5-LO product formation 3.8- and 1.9-fold in the absence and presence of 1 mM Ca^{2+}, respectively. Other BAs were less potent. When the AKBA concentration was increased to 100 μM, no 5-LO products were detectable, possibly due to direct inhibitory effects on the 5-LO enzyme. Inhibitor experiments excluded a role for MAPK in the induction of 5-LO product formation.

Since AKBA was identified as potent activator of ROS generation and Ca^{2+} mobilisation, and sustained exposure of these determinants to 5-LO inactivates the enzyme [91], we speculated whether a prolonged preincubation (30 min) with AKBA would still lead to enhanced LT formation. Interestingly, no significant upregulation could be observed; instead, AKBA concentration-dependently inhibited 5-LO product accumulation. This effect was more pronounced in the presence of Ca^{2+} (IC_{50} = 8 μM) rather than in its absence (IC_{50} = 50 μM).

It had been speculated that AKBA might act via a not yet identified GPCR to induce cellular signalling pathways. Involvement of a putative $G\alpha_i$ protein was therefore evaluated by means of pertussis toxin (PTx). We observed that PTx attenuated the AKBA-induced stimulatory effects regarding Ca^{2+} mobilisation (~70 % inhibition), ERK activation (~60 % inhibition), ROS formation (~20 % inhibition), and 5-LO activation (~8 % inhibition). The efficacy of PTx was verified in control experiments using $G\alpha_i$-dependent stimuli such as fMLP.

Finally, we determined the effects of AKBA in related cells of the haematopoietic lineage, i.e. monocytic Mono Mac 6 cells, B-lymphocytic BL-41-E-95-A cells and granulocytic HL-60 cells. No Ca^{2+} mobilisation, MAPK activation, or ROS formation induced by AKBA was observed in monocytic and B-lymphocytic cells. In contrast, HL-60 cells

showed Ca^{2+} elevations, MAPK (ERK2, p38) activation, and ROS generation in analogy to PMNL exposed to AKBA (30 μM).

Personal contributions: Lucigenin assay, parts of ROS generation assay, PTx experiments, parts of 5-LO product formation measurements, preparation of text and figures for the manuscript (~40 % of total work).

Discussion:

In this study, we could demonstrate that BAs, particularly those bearing a 11-keto moiety, exert potent stimulatory effects on human PMNL. The induction of Ca^{2+} mobilisation and MAPK phosphorylation is in line with the findings made with 3-oxo-TA in PMNL before [234]. Since Ca^{2+} and MAPK may promote $cPLA_2$ activation and subsequent LT formation, it is conclusive that AKBA also induces the release of AA and the biosynthesis of LTs by 5-LO. Moreover, Ca^{2+} promotes NADPH oxidase activation, and consequently, AKBA strongly stimulates ROS formation. Thus, we show that 11-keto-BAs (but hardly those BAs lacking an 11-keto-moiety) indeed activate functional responses in leukocytes, i.e. PMNL and granulocytic HL-60 cells. The physiological meaning of this agonism is unknown, especially since the concentrations required to elicit agonistic effects of AKBA (10-30 μM) are not achieved in the blood plasma after standard dosage of *B. spec* extracts. However, due to their lipophilic structure, BAs might accumulate in local intracellular membrane compartments, thereby possibly occurring in concentrations related to those in our experiments. Hence, investigation of signalling molecules evoked by AKBA is reasonable.

Similarities in kinetics and utilisation of signalling molecules (MAPK) by AKBA and common chemotactic ligands such as fMLP, PAF, or LTB_4, might justify the hypothesis that AKBA transduces signals in a similar way to these GPCR agonists. That issue was investigated using the $G\alpha_i$ protein inhibitor PTx. Our results indicate that a heterotrimeric G protein mediates the effects of AKBA in PMNL, especially the Ca^{2+} mobilisation and ERK activation. In contrast, other mediators than $G\alpha_i$ proteins are potential transducers of the AKBA-induced p38 MAPK and 5-LO activation. In conclusion, the participation of a G protein in signal transduction pathways initialised by AKBA can be appreciated, but whether interference of AKBA with the signalling cascades occurs at the stage of a GPCR, G proteins, or at regulators of G protein signalling (RGS), is still unclear.

Downstream of the initial AKBA target, various kinases appear to play a central role in further signal transduction. Comparable differential inhibitory effects as for PTx were evident for the PI-3 K inhibitor wortmannin, which suggests an important but not essential

involvement of PI-3 K in AKBA signalling, depending on the functional response that was assayed. PI-3 K converts PIP_2 into PIP_3, thus altering the abundance of phosphatidylinositides in cellular membranes with impact on the membrane binding capacity of proteins bearing PH (pleckstrin homology) domains. A precise relation of PI-3 K to the 5-LO pathway has not been established so far. Nevertheless, PI-3 K, which can be targeted by G protein subunits (e.g. $G_i\beta\gamma$), may influence the activity of PLC isoforms that rely on a supply of PIP_2, and also modulate downstream MAPK activation. Via soluble tyrosine kinases from the Src family, PI-3 K activates the Ras-Raf-MEK-ERK cascade; therefore, it is conclusive that the inhibitory effects of PI-3 K and MEK/ERK inhibitors (wortmannin and U0126, respectively) in this work often correlate with each other. One example is the induction of ROS formation by AKBA. ROS, predominantly superoxide anions, are part of the unspecific host defence against pathogens. The main source for ROS is the NADPH oxidase, which can be activated via Ca^{2+} and PKC. In our control experiments, we demonstrated the absolute requirement for PKC and a partial contribution of ERK in PMA-induced ROS generation. Contrasting these findings, the AKBA-induced ROS formation was not dependent on PKC but rather on PI-3 K and partly on ERK. In either case, Ca^{2+} was a prerequisite for ROS generation.

The release of AA was likewise dependent on Ca^{2+} and ERK activity. Thus, it appears reasonable that $cPLA_2$ activation occurs downstream of Ca^{2+} mobilisation and ERK activation. However, we found a substantial increase in 5-LO product formation also in the absence of Ca^{2+}, and furthermore, this increase was not dependent on MAPK (ERK and p38). Notably, the enhancement (fold increase) of LT formation was most pronounced when Ca^{2+} was chelated (3.8 fold) as compared to the presence of Ca^{2+} (1.9 fold), clearly indicating that additional signalling routes govern AKBA-induced LT formation. Taken together, these data indicate that BAs (foremost AKBA) are potent activators of pro-inflammatory responses in PMNL, and that Ca^{2+}, PI-3 K and ERK are major determinants of AKBA-induced signal transduction. The respective molecular target(s) for AKBA has not yet been solved, although regulation at the level of G proteins or GPCRs is conceivable. Despite these potent activating effects on inflammatory cells, pre-exposure to AKBA may reveal anti-inflammatory actions of the compound. Our experiments performed with cells incubated with AKBA for 20-30 min prior stimulation with pro-inflammatory agonists (such as AA) indicate that the capacity of cells to form LTs is remarkably reduced. This may be due to suicide inactivation of the enzyme [91], a consequence of elevated Ca^{2+} and ROS levels caused by AKBA. Thus, sustained exposure of cells to AKBA, a setting that more closely reflects the situation in the human body after

ingestion of *B. spec* medicine, may eventually lead to decreased levels of pro-inflammatory LTs.

In conclusion, elucidation of the signalling molecules involved in the agonistic action of AKBA in inflammatory cells as well as knowledge of the functional responses elicited by AKBA may help to better understand the molecular and cellular effects of AKBA, but identification of precise molecular targets is missing. Moreover, the causal relation with the observed anti-inflammatory action of *B. spec* extracts in disease treatment still has to be established.

4.2. **Paper II:** Induction of central signalling pathways and select functional effects in human platelets by β-boswellic acid

Background and aims:

Platelets are relevant cells in the onset of an inflammatory response, particularly after tissue injury or vessel wounding (section 2.2.3). Thus, there was reason to assess the influence of BAs on platelet signalling, in order to evaluate whether platelet physiology is modified by BAs. Previous studies using several types of leukocytes had revealed that BAs evoke differential effects, depending on the cell type ([235] and Paper I). We observed that 11-keto-BAs were most potent in the activation of intracellular signalling cascades involving Ca^{2+} mobilisation, PI-3 K and ERK activation, as well as $cPLA_2$ activation, in human PMNL. Other related cells such as B-lymphocytic and monocytic cells could not be activated by BAs (paper I). The cellular effects in PMNL resulted in enhanced formation of ROS and LT, which are generally regarded as factors that promote an inflammatory response in phagocytes. How these findings relate to the anti-inflammatory action of BAs in disease models is unknown, but we reasoned that other cells (relevant in inflammation) might contribute to the physiological effects of BAs. Therefore, we decided to investigate the response of human platelets upon exposure of BAs.

Interestingly, our previous studies revealed that AKBA and KBA, rather than BAs lacking the 11-keto moiety (11-methylene-BAs), are the most effective BA members regarding modulation of cellular physiology. Only in HL-60 cells, Aβ-BA and β-BA also exerted agonistic effects. Nonetheless, reports in the literature in some selected cases also identified 11-methylene-BAs as the responsible BA isoforms for the observed effect [231,

244]. We therefore devoted particular attention to the structure-activity relationship of the four major β-configurated BAs regarding platelet functional responses.

Results:

In comparison to PMNL, platelets were more sensitive towards BA-induced intracellular Ca^{2+} mobilisation. Furthermore, platelets showed an inverse structure-activity relationship for BA agonism. 11-Methylene-BAs evoked substantial Ca^{2+} mobilisation already at 10 μM (increase of ~400 nM $[Ca^{2+}]_i$), with β-BA being the most potent analogue. This response was transient, equally potent to the strong platelet agonist thrombin (0.5 U/ml), and further increased up to 100 μM β-BA. In contrast, 11-keto-BAs displayed a non-significant Ca^{2+} mobilisation at 10 μM and required higher concentrations (30-100 μM) to evoke a notable but delayed response (compared to thrombin or β-BA). Experiments in the absence of extracellular Ca^{2+} also revealed that the Ca^{2+} elevation by β-BA consisted of an intracellular (~37 %) and an extracellular component, presumably via SOCE mechanisms.

Inhibitor studies demonstrated that the β-BA- and thrombin-evoked Ca^{2+} signals were essentially dependent on PLC (inhibition by U-73122 and 2-aminoethoxyphenylborate (2-APB)) and Src family kinases (inhibition by PP2 and SU6656). Despite the similarities between thrombin and β-BA, the putative receptor for β-BA is still unknown. Thus, several receptor antagonists were employed for their ability to interfere with β-BA signalling. Neither purinergic $P2X_1$ and $P2Y_{1,12}$ receptor antagonists (NF-449/MRS-2179) nor PAF receptor (WEB 2086) and thrombin antagonists (argatroban) significantly attenuated the β-BA-induced Ca^{2+} response, excluding these receptors as targets for β-BA. Further studies will have to identify the molecular target transducing β-BA signalling.

Besides elevation of intracellular Ca^{2+} levels, 11-methylene-BAs induced p38 MAPK and ERK phosphorylation. p38 MAPK was also activated by 11-keto-BAs. The fast and potent effect of β-BA on ERK phosphorylation (EC_{50} = 10-30 μM, maximal after 1 min) was also found in a slightly attenuated fashion in Ca^{2+}-depleted cells with similar kinetics, implying the existence of a Ca^{2+}-independent activation pathway. Moreover, β-BA but not AKBA induced Akt activation with a maximum after 5 min in a PI-3 K-(inhibitor: wortmannin) and PLC-dependent (inhibitor: U-73122) manner. Chelation of Ca^{2+} (BAPTA/EDTA) abolished the β-BA effect on Akt phosphorylation, indicating that this event is located downstream of Ca^{2+} mobilisation in the signalling cascade.

A cell viability assay demonstrated no immediate cytotoxicity of β-BA and AKBA (30 μM, each, incubation for 15 min). BAs were then evaluated regarding functional responses of platelets. β-BA was shown to moderately induce the release of endogenous AA, which

is a marker of platelet activation due to subsequent generation of bioactive eicosanoids. However, β-BA only slowly and weakly induced platelet aggregation in calcified buffer, displaying no endogenous (Ca^{2+}-independent) pro-aggregatory action. AKBA was totally ineffective. In that regard, BAs strongly differed from the potent agonists collagen and ADP that immediately cause aggregation. Likewise, β-BA did not induce the externalisation of the platelet activation markers PAC-1 and CD62, whereas thrombin receptor-activated peptide (TRAP, used as positive control) did.

Another parameter that displays platelet activation is the capacity of agonists to evoke thrombin generation. β-BA (but not AKBA) strongly induced the endogenous thrombin potential (ETP), almost comparably potent to collagen. In the absence of Ca^{2+}, there was only a slight activation by β-BA but none of collagen.

Personal contributions: All experimental work except for parts of platelet functional assays. Functional assays were performed together with Lars Tausch. Major contributions to the preparation of the manuscript (~90 % of total work).

Discussion:

Our experiments with human platelets revealed a striking difference in the structure-activity relationship of BAs concerning cell activation as compared to PMNL and HL-60 cells. We found that 11-methylene-BAs (β-BA) were superior to 11-keto-BAs (e.g. AKBA) with respect to the induction of several functional effects in platelets, and that 11-methylene-BAs were often similarly potent as strong platelet agonists such as thrombin or collagen. Nevertheless, the pattern of β-BA-induced platelet activation is unique and differs in some aspects to that of thrombin or collagen, indicating that distinct signalling routes are operative. For example, intracellular Ca^{2+} mobilisation induced by β-BA is significantly delayed as compared to thrombin (peaking ~25 s after application in contrast to ~3-5 s for thrombin), allowing the conclusion that β-BA does not directly target the (G protein-)PLC-IP_3 route but might instead activate an upstream member of an as yet unidentified signalling cascade. The receptors for ADP, PAF, and thrombin were ruled out as potential signal transducers for β-BA, but Src family kinases and PLC were identified as indispensable mediators of β-BA signalling. Presumably, Src family kinases might be activated as direct or secondary targets of β-BA, transducing the signal to U-73122-sensitive PLC-γ isoforms (which are known targets of Src family kinases, see [53]), resulting in IP_3-mediated Ca^{2+} mobilisation. Whether an endogenous mediator is first generated upon exposure of platelets to β-BA, or β-BA targets an adhesion receptor linked to soluble tyrosine kinases (Src) is currently under investigation.

An involvement of a signalling cascade involving PLC/Ca^{2+} and PI-3 K can be appreciated in the activation of Akt, an anti-apoptotic protein kinase with multiple cellular functions. Akt activation by β-BA, as reported here, is yet in contrast to many reports in the literature stating general pro-apoptotic capacity of BAs in diverse cell lines such as human leukaemia cells (see for example [240, 245], and section 2.6.6.). Although this potential contradiction can not be explained by now, it has to be noted that most pro-apoptotic effects of BAs were mediated by AKBA and less by 11-methylene-BAs, pointing out specific effects of BAs depending on the structure. Along these lines, when dissecting the AKBA effects from those of β-BA, we found that AKBA did not stimulate intracellular Ca^{2+} mobilisation with similar potency, did not activate Akt, and was inefficient in the induction of functional responses such as aggregation, thrombin generation, or adhesion receptor externalisation. All BAs, though, induced p38 MAPK and ERK activation (concerning ERK, β-BA was most potent), whose functional implication is not readily apparent. The Ca^{2+}-independent effects of β-BA on ERK activation and thrombin generation indicate that additional signalling routes are initiated by β-BA that will have to be further defined.

In several assays performed in this study, β-BA proved to be efficient already at concentrations of 3-10 μM. This is in the range of physiologically achievable plasma levels of β-BA (10 μM), determined after oral application of 4 x 786 mg *B. serrata* extract/day (H15$^{(R)}$) within 10 days [253], thus highlighting the pharmacological relevance of our results *in vivo*. In contrast, AKBA was much less concentrated in plasma (0.1 μM), probably due to deacetylation processes and a generally lower abundance in crude *B. serrata* extracts as compared to β-BA [194].

Despite the strong agonistic effects of β-BA in terms of Ca^{2+} mobilisation, MAPK and Akt activation, AA release, and thrombin generation, there is a curious lack of activity regarding aggregation and externalisation of pro-coagulant adhesion receptors. On the basis of these findings, β-BA can not be considered as an equally potent platelet agonist like thrombin or collagen. Obviously, the failure of β-BA to cause rapid aggregation and induce adhesion receptor expression is more compatible with physiological evidence of anti-inflammatory or immunomodulatory properties of *B. spec* preparations observed in disease treatment. It may be speculated that β-BA has additional targets, putatively within pro-coagulation signalling routes in the platelet, that may counteract its agonistic activity on Ca^{2+} homeostasis and other responses. Thus, the search for additional molecular targets for BAs is ongoing.

4.3. **Paper III:** Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase

Background and aims:

In the previous report (paper II), we demonstrated that β-BA activates selected functional effects in human platelets in a specific mode, including robust mobilisation of intracellular Ca^{2+}, activation of MAPK and Akt, and liberation of AA. Ca^{2+} and MAPK are determinants of $cPLA_2$ activation, eventually leading to enhanced activity of the enzyme [65]. Along these lines, we reported earlier that 11-keto-BAs promoted Ca^{2+} elevations, MAPK activation, ROS formation, and AA release in human PMNL (paper I). The release of AA was essentially dependent on the presence of Ca^{2+} and relied partially on ERK activity, suggesting a signalling pathway encompassing Ca^{2+} elevations and ERK activity upstream of $cPLA_2$ activation. Concomitant to $cPLA_2$ induction, AKBA enhanced the formation of pro-inflammatory LTs by 5-LO.

Regulation of cellular 5-LO activation is complex, involving peroxides, Ca^{2+}, phospholipids, and phosphorylation by MK-2 or ERK2 (for review, see [96]). Upon exposure to PMNL, AKBA indeed positively influenced most of these means, so subsequent activation of 5-LO in addition to $cPLA_2$ was consistent. Nonetheless, AKBA had been reported as a selective 5-LO inhibitor before [217]. Although the molecular inhibition of the purified enzyme was later shown to require high AKBA concentrations (50 μM, [221, 222]), it may be that this inhibition partly compromised the stimulatory effects of AKBA on LT formation.

Platelets do not express 5-LO, but contain the closely related platelet-type (p) 12-LO. There is little information about the regulation of p12-LO activation, at least Ca^{2+} appears to play a role in facilitating membrane association [64] (also see section 2.4.2.2.). We previously observed that 11-methylene-BAs are potent inducers of platelet Ca^{2+} mobilisation and AA release, and here we investigated the effects of β-BA on p12-LO product formation. Also the kinetics of 12-H(P)ETE accumulation were studied. On the basis of the known signalling molecules transducing β-BA effects in human platelets (paper II), we further attempted to determine the participation of these signalling routes in β-BA-induced lipid metabolism.

Results:

First, the induction of PLA_2 activity by BAs was investigated in more detail. Despite divergent effects of 11-methylene- and 11-keto BAs in other assays, in the presence of Ca^{2+}, β-BA and AKBA caused a similar concentration-dependent increase in AA release that was significant at concentrations ≥10 μM. At 30 μM, BAs were almost as potent as the strong agonists thrombin (2 U/ml) and Ca^{2+}-ionophore A23187 (5 μM). Potent AA liberation by BAs was also observed in Ca^{2+}-depleted platelets, indicating that Ca^{2+}-independent signalling pathways are induced by BAs (see papers I and II). In the absence of Ca^{2+}, BAs were superior over thrombin and A23187.

AA is the substrate for p12-LO, hence the formation of the p12-LO metabolite 12-H(P)ETE was assayed. As expected, BAs promoted 12-H(P)ETE accumulation, but with rather different potency. β-BA evoked a concentration-dependent increase in p12-LO products, that (at 30 μM) was almost equally potent as thrombin (2 U/ml). In contrast, AKBA exerted only a weak effect on p12-LO product synthesis, and at concentrations >30 μM, it was no longer active. Thus, regarding p12-LO activity, a remarkable difference between AKBA and β-BA became apparent that was not seen in the release of AA.

Since we demonstrated Ca^{2+}-independent release of AA by BAs, it appeared possible that BAs also cause Ca^{2+}-independent accumulation of 12-H(P)ETE. In fact, we found enhanced 12-H(P)ETE synthesis in the absence of Ca^{2+}, again with much higher potency for β-BA as compared to AKBA. This was a unique finding as the potent platelet agonist thrombin was ineffective under these conditions.

Detailed kinetic studies revealed that fundamentally different mechanisms apparently underlie the β-BA-induced p12-LO activation. In the presence of intracellular Ca^{2+}, 12-H(P)ETE formation is rapid (half maximal product accumulation within 37 s), thus being equally fast as the direct conversion of p12-LO substrate AA, and considerably faster than that of thrombin. In the absence of Ca^{2+}, however, p12-LO product formation induced by β-BA was delayed for approximately 3 min until it started to continuously increase for up to 2 hours. No comparable effect was found for any other applied stimulus.

Dissecting the signalling pathways that mediate the β-BA effects on lipid metabolism (AA release and 12-H(P)ETE formation), we identified Src family kinases and PI-3 K as important signalling molecules as long as Ca^{2+} was present. No role for p38 MAPK or ERK was evident. However, when Ca^{2+} was omitted, none of the examined signalling molecules (Src kinases, PI-3 K, ERK, p38 MAPK) apparently contributed, so other unknown signalling routes seemingly mediate the β-BA effects.

The finding of Ca^{2+}-independent activation of p12-LO activity prompted us to investigate putative direct interaction of BAs with p12-LO. Accordingly, we incubated platelet supernatants (rich in soluble p12-LO) with β-BA and AKBA. Whereas β-BA was found to activate p12-LO approximately 2-fold at a threshold concentration of 10 μM in Ca^{2+}-free buffer, AKBA concentration-dependently inhibited p12-LO product formation *in vitro*. The IC_{50} for AKBA was determined at ~15 μM in the presence of Ca^{2+} and at ~50 μM in the absence of Ca^{2+}, the difference in IC_{50}s due to enhanced basal conversion by p12-LO in Ca^{2+}-containing buffer. Interaction of AKBA with p12-LO was then confirmed in a protein fishing assay using platelet homogenates as protein source and immobilised KBA (linked to sepharose beads) as bait. We selectively precipitated p12-LO as compared to control samples using pure sepharose beads, thus supporting the hypothesis of a direct interaction between AKBA and p12-LO. Along these lines, *in vitro* $cPLA_2$ activation could not be measured, and $cPLA_2$ was not precipitated in the protein fishing assay. Hence, we conclude that BAs interfere with lipid signalling primarily at the stage of p12-LO.

Personal contributions: All experimental work plus manuscript preparation. Parts of additional protein fishing and AA release measurements were conducted by Lars Tausch. Together ~90% of total work.

Discussion:

Several platelet stimuli (thrombin, PAF, thromboxane) cause a release of AA, leading to the formation of endogenous mediators via COX and p12-LO [254]. $cPLA_2$ activation is promoted by Ca^{2+} [255], MAPK phosphorylation [65], and PIP_2 interaction [80]. In contrast, only Ca^{2+} was described as a stimulating factor of p12-LO [64], whereas no other regulators have been clearly defined. We previously reported strong stimulating effects of 11-methylene-BAs in terms of Ca^{2+} mobilisation and MAPK activation in human platelets. Based on these findings, we expanded our investigations toward platelet lipid metabolism. Not surprisingly, β-BA induced the release of AA and its subsequent conversion into 12-H(P)ETE, comparable to potent platelet agonists (thrombin, A23187). More surprisingly, β-BA also promoted $cPLA_2$ activation and 12-LO product formation in the absence of Ca^{2+}. So far no agonists are known which are able to induce this cascade in a Ca^{2+}-independent manner. The underlying mechanism appears to deviate from the one involving Ca^{2+}, since, in the first case, it took significantly longer to accumulate substantial amounts of 12-H(P)ETE, whereas in the second case, none of the known signalling molecules mediating β-BA effects regarding Ca^{2+} mobilisation and AA release

in the presence of Ca^{2+} apparently contributed when cells were depleted of Ca^{2+}. *In vitro* experiments with soluble p12-LO revealed that the capacity of β-BA to activate p12-LO at least in part relies on direct stimulation of the enzyme. No activation of $cPLA_2$ was found until now. Nevertheless, additional unknown factors seem to transduce the activation of $cPLA_2$ by β-BA when Ca^{2+} is missing. On the other hand, when Ca^{2+} is present, $cPLA_2$ and 12-LO activities are probably located downstream of the major activating events induced by β-BA (Ca^{2+} mobilisation, PI-3 K and MAPK activation), which were described in the previous publication (paper II).

AKBA paralleled the effects of β-BA in respect to AA release, both in the presence or absence of Ca^{2+}. However, striking differences were apparent regarding p12-LO activation, where AKBA caused only a minor (and slow) enhancement of 12-H(P)ETE synthesis, accompanied by total inefficacy at 100 μM. This discrepancy can be attributed to direct inhibition of p12-LO, an effect that was verified in a protein fishing approach yielding p12-LO as selective binding partner for immobilised KBA. Thus, despite robust release of AA, further conversion to 12-H(P)ETE is blocked by direct inhibition of p12-LO. These results disprove the traditional paradigm that AKBA is a selective inhibitor of 5-LO without affecting p12-LO and COX enzymes [217]. Comparing IC_{50} values, p12-LO (IC_{50} approx. 15 μM) is even more susceptible to AKBA inhibition than human 5-LO (IC_{50} approx. 50 μM) under similar experimental conditions.

The pharmacological implications of our results for platelets *in vivo* are speculative. Enhanced 12-H(P)ETE formation upon exposure to β-BA might promote or maintain pro-inflammatory processes, including leukocyte chemotaxis and motility, angiogenesis, and tumour metastasis [172-174]. Even so, 12-HPETE might also be involved in an autocrine loop, eventually causing platelet silencing by inhibition of main platelet functions [176]. That scenario, although hypothetic, would better match the beneficial effects of *B. spec* preparations observed in the treatment of inflammatory diseases. Certainly, the validity of this model will have to be verified in further experiments.

4.4. **Paper IV:** Immobilisation of Boswellic acids at EAH Sepharose™ for "target fishing"

Background and aims:

This publication describes the chemical synthesis of the immobilised KBA construct that was applied in the previous paper (III).

To date, two publications have appeared that utilised modified BAs to determine the molecular interaction with different target molecules. In the first report [226], AKBA was derivatised at its 3-*O*-acetyl group with radio-labelled 4-azido-5-^{125}iodo-salicyloyl-β-alanine to yield azido-^{125}I-KBA. This molecule was structurally similar to AKBA with exception for a more bulky alanyl ester as compared to the original acetyl group. The synthesis of this construct was not stated. Azido-^{125}I-KBA inhibited 5-LO in intact PMNL as potently as AKBA. Furthermore, amyrin modulated 5-LO inhibition by the novel construct in a similar way as with AKBA. The aim of that study was to identify and characterise the binding site of AKBA at 5-LO. Accordingly, azido-^{125}I-KBA specifically labelled 5-LO in a Ca^{2+}-dependent manner when irradiated with UV light (254 nm), and binding was competitively antagonised by the presence of other related PTs including AKBA as well as AA. In contrast, competitive 5-LO inhibitors (ZM-230,487) did not interfere with photolabelling. The authors concluded that AKBA binds to 5-LO at a regulatory site (that also binds AA) distinct of the substrate binding site [226].

A second study used Aα-BA as template for an immobilisation to surface plasmon resonance (SPR) sensor chips [231]. In a first step, Aα-BA was deacetylated and coupled to 6-aminocaproic acid anhydride to yield 3-*O*-(6-aminocaproyl-)-α-BA. In a second step, this molecule was biotinylated, resulting in a construct termed 3-*O*-(*N*-(+)-biotinyl-6-aminocaproyl)-α-BA. The conjugate was bound to neutravidin and then immobilised on the SPR surface. In biological tests, the soluble conjugate was able to inhibit human topoisomerase activity in relaxation assays in the same way as natural Aα-BA (IC50 = 12 and 2 μM for topoisomerases I and IIα, respectively). The immobilised compound specifically and potently interacted with human topoisomerases through high-affinity binding sites in a DNA-competitive mode [231].

Thus, since modified BAs proved to be valuable without remarkable loss of efficiency in at least two different reports, we attempted to create a novel construct using the 3-OH moiety of KBA as starting point for chemical modification. In parallel, this synthesis was carried out with β-BA. It was our aim to couple the BAs to an insoluble biocompatible resin via a linker molecule. With this construct, we attempt to selectively precipitate binding partners of KBA (β-BA) present in cellular samples.

Results:

As insoluble resin we chose EAH Sepharose 4B^{TM}, which is a frequently applied and rather inert resin in biochemical assays. Glutaric anhydride was applied as reactant for coupling to the 3-hydroxyl group of KBA or β-BA, yielding a glutaric acid half ester

termed glutaroyl-KBA (glutaroyl-β-BA). The remaining carboxyl group of the glutaroyl moiety reacted with the amino group of EAH Sepharose 4B™ to give immobilised glutaroyl-KBA (glutaroyl-β-BA), designated KBA-sepharose (β-BA-sepharose).

Experimentally, glutaroyl-BAs were prepared using 4-pyrrolidino-pyridine as catalyst and pyridine as solvent. The product was crystallised and subjected to X-ray spectroscopy, enabling the calculation of a three dimensional model. Due to poor solubility of glutaroyl-BAs in water, the coupling to EAH Sepharose 4B™ had to be conducted under heterogeneous conditions in a water/dioxane 50/50 (v/v) mixture. The success of the coupling reaction was determined by recovering the calculated excess of glutaroyl-BA. For detailed technical parameters of the synthesis reaction as well as particular experimental handicaps please refer to the original publication (paper IV).

Personal contributions: Idea and selection of reaction educts, biochemical application (~10%).

Discussion:

Immobilised BAs are considered as a valuable tool for the selective precipitation of potential target molecules from protein pools. In ongoing biochemical studies, we will attempt to pull down proteins that specifically bind to the immobilised β-BA or KBA. Naturally, this approach does not enable all existing (but not yet identified) BA targets to be retrieved, because masking of the 3-*O*-acetyl group will exclude all those proteins that specifically interact with this portion of BAs. From a chemical point of view, this was however the only readily accessible functional residue of the BA molecule for chemical modification, and was therefore chosen as starting point for the immobilisation. Glutaric acid was selected as a linker of suitable length, on the one hand providing sufficient space between the sepharose matrix and the KBA molecule to allow interaction with bulky binding partners, on the other hand being short enough to prevent self-cyclisation via the two carboxyl groups.

Technically, protein pull-downs are separated by gel electrophoresis and after comparison with pull-downs utilising crude EAH sepharose beads (lacking BAs as bait, negative control), the respective proteins are selected and identified by MALDI mass spectrometry or, as performed in paper III, by western blotting and visualisation via specific antibodies.

In paper III, we applied KBA-sepharose in a protein pull-down assay to precipitate potential target molecules from platelet lysates. Whereas p12-LO was successfully and selectively extracted by KBA-sepharose from the protein mixture, no specific interaction was found for KBA-sepharose and $cPLA_2$ from platelets (no detectable binding), as well

as for KBA-sepharose and 5-LO from human PMNL lysates (paper III). Thus, although 5-LO was once shown to be a molecular target for AKBA [226], this interacting could not be verified. Two reasons can explain this finding. Either, the molecular interaction of KBA to 5-LO is too weak to yield significant binding, or the 3-*O*-acetyl moiety in its unmasked form is a critical determinant of the KBA-5-LO binding.

After the successful application of this construct in p12-LO precipitation, experiments to retrieve additional as yet unrecognised binding partners for KBA-sepharose and β-BA-sepharose have started. Presumably, systematic analysis of fished proteins via 1-D or 2-D gel electrophoresis followed by Coomassie staining and MALDI-based protein identification will yield a variety of novel binding partners for BAs, whose physiological relevance will be evaluated in subsequent studies.

4.5. **Paper V:** 3-*O*-Acetyl-11-keto-boswellic acid decreases basal intracellular Ca^{2+} levels and inhibits agonist-induced Ca^{2+} mobilisation and MAP kinase activation in human monocytic cells

Background and aims:

In the previous publications, multifaceted aspects of BA action on haematopoietic cells have been described. Moreover, the literature provides a large number of clinical and molecular/cellular studies devoted to elucidate the effects of *B. spec* preparations or isolated BAs, and results have been almost as diverse as the number of studies themselves (for review, see [252]). Thus, it becomes increasingly clear that the actions of BAs are very selective, depending on the cell type as well as on the structure of the BA analogue. In our papers discussed above, we demonstrated opposite structure-activity relationships of 11-keto-BAs (AKBA) and 11-methylene-BAs (β-BA) in human PMNL and human platelets, respectively. In PMNL, AKBA was found to be the most potent BA analogue, capable to induce cellular activation as well as functional responses. On the other hand, platelets were most strongly activated by β-BA. 11-keto-BAs acted more weakly and by different mechanisms, partly mediated by direct inhibitory effects on p12-LO. Taken together, in these studies cell-activating effects of BAs were evident, leading to functional responses with mainly pro-inflammatory characteristics. Which processes are eventually responsible for the anti-inflammatory action of *B. spec* extracts or BAs in disease models, remains to be answered. A growing number of researchers attempt to reveal the basis of the anti-inflammatory effects of BAs, and recently inhibition of human topoisomerases

[231] and/or IκB kinases (IKK) [223] have been suggested as responsible mechanisms. Still, convincing evidence capable to fully explain *B. spec* actions on a cellular and molecular level is lacking.

Platelets play an important role in tissue repair and wounding, whereas leukocytes have additional tasks in host defence mechanisms. At sites of infection, PMNL govern the acute inflammatory response, until they are gradually replaced by macrophages, derived from monocytes [2, 3]. Hence, in chronic inflammation macrophages play a dominant role. On this account, in the following study we addressed the issue whether monocyte/macrophage physiology (represented by the monocyte-macrophage cell line Mono Mac (MM) 6) is affected by BA treatment.

Results:

When resting MM6 cells were exposed to BAs, they responded with a sudden decrease of intracellular Ca^{2+} levels. This response was most pronounced for AKBA, with a significant drop of $[Ca^{2+}]_i$ already at 1 μM, followed by KBA, Aβ-BA, and β-BA. The 11-methylene analogues did not significantly reduce resting $[Ca^{2+}]_i$ levels even at a concentration of 30 μM, indicating that the 11-keto moiety is of crucial importance for this effect. A similar drop in $[Ca^{2+}]_i$ as seen for AKBA (30 μM ; from approx. 160 to 80 nM) was determined after application of the PLC inhibitor U-73122 (3 μM; from approx. 160 to 90 nM). Since U-73122 and AKBA gave no additive effects, we initially suspected AKBA to act via PLC inhibition (see below).

Next, we investigated whether AKBA also antagonises $[Ca^{2+}]_i$ elevations evoked by physiological (fMLP, PAF) and non-physiological (thapsigargin (TG), the PLC activator *m*-3M3FBS, and ionomycin) agonists. AKBA added 50 s after PAF (0.1 μM) immediately caused a drop of the increases in intracellular Ca^{2+} level, attaining almost equally low $[Ca^{2+}]_i$ levels as after addition to resting cells. This effect was again mimicked by U-73122 in a concentration-dependent fashion. Likewise, pre-exposure of MM6 cells to AKBA (30 μM, 20 s or 20 min) significantly attenuated the subsequent $[Ca^{2+}]_i$ elevation elicited by PAF, fMLP, and *m*-3M3FBS. Notably, no attenuation of Ca^{2+} mobilisation was observed when agonists that circumvent the PLC/IP_3 route, namely TG and ionomycin, were utilised. Evaluating the influence of different pre-incubation periods (20 s vs. 20 min) with AKBA and U-73122 on Ca^{2+} homeostasis, we found that AKBA but not U-73122 caused sustained depression of $[Ca^{2+}]_i$ levels. The suppressive effects of AKBA on Ca^{2+} elevations induced by PAF were confirmed in primary human monocytes isolated from leukocyte concentrates.

Ca^{2+} mobilisation induced by receptor agonists (PAF, fMLP) as well as by *m*-3M3FBS and the SERCA inhibitor TG consists of an intracellular release from internal storage sites (endoplasmatic reticulum, ER), and influx from the extracellular milieu via SOCE mechanisms [256]. Thus, we asked whether AKBA also compromises the intracellular portion of agonist-induced Ca^{2+} mobilisation. Indeed, AKBA attenuated Ca^{2+} mobilisation from intracellular stores with slightly reduced potency, when added to MM6 cells 20 s before the agonist. As AKBA also affected the Ca^{2+} release by TG, which does not signal via PLC and IP_3, inhibition of PLC was excluded as sole mechanism mediating the effects of AKBA on Ca^{2+} homeostasis.

In contrast, when a putative interaction of AKBA with PLC was tested in an assay quantifying the amount of IP_3 generated by PLC in intact MM6 cells, we only found minor (non-significant) attenuation of *m*-3M3FBS-induced PLC activity by AKBA (30 μM) but strong inhibition, as expected, by the PLC inhibitor U-73122 (5 μM). Intriguingly, Aβ-BA was superior as compared to AKBA regarding PLC inhibition. The weak effect of AKBA was accompanied by the finding that AKBA concomitantly caused a significant increase on PIP_2 turnover in resting (unstimulated) MM6 cells. Thus, AKBA appears to stimulate PLC activity rather than inhibiting it, ruling out PLC inhibition as primary mechanism of AKBA-induced $[Ca^{2+}]_i$ drops.

The potential mode of action exerted by AKBA was investigated using pharmacological inhibitors of various Ca^{2+}-regulating systems of the plasma membrane. We observed that inhibitors of non-selective cation channels (LOE908, SKF96365) and SOCE channels (2-APB, SKF96365), but not inhibitors of voltage-gated Ca^{2+} channels or the Na^+-Ca^{2+} exchanger, mimicked the effect of AKBA.

Finally, we expanded this antagonism study to another read-out system, namely the activation of members of the MAPK family (ERKs, p38). AKBA antagonised both resting ERK activity and agonist (fMLP)-elevated ERK and p38 MAPK phosphorylation when MM6 cells were pre-exposed to AKBA for 5 min. Similar effects were recorded for U-73122, whereas Aβ-BA was inactive. Collectively, AKBA is the most potent BA analogue, causing interference with primary signalling routes in human monocytic cells, visualised by reduced or suppressed Ca^{2+} elevations as well as impaired MAPK activation elicited by pro-inflammatory stimuli.

Personal contributions: All experimental work except for parts of Western Blotting experiments and Ca^{2+} measurements, manuscript and figure preparation (~95% of total work).

Discussion:

The activation of monocytes by pro-inflammatory stimuli occurs frequently at sites of infections or tissue injury. The bacterial tripeptide fMLP represents an exogenous agonist that acts on specific fMLP receptors on leukocytes, thereby causing cell activation connected to the synthesis of pro-inflammatory mediators as well as pathogen-directed ROS [10, 257]. However, in chronic inflammation, persistent presence of macrophages at inflamed sites may eventually result in destruction of surrounding healthy tissue, therefore pharmacological intervention of macrophage activation is reasonable. *B. spec* preparations have proved to be effective in the traditional treatment of chronic inflammatory disorders for centuries [258], but the molecular mechanism of BA action has not been resolved yet. Thus, this study was designed to address the effects of BAs on monocyte/macrophage physiology. The situation at a site of (chronic) inflammation experienced by monocytes was mimicked by the application of natural chemokines like fMLP and PAF.

Our data show that AKBA rather than any other BA was effective in attenuating agonist-induced Ca^{2+} elevations as well as reducing basal $[Ca^{2+}]_i$ levels in resting MM6 cells. At the same time, AKBA interfered with MAPK activation, causing a reduction of activated (phosphorylated) ERK and p38 MAPK under pro-inflammatory conditions (stimulation with fMLP). Hence, pharmacological intervention with *B. spec* extracts targeting chronic inflammatory diseases might indeed (partly) rely on reduced monocyte activation, conferred by AKBA. Other potential mechanisms explaining the anti-inflammatory action of BAs have also been suggested. For instance, BAs were reported to down-regulate expression of the pro-inflammatory cytokine TNFα in activated human monocytes via inhibition of IKK [223]. Moreover, AKBA was shown to reduce expression of the transcription factor NF-κB in stimulated tumour cells, abrogating the expression of pro-inflammatory and anti-apoptotic gene products [236]. All these studies add pieces of evidence that may ultimately allow to explain the anti-inflammatory effects of *B. spec* extracts *in vivo*.

In our study, we also investigated the molecular basis of the antagonistic effects observed after AKBA treatment. Although initial results pointed toward PLC as principal target for AKBA, subsequent experiments excluded a central role for PLC in the mediation of AKBA effects. AKBA exerted a much more sustained attenuation of agonist-induced Ca^{2+} mobilisation than U-73122, indicating different modulation of cellular physiology. It also antagonised the intracellular Ca^{2+} release elicited by the SERCA inhibitor TG, which does not act via PLC. Lastly, by analysis of cellular IP_3 levels in response to AKBA and/or U-73122, we observed that AKBA even activates PLC, whereas U-73122 inhibited PIP_2

turnover. Thus, we conclude that AKBA and U-73122 target different structures involved in the regulation of Ca^{2+} homeostasis and MAPK activation.

In attempts to elucidate the molecular mechanism of the AKBA-induced Ca^{2+} depression, we applied various inhibitors of the machinery controlling cellular Ca^{2+} homeostasis. In principle, enhanced Ca^{2+} export or intracellular storage via Ca^{2+}-ATPases are possible mechanisms, otherwise attenuated influx via Ca^{2+}- or unspecific cation channels could be responsible for the observed effects [259, 260]. Our results indicate non-selective cation channels and/or SOCE channels probably modulated by AKBA in a direct or indirect fashion. We propose that AKBA might reduce the open probability of these channels, meanwhile Ca^{2+} extrusion via ATPases is ongoing, leading in sum to a net export of Ca^{2+} that was observed in our experiments. Evidently, more detailed studies will have to address this issue in the future.

5 Summary

Traditional ayurvedic medicine in India, but also folk medicine in China and Africa has long included BAs in the therapy of inflammatory diseases. Besides phenomenological descriptions of the beneficial effects conferred by application of *B. spec* extracts (frankincense) in arthritis or joint diseases, clinical studies have addressed the question of efficacy of *B. spec* preparations in selected disease types. Today, only one approved medicine derived from *B. serrata* gum resin is available on the market (H15® Gufic), and the distribution is restricted to India and a part of Switzerland. Regardless of the restricted availability of *B. spec* medicine, there is growing demand for natural, well tolerable products with beneficial implications in certain inflammatory disorders. Consequently, medical researchers continue to evaluate the efficacy of various *B. spec* preparations (most trials are carried out using H15® Gufic) in animal studies or clinical trials. On the other hand, basic scientists are trying to identify the molecular mechanisms by which the effects of BAs, the main ingredients of *B. spec* extracts, are transduced.

Promising results came from some clinical studies that demonstrated efficiency of *B. spec* preparations in the treatment of asthma, osteoarthritis, colitis ulcerosa and inflammatory bowel diseases. Apart from several inflammation-related disorders, a potential future indication for *B. spec* treatments are brain tumours, as implied by two approaches demonstrating efficacy in clinical trials. Up to now, clinical research has been carried out using *B. spec* extracts or mixtures of the main ingredients, the BAs. Molecular research, in contrast, is increasingly focusing on the effects of solitary BAs, thereby simplifying the experimental setup and allowing to draw verifiable conclusions regarding BA actions. Based on the hypothesis that BAs are the main active principles of *B. spec* extracts responsible for the pharmacological efficacy, the aim of these studies is to specify the mode of BA action on a molecular and cellular level. Eventually, elucidation of the mechanisms should help to better understand the phenomenological effects of *B. spec* preparations *in vivo*, and facilitate the development of novel drugs containing a defined composition of BA(s).

Molecular targets for BAs have already been identified in several reports. Initially, abrogation of LT biosynthesis by neutrophils, presumably due to inhibition of the responsible enzyme 5-LO by AKBA, was suggested as responsible mechanism for the anti-inflammatory effects of *B. spec*. Although that hypothesis constituted an attractive model to explain *B. spec* actions, doubts could not be entirely smoothed out whether 5-LO inhibition would be the predominant mechanism conferring the anti-inflammatory effects.

The scepticism was mainly due to high AKBA concentrations necessary to cause sufficient 5-LO inhibition in *in vitro* assays on the one hand, and to relatively low plasma levels determined for AKBA after ingestion of *B. spec* preparations on the other hand. Accordingly, researchers were prompted to search for additional mechanisms and targets that might provide more satisfying explanations concerning the mode of action of BAs. In fact, a number of additional molecular targets have been reported for BAs, such as topoisomerases, human leukocyte elastase, cytochrome p450 enzymes, or IκB kinase.

Defining the interference of BAs with molecular targets *in vitro* is certainly important, but sole focusing on molecular interactions of BAs with putative binding partners ignores the fact that an interplay of several intra- and intercellular parameters in intact cells or tissues might form the basis of the eventual efficacy of BAs. Following this line, in the present work a more integrated approach using intact cells related to inflammation (PMNL, monocytes/macrophages, platelets) was pursued in order to evaluate the effects of BAs on the cellular physiology, including signalling pathways and functional cellular responses. Based on interesting findings made in this respect, we finally switched toward the characterisation of molecular interactions of BAs with some putative binding partners.

Initial studies using AKBA and human PMNL in this group indeed revealed remarkable effects of AKBA on PMNL signalling events. Exposure of PMNL to BAs resulted in a prominent transient mobilisation of intracellular Ca^{2+}, comparable to that evoked by the chemotactic agent fMLP. Moreover, enhanced phosphorylation of the MAPK members p38 and ERK2 was stated, and signalling molecules involved in the activation pathway were defined (Ca^{2+}, PI-3 K). Structure-activity comparisons showed that AKBA was the most potent BA analogue, whereas 11-methylene-BAs were rather inactive. The finding of substantial activating properties exhibited by AKBA was however hardly compatible with the anti-inflammatory effects observed in disease treatment. Thus, the physiological significance of the reported results was unclear.

This thesis was started with a project attempting to define the cellular effects of BAs on PMNL in more detail, and to relate the findings to cellular functional responses of PMNL. As demonstrated in paper I, we discovered a relation between the previously reported Ca^{2+} mobilisation/MAPK activation and PMNL functional effects such as formation of ROS, release of AA, and biosynthesis of LTs. Upon exposure to AKBA, PMNL synthesised large amounts of ROS, which represent a pivotal unspecific host defence mechanism. Likewise, AKBA promoted the release of AA, a precursor for pro-inflammatory LTs as well as prostanoids. Elevated hydroperoxide levels, Ca^{2+}, and the availability of substrate (AA) generally determine 5-LO catalysis, resulting in the formation of LTs. Accordingly,

we observed that AKBA caused enhanced biosynthesis of 5-LO products. In conclusion, AKBA showed pro-inflammatory characteristics despite its anti-inflammatory effects *in vivo* that led to PMNL activation rather than inhibition. Comparable results were obtained with a granulocytic human leukaemia cell line (HL-60). In contrast, a B-lymphocytic and monocytic cell line did not respond to AKBA, emphasising specific cell type dependency of BA actions.

Pharmacological inhibitors were employed to unravel critical determinants for the observed functional effects. We found that AKBA signals through a putative $G\alpha_i$ protein, Ca^{2+} mobilisation, PI-3 K, and ERKs. Thus, we gained insight into the intracellular signalling machinery stimulated by AKBA, culminating in PMNL activation. Furthermore, we confirmed AKBA to be the most potent BA analogue, since BAs lacking the 11-keto group were virtually ineffective.

PMNL are not the only type of leukocytes implicated in inflammatory processes, hence we switched to other haematopoietic cell types to assess the effects of BAs on cellular physiology. In papers II and III, we extensively examined how and by what means BAs affect signalling routes and functional responses of human platelets. This cell type was selected as a model system since platelets are involved in the onset of local inflammatory reactions after tissue injury and vessel wounding. Furthermore, platelets intimately interact with several leukocyte types, thereby modulating leukocyte activation via receptor-ligand interactions as well as via secretion of lipid mediators. The most striking discovery of our first platelet study was an inverse structure-activity relationship for 11-methylene-BAs and 11-keto-BAs compared to previous results with PMNL. BAs lacking the 11-keto moiety (β-BA, Aβ-BA) were pronounced activators of platelets, displayed by robust transient Ca^{2+} mobilisation, MAPK (p38, ERK) and Akt activation, AA release, thrombin generation and (moderate) aggregation. In this regard, the potency of β-BA (10-30 μM) often corresponded to that of the strong platelet agonists thrombin, collagen, and Ca^{2+} ionophore A23187.

β-BA also strongly stimulated the generation of the platelet-type 12-lipoxygenase (p12-LO) product 12-H(P)ETE, a chemotactic factor for leukocytes. Notably, the concentrations for β-BA required to provoke these effects were in the range of achievable plasma levels in humans (10 μM), thus being of physiological relevance. 11-Keto-BAs were only weak activators of some platelet functions. The most intriguing difference between the 11-methylene-BA and 11-keto-BA effects was obvious in the modulation of p12-LO product formation. With increasing concentrations, 11-keto-BAs failed to stimulate p12-LO, although both AKBA and β-BA had been shown to concomitantly

cause the release of AA. This effect, published in paper III, was due to direct inhibition of the p12-LO enzyme by AKBA. Two strategies confirmed this finding. First, in cell-free assays using cytosolic fractions of platelets as source for p12-LO, AKBA inhibited the substrate conversion by the enzyme. Second, in a protein pull-down assay using immobilised AKBA as bait, we selectively precipitated p12-LO from platelet lysates. The synthesis of immobilised BAs is documented in paper IV.

It is of interest that the remarkable stimulation of platelet lipid signalling by β-BA was also found at conditions where virtually no other known agonist was active, that is, when Ca^{2+} was missing. Along these lines, we state that all BAs cause the release of substantial amounts of AA, and β-BA induces slow but continuous p12-LO product accumulation in the absence of Ca^{2+}. Thus, with β-BA we identified a novel agonist for platelet lipid metabolism that acts independently of Ca^{2+}.

In line with previous studies, we also focused on the elucidation of intracellular signalling events transducing the activating BA effects in human platelets. Under normal conditions where Ca^{2+} was present, the observed effects clearly involved Src family kinases as putative signal transducers toward PLCγ, as well as PI-3 K and ERKs. None of these proteins however participated in the transduction of Ca^{2+}-independent β-BA effects. Thus, platelet activation by BAs is manifold, diverse, and characterised by the utilisation of several signalling routes. Nevertheless, our platelet studies summarise remarkable agonistic effects of BAs, especially of 11-methylene-BAs, on platelet physiology, whose consequences in physiological settings will have to be investigated in upcoming studies. The fact that BAs induced only selected functional responses in platelets, accompanied by the inhibition of p12-LO by AKBA, might indicate that exposure of platelets to BAs does not simply cause irreversible platelet activation. Rather, induction of specific effects may cause a kind of fine-tuning of the involved cells in the onset of an inflammatory response, presumably connected to the anti-inflammatory functionality of BAs by an as yet unrecognised mechanism.

Turning focus to human monocytes, anti-inflammatory implications of BAs were apparent. In clinical trials and folk medicine, *B. spec* preparations were demonstrated to be efficient in chronic inflammatory disorders rather than in acute states of inflammation. Since monocytes (that differentiate into macrophages) are the primary type of leukocytes implicated in the maintenance of chronic inflammation, the necessity to address BA effects on monocyte/macrophage physiology was evident.

The human monocytic cell line MM6 was chosen as experimental model because the purification of primary monocytes from blood samples is difficult and laborious.

Nevertheless, the most important findings attained with MM6 cells were later confirmed with primary monocytes. In the course of that study (paper V), it became apparent that monocytes respond in a very distinct manner to BA treatment compared to PMNL or platelets. As reported before with PMNL, in MM6 cells AKBA was the most potent BA analogue, consistent with the fact that both cell types are leukocytes. However, Ca^{2+} homeostasis of MM6 cells was downregulated after application of AKBA, visualised by depressed intracellular Ca^{2+} levels of resting and agonist-treated cells as well as by attenuation of agonist-induced Ca^{2+} transients after pre-exposure to AKBA. The suppressive effect on Ca^{2+} mobilisation by AKBA was long-lasting, and BAs lacking the 11-keto-moiety were virtually ineffective. Similar attenuating effects were found when MAPK phosphorylation levels were assessed after stimulation with the chemotactic agonist fMLP.

In that study, also the molecular basis of the antagonistic effects of AKBA was investigated. In contrast to initial assumptions, PLC inhibition was not the responsible mechanism, since AKBA even enhanced PIP_2 turnover. The responses to AKBA were always compared to those of the PLC inhibitor U-73122, and U-73122 strongly suppressed PLC activity although both agents (AKBA, U-73122) showed comparable effects on intracellular Ca^{2+} levels. Based on inhibitor experiments, several regulators of cellular ion homeostasis were assayed for a putative role in the transduction of AKBA effects. We identified two regulators of Ca^{2+} influx, non-selective cation channels as well as the SOCE channel system, as likely targets for the action of AKBA. Partial inhibition of these channels would then result in a net efflux of intracellular Ca^{2+} due to continuous activity of extrusion pumps.

Collectively, we report that AKBA exerts a form of ‘silencing’ effect on monocyte physiology, resulting in attenuation of cellular activation elicited by pro-inflammatory external stimuli. This interference occurs at the stage of crucial signalling parameters within the cell, namely Ca^{2+} mobilisation and MAPK activation. As a consequence, monocyte activation might be impaired when these cells are exposed to AKBA under physiological conditions in the plasma and tissues; hence, the effects described in this paper might provide a mechanistic basis underlying the well-known anti-inflammatory features of AKBA.

In conclusion, the work included in this thesis yielded substantial additional information to the growing knowledge of the cellular effects exerted by BAs, with particular focus on three cell types involved in inflammation and injury (PMNL, monocytes, platelets). Now,

we have gained a more differentiated perception of the intracellular signalling events as well as the functional responses activated in leukocytes and platelets, the pivotal regulators of inflammation. It has to be noted that the physiological significance of the reported findings is not always evident, but at least in case of the platelet and the monocyte studies, results were obtained at BA concentrations that can be accomplished *in vivo*. Besides description of cellular effects, target identification for BAs was an intended aim of the investigations. In the course of this thesis, p12-LO was discovered as novel target for 11-keto-BAs, but certainly more targets will be identified in future studies. A useful tool for selective fishing of binding partners will be the immobilised BA construct described in paper IV. Broad scientific interest for BAs and *B. spec* effects has grown within the recent years, as reflected by the increasing number of scientific studies in renowned journals, so exciting novel insights into molecular, cellular and clinical BA effects are likely to come up in the near future.

6 Zusammenfassung

Die traditionelle ayurvedische Medizin in Indien, aber auch die Naturmedizin in China und Afrika stützt sich hinsichtlich der Behandlung entzündlicher Erkrankungen seit langer Zeit auf Boswelliasäuren (BAs). Neben empirischen Beschreibungen der positiven Effekte, die durch Anwendung von *B. spec*-Extrakten (Weihrauch) bei Arthritis oder Gelenkerkrankungen erhalten werden konnten, wurden auch in klinischen Studien die Wirksamkeit von *B. spec*-Präparationen auf verschiedene Krankheitsformen untersucht. Heutzutage ist jedoch nur ein (regional) zugelassenes Präparat auf dem Markt erhältlich (H15® Gufic), welches aus *B. serrata*-Rohharz hergestellt wird. Der Vertrieb beschränkt sich auf Indien und einen Teil der Schweiz. Ungeachtet der geringen Verfügbarkeit von Weihrauchprodukten gibt es jedoch eine wachsende Nachfrage nach pflanzlichen, gut verträglichen Medikamenten zur Behandlung entzündlicher Krankheiten. Um die Wirksamkeit von BAs zu belegen, werden weiterhin verschiedenste Weihrauchpräparationen (im Regelfall H15® Gufic) in Tierexperimenten oder klinischen Studien getestet. Parallel dazu arbeiten Grundlagenforscher an der Aufklärung der molekularen Mechanismen, welche den Wirkungen von BAs, den Hauptinhaltsstoffen von Weihrauchextrakten, zugrunde liegen.

Vielversprechende Ergebnisse lieferten einige klinische Studien, welche die Wirksamkeit von *B. spec*-Präparationen in der Behandlung von Asthma, Osteoarthritis, Colitis Ulcerosa, und entzündlichen Darmerkrankungen untersuchten. Neben Entzündungsprozessen stellen Gehirntumore eine potentielle Indikation für die Behandlung mit Boswelliaextrakten dar. Dies wird durch zwei klinische Studien nahe gelegt. Bis heute wurden solche klinischen Untersuchungen hauptsächlich mit Weihrauchextrakten oder Mischungen aus den Hauptinhaltsstoffen, den BAs, durchgeführt. Die molekulare Forschung konzentriert sich hingegen auf die Analyse der Effekte von reinen BAs, wodurch experimentelle Ansätze vereinfacht und nachprüfbare Schlussfolgerungen ermöglicht werden. Auf Grundlage der Hypothese, dass BAs die pharmakologisch relevanten Inhaltsstoffe von Weihrauchextrakten sind, haben wissenschaftliche Studien die Ermittlung des Wirkmechanismus von BAs auf molekularer und zellulärer Ebene zum Ziel. Auf lange Sicht sollte die Aufklärung der zugrunde liegenden Mechanismen das Verständnis für die nutzbringenden Effekte von Weihrauchpräparaten *in vivo* erleichtern und die Entwicklung neuer Medikamente mit einer definierten Zusammensetzung von BAs begünstigen.

In einigen Publikationen wurden bereits molekulare Targets von BAs beschrieben. Ursprünglich war die Hemmung der LT-Biosynthese in Neutrophilen, vermutlich über eine AKBA-vermittelte Inhibition des verantwortlichen Enzyms (5-LO), als verantwortlicher Mechanismus für die anti-entzündlichen Effekte von Weihrauch vorgeschlagen worden. Damit war ein attraktives Modell geschaffen worden, das die Effekte von *B. spec* erklären konnte. Allerdings kamen bald Zweifel auf, ob die beobachteten Effekte allein durch eine 5-LO-Hemmung begründet werden können. Die Zweifel basierten unter anderem auf den hohen AKBA-Konzentrationen, die in *in vitro*-Studien notwendig waren, um ausreichende 5-LO-Hemmung zu erzielen, sowie den relativ niedrigen gemessenen Plasmakonzentrationen für AKBA nach Einnahme von Weihrauchmedikation. Aus diesem Grund haben Forscher kontinuierlich nach weiteren Mechanismen und Targets gesucht, welche die Wirkungen von BAs hinreichender erklären können. Infolgedessen wurde eine Anzahl zusätzlicher molekularer Targets für BAs identifiziert, darunter Topoisomerasen, humane Leukozyten-Elastase, CYP-Enzyme, oder die IκB Kinase.

Die Charakterisierung der Wechselwirkung von BAs mit molekularen Zielstrukturen in *in vitro*-Ansätzen ist zweifellos wichtig, jedoch lässt die Beschränkung auf molekulare Interaktionen von BAs mit potenziellen Bindungspartnern die Tatsache außer Acht, dass das Wechselspiel vielerlei intra- und interzellulärer Parameter in intakten Zellen und Geweben wesentlich zum Wirkmechanismus von BAs beitragen könnte. Basierend auf dieser Hypothese wurde in der vorliegenden Arbeit ein eher „ganzheitlicher“ Ansatz verfolgt und intakte Zellen als Modellsystem benutzt, die in Enzündungsprozessen beteiligt sind (PMNL, Monozyten/Makrophagen, Thrombozyten), und die Effekte von BAs auf die zelluläre Physiologie inklusive Signalkaskaden und funktionellen Antworten hin untersucht. Auf der Grundlage der erhaltenen Ergebnisse wurde im letzten Teil dieser Arbeit die Charakterisierung molekularer Wechselwirkungen von BAs mit einigen möglichen Bindungspartnern eingehender beleuchtet.

Anfängliche Studien mit AKBA und humanen PMNL in dieser Arbeitsgruppe zeigten in der Tat bemerkenswerte Auswirkungen von AKBA auf die Signalwege in PMNL. Die Zugabe von AKBA verursachte unter anderem eine prominente transiente Mobilisierung von intrazellulärem Ca^{2+}, vergleichbar mit der Wirkung des chemotaktischen Liganden fMLP. Weiterhin konnte eine verstärkte Phosphorylierung der MAPK p38 und ERK2 festgestellt werden, und eine Definierung der beteiligten Signalwegsmoleküle (Ca^{2+}, PI-3 K) wurde versucht. Struktur-Wirkungs-Beziehungen ergaben, dass AKBA die potenteste BA darstellte, wohingegen 11-Methylen-BAs eher inaktiv waren. Die Entdeckung

substanzieller aktivierender Eigenschaften durch AKBA stand jedoch im Widerspruch zu den beoachteten anti-entzündlichen Wirkungen in der Behandlung von Krankheiten, und die physiologische Bedeutung der berichteten Ergebnisse blieb zunächst unklar.

Die vorliegende Arbeit wurde daher mit einem Projekt begonnen, welches die zellulären Effekte von BAs in PMNL im Detail analysieren sowie einen Bezug zu funktionellen Antworten von PMNL herstellen sollte. Wie in Paper I gezeigt wird, konnte eine Verbindung zwischen den zuvor berichteten Effekten bezüglich Ca^{2+}-Mobilisierung/MAPK-Aktivierung und funktionellen Antworten wie ROS-Bildung, Arachidonsäure (AA)-Freisetzung, und LT-Biosynthese hergestellt werden. Nach Zugabe von AKBA bildeten PMNL große Mengen an Sauerstoffradikalen, welche einen primären Abwehrmechanismus der unspezifischen Immunabwehr darstellen. In gleichem Maße bewirkte AKBA eine Freisetzung von AA, den Vorläufer entzündungsfördernder LT sowie Prostanoide. Erhöhte Hydroperoxid-Konzentrationen, Ca^{2+}, und die Verfügbarkeit von Substrat (AA) stimulieren allgemein die 5-LO-Katalyse mit dem Ergebnis der Bildung von LT. Dementsprechend konnte eine verstärkte Biosynthese von 5-LO-Produkten beobachtet werden. Insgesamt zeigte AKBA trotz seiner entzündungshemmenden Effekte in der Medizin hier pro-entzündliche Eigenschaften, welche eher eine PMNL-Aktivierung als eine Hemmung bewirkten. Vergleichbare Ergebnisse wurden bei Verwendung der granulozytären humanen Leukämie-Zelllinie HL-60 erhalten. Im Gegensatz dazu reagierten eine B-lymphozytäre und eine monozytäre Zelllinie nicht auf AKBA, was die spezifischen zelltyp-abhängigen Effekte von BAs verdeutlicht.

Mit Hilfe von pharmakologischen Inhibitoren wurden beteiligte Signalmoleküle der beobachteten Zellantworten ermittelt. Es zeigte sich, dass in der durch AKBA eingeleiteten Signaltransduktion ein putatives $G\alpha_i$ Protein, Ca^{2+}-Mobilisierung, PI-3 K und in den meisten Fällen auch ERKs beteiligt sind. Auf diesem Wege erhielten wir Einblicke in das intrazelluläre Signaltransduktions-Netzwerk, welches durch AKBA stimuliert wird. Weiterhin wurde AKBA als potenteste BA bestätigt, da BAs ohne 11-Keto-Gruppe praktisch inaktiv waren.

In Entzündungsprozessen spielen nicht nur PMNL eine wichtige Rolle, daher befassten wir uns ebenso mit anderen hämatopoetischen Zelltypen, um die Einflüsse von BAs auf die zelluläre Physiologie zu beleuchten. Die Publikationen II und III enthalten eine umfassende Untersuchung über die Art und Weise, wie BAs die Signalwege und funktionellen Zellantworten in humanen Thrombozyten beeinflussen. Dieser Zelltyp wurde deshalb als Modellsystem gewählt, weil Thrombozyten zu Beginn einer lokalen

entzündlichen Reaktion nach Verletzung von Gewebe oder Blutgefäßen eine wichtige Rolle spielen. Darüberhinaus interagieren Plättchen sehr eng mit mehreren Leukozytenarten und modulieren die Leukozytenaktivierung über Rezeptor-Ligand-Wechselwirkungen sowie anhand der Sekretion von Lipidmediatoren. Das bemerkenswerteste Ergebnis unserer ersten Thrombozytenstudie war die umgekehrte Struktur-Wirkungs-Beziehung für 11-Keto-BAs und 11-Methylen-BAs im Vergleich zu früheren Berichten. BAs ohne 11-Keto-Gruppe (β-BA, Aβ-BA) waren ausgeprägte Aktivatoren der Plättchenphysiologie, erkennbar an einer starken Ca^{2+}-Mobilisierung, MAPK (p38, ERK) und Akt-Aktivierung, AA-Freisetzung, Thrombin-Generierung und (moderaten) Aggregation. In dieser Hinsicht entsprach die Wirksamkeit von β-BA (10-30 μM) zumeist der Potenz der starken Plättchen-Stimuli Thrombin, Kollagen und Ca^{2+}-Ionophor A23187.

β-BA stimulierte außerdem die Bildung des p12-LO-Produkts 12-H(P)ETE, eines chemotaktischen Faktors für Leukozyten. Interessanterweise waren Konzentrationen um 10 μM, die im Bereich der physiologisch erreichbaren Plasmaspiegel im Menschen liegen, bereits ausreichend, um die beschriebenen Effekte von β-BA auszulösen. 11-Keto-BAs stellten sich im Unterschied zu PMNL als nur schwache Aktivatoren einiger zellulärer Funktionen dar. Der auffälligste Unterschied zwischen 11-Methylen- und 11-Keto-BAs wurde in Bezug auf die p12-LO-Produktspiegel deutlich. Mit zunehmenden BA-Konzentrationen stimulierten 11-Keto-BAs die p12-LO nur bis zu einer gewissen Grenze, obwohl sie gleichzeitig zu einer verstärkten AA-Freisetzung führen. Dieser Effekt (veröffentlicht in Paper III) wird auf die direkte Hemmung des p12-LO-Enzyms durch AKBA zurückgeführt. Zwei Strategien konnten dieses Ergebnis belegen. Erstens, im zellfreien System, unter Verwendung von zytosolischen Fraktionen aus Blutplättchen als p12-LO-Quelle, hemmte AKBA die Substratumwandlung durch das Enzym. Zweitens konnte p12-LO in einem Pulldown-Verfahren, welches immobilisierte KBA als Anker nutzte, selektiv aus Plättchen-Lysaten präzipitiert werden. Die Synthese der immobilisierten BAs ist in Paper IV beschrieben.

Hervorzuheben ist, dass der bemerkenswerte Agonismus von β-BA auf das Lipid-Signalling in Plättchen auch unter Bedingungen gefunden wurde, bei denen kein anderer bekannter Stimulus wirksam ist, nämlich dann, wenn Ca^{2+} fehlt. Unter diesen Messbedingungen wurde die Freisetzung beträchtlicher Mengen an AA durch alle BAs registriert, und β-BA induzierte zudem eine langsame, aber kontinuierliche Bildung von p12-LO-Produkten. Daher ist festzustellen, dass mit β-BA ein neuer Agonist für den

Lipidmetabolismus in Blutplättchen identifiziert wurde, welcher unabhängig von Ca^{2+} wirkt.

In Analogie zu vorhergehenden Studien wurden auch in Plättchen die intrazellulären Signaltransduktions-Ereignisse untersucht, welche den aktivierenden Effekten von BAs zugrunde liegen. Unter normalen Bedingungen, wenn Ca^{2+} zugegen ist, sind Src Kinasen, PI-3 K und ERKs an der PLCγ-Aktivierung beteiligt. Keines dieser Proteine war indessen in die Vermittlung der Ca^{2+}-unabhängigen Effekte von β-BA involviert. Somit kann die Thrombozyten-Aktivierung durch BAs als vielfältig beschrieben und durch die Verwendung mehrerer Signalwege gut charakterisiert werden. Weiterhin fasst unsere Studie auffällige agonistische Effekte von BAs, speziell von 11-Methylen-BAs, auf die Plättchenphysiologie zusammen, deren Konsequenzen im physiologischen System im Rahmen von zukünftigen Studien erforscht werden sollen. Die Tatsache, dass BAs nur bestimmte funktionelle Antworten in Plättchen induzieren (unter Berücksichtigung der p12-LO-Hemmung durch AKBA), deutet darauf hin, dass Zugabe von BAs zu Thrombozyten nicht nur schlicht eine irreversible Aktivierung der Zellen bewirkt. Im Gegenteil, die Induktion selektiver Effekte könnte eine Art Feinregulierung der beteiligten Zellen zu Beginn einer Entzündungsreaktion verursachen, die mit der anti-entzündlichen Funktionalität der BAs über noch ungeklärte Mechanismen in Zusammenhang stehen könnte.

In Experimenten mit Monozyten waren mögliche entzündungshemmende Wirkungen von BAs leichter einsichtig. In klinischen Studien und der Naturmedizin hatten *B. spec*-Präparationen ihre Wirksamkeit eher in chronischen als in akuten entzündlichen Zuständen gezeigt. Da Monozyten (die zu Makrophagen differenzieren können) die primäre Leukozytenart in der Aufrechterhaltung chronisch entzündlicher Zustände ist, liegt die Notwendigkeit einer Auseinandersetzung mit den BA-Effekten auf die Monozyten/Makrophagen-Physiologie auf der Hand.

Die humane Monozyten-Zelllinie MM6 wurde als experimentelles Modellsystem ausgewählt, da die Aufreinigung von primären Monozyten aus Blutproben aufwendig und schwierig ist. Dennoch wurden primäre Monozyten später zur Bestätigung der wichtigsten Erkenntnisse eingesetzt. Im Zuge dieser Studie (Paper V) wurde offenbar, dass Monozyten, verglichen mit PMNL oder Thrombozyten, in sehr verschiedener Weise auf BA-Behandlung reagieren. Wie zuvor in PMNL stellte AKBA die potenteste BA dar, was damit in Einklang steht, dass beide Zellarten Leukozyten sind. Dennoch wurde die Ca^{2+}-Homöostase in MM6 Zellen nach Behandlung mit AKBA herunterreguliert. Dies spiegelte sich in erniedrigten $[Ca^{2+}]_i$-Spiegeln von ruhenden oder agonist-stimulierten Zellen sowie

durch die Abschwächung agonist-induzierter Ca^{2+}-Erhöhungen nach vorheriger AKBA-Zugabe wider. Der unterdrückende Effekt auf die Ca^{2+}-Mobilisierung durch AKBA war langanhaltend, und BAs ohne 11-Keto-Gruppe waren praktisch unwirksam. Ähnliche abschwächende Effekte wurden hinsichtlich der MAPK Phosphorylierungs-Spiegel nach Stimulation mit dem chemotaktischen Agonisten fMLP beobachtet.

In dieser Studie wurde weiterhin die molekulare Basis der antagonistischen AKBA-Effekte erkundet. Im Gegensatz zur anfänglichen Vermutung war eine Hemmung der PLC nicht der verantwortliche Mechanismus, da AKBA die PIP_2-Umwandlung sogar erhöhte. Die zellulären Antworten auf AKBA wurden jeweils mit denen des PLC-Inhibitors U-73122 verglichen, und U-73122 blockierte erwartungsgemäß die PLC-Aktivität, obwohl beide Agonisten (AKBA, U-73122) vergleichbare Effekte auf die intrazellulären Ca^{2+}-Spiegel hatten. Mittels Inhibitorexperimenten wurden mehrere Schaltstellen der zellulären Ca^{2+}-Homöostase auf eine Mitwirkung an den durch AKBA ausgelösten Effekten hin untersucht. Wir konnten dabei zwei Regulatoren des Ca^{2+}-Einstroms als wahrscheinliche Targets für AKBA-Wirkungen identifizieren, nämlich sowohl nicht-selektive Kationen-Kanäle als auch SOCE Kanäle. Eine teilweise Hemmung dieser Kanäle würde dann in einem Netto-Ausstrom von intrazellulärem Ca^{2+} bei gleichzeitiger kontinuierlicher Aktivität der Extrusions-Pumpen resultieren.

Zusammenfassend dokumentiert Paper V somit eine Anzahl ‚supprimierender' Effekte von AKBA auf die Monozyten-Physiologie, die sich in einer Abschwächung der zellulären Aktivierung durch pro-entzündliche Stimuli ausdrückt. Diese Interferenz findet auf der Ebene wichtiger Signaltransduktionswege innerhalb der Zelle statt, der Regulation des Ca^{2+}-Spiegels sowie der MAPK-Aktivierung. Demzufolge wird die Monozyten-Aktivierung verhindert, wenn die Zellen in Plasma oder Gewebe AKBA ausgesetzt waren. Damit können die hier vorgestellten Effekte eine Basis für die bekannten anti-entzündlichen Eigenschaften von AKBA sein.

Als Fazit kann festgestellt werden, dass die vorliegende Arbeit wesentliche neue Informationen zum wachsenden Kenntnisstand über die zellulären Effekte von BAs beiträgt, mit besonderem Augenmerk auf drei im Entzündungsgeschehen und bei Verletzungen beteiligte Zellarten (PMNL, Monozyten, Thrombozyten). Infolgedessen sind wir zu einer differenzierteren Wahrnehmung sowohl der Einflüsse auf intrazelluläre Signaltransduktionswege als auch der funktionellen Zellantworten von Leukozyten und Blutplättchen gelangt. Zwar ist die physiologische Bedeutung der vorgestellten Befunde nicht immer auf Anhieb offensichtlich, jedoch wurden unsere Ergebnisse zumindest im

Falle der Thrombozyten- und Monozyten-Studien mit BA-Konzentrationen erzielt, die *in vivo* erreichbar sind. Neben der Beschreibung von zellulären Effekten stellte auch die Targetidentifizierung von BAs ein erklärtes Ziel der Untersuchungen dar. Im Zuge der Arbeit wurde daher p12-LO als neues Target von 11-Keto-BAs entdeckt. Sicherlich wird es zukünftig zur Identifikation weiterer Targets kommen, beispielsweise mittels selektivem „Fischen“ nach Bindungspartnern auf der Grundlage immobilisierter BAs (Konstrukt vorgestellt in Paper IV).

Das breite wissenschaftliche Interesse an BAs und *B. spec*-Effekten hat innerhalb der letzten Jahre zugenommen, was sich in der steigenden Zahl wissenschaftlicher Studien in renommierten Fachzeitschriften widerspiegelt. So ist in absehbarer Zeit mit weiteren interessanten Einblicken in die molekularen, zellulären und klinischen Effekte von BAs zu rechnen.

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8 Appendix (Paper I-V)

Paper I

Altmann, A.; Poeckel, 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 leucocytes. *Br J Pharmacol* **2004**, 141, (2), 223-232.

Paper II

Poeckel, D.; Tausch, L.; Altmann, A.; Feisst, C.; Klinkhardt, U.; Graff, J.; Harder, S.; Werz, O.; Induction of central signalling pathways and select functional effects in human platelets by β-boswellic acid. *Br J Pharmacol* **2005**, 146, (4), 514-524.

Paper III

Poeckel, D.; Tausch, L.; Kather, N.; Jauch, J.; Werz, O.; Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase. *Mol Pharmacol* **2006**, 70, (3), 1071-1078.

Paper IV

Kather, N.; Tausch, L.; Poeckel, D.; Werz, O.; Herdtweck, E.; Jauch, J.; Immobilization of Boswellic acids at EAH Sepharose™ for "target fishing". *Tetrahedron* **2006**, submitted.

Paper V

Poeckel, D.; Tausch, L.; George, S.; Jauch, J.; Werz, O.; 3-O-Acetyl-11-keto-boswellic acid decreases basal intracellular Ca^{2+} levels and inhibits agonist-induced Ca^{2+} mobilisation and MAP kinase activation in human monocytic cells. *J Pharmacol Exp Ther* **2006**, 316, (1), 224-232.

Papers I-V

Paper I

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Coupling of boswellic acid-induced Ca^{2+} mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes

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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.
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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).

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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

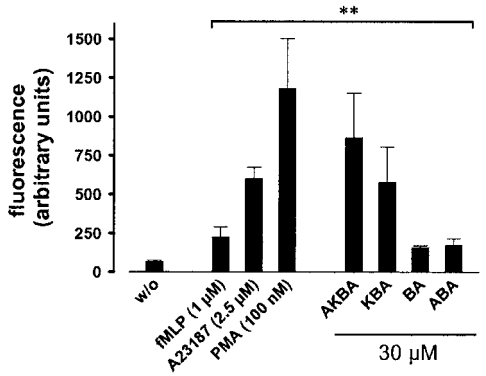

Figure 1 11-Keto-BAs induce the generation of ROS in PMNL. Freshly isolated PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with DCF-DA (1 μg ml^{-1}) for 2 min at 37°C, prior addition of the indicated stimuli. The generation of peroxides was measured as described. Data determined 5 min after addition 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

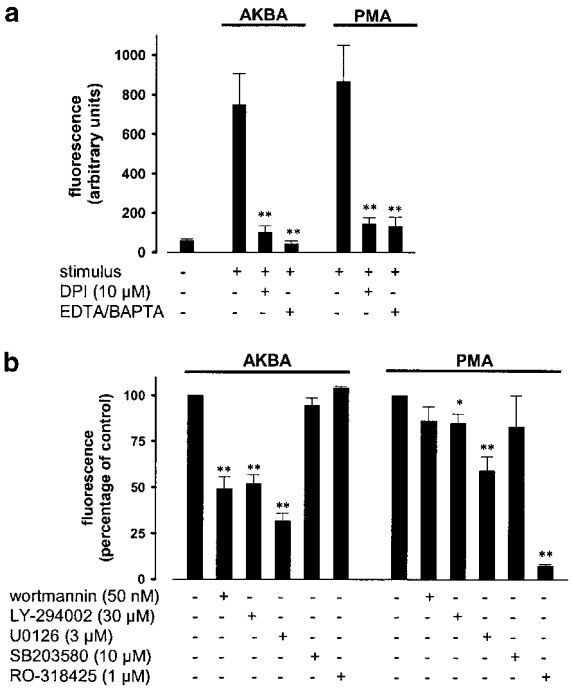

Figure 2 AKBA-induced ROS formation involves NADPH oxidase, PI 3-K, and p42/44^{MAPK}, and requires Ca^{2+}. PMNL (5×10^6 in 1 ml PGC buffer) were preincubated with the indicated compounds for 20 min at RT, prior addition of DCF-DA (1 μg ml^{-1}). After another 2 min, AKBA (30 μM) or PMA (100 nM) were 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

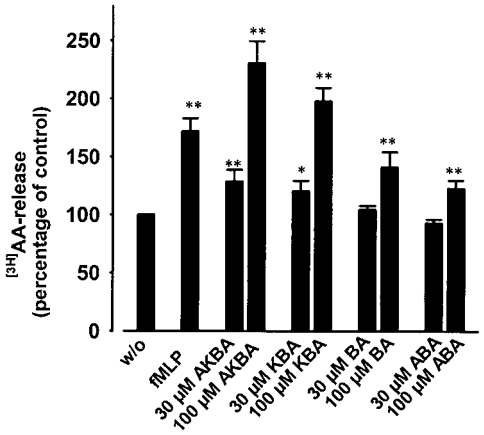

Figure 3 11-Keto-BAs elicit the liberation of AA in PMNL. Freshly isolated PMNL (2×10^6 in 1 ml RPMI 1640 medium) were prelabelled with 0.25 μCi ml^{-1} [^{3}H]AA for 120 min at 37°C and 5% CO_2. After unincorporated [^{3}H]AA was removed, cells (5×10^6 in 1 ml PGC buffer, containing 2 mg ml^{-1} fatty 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; $^*P<0.05$; $^{**}P<0.01$.

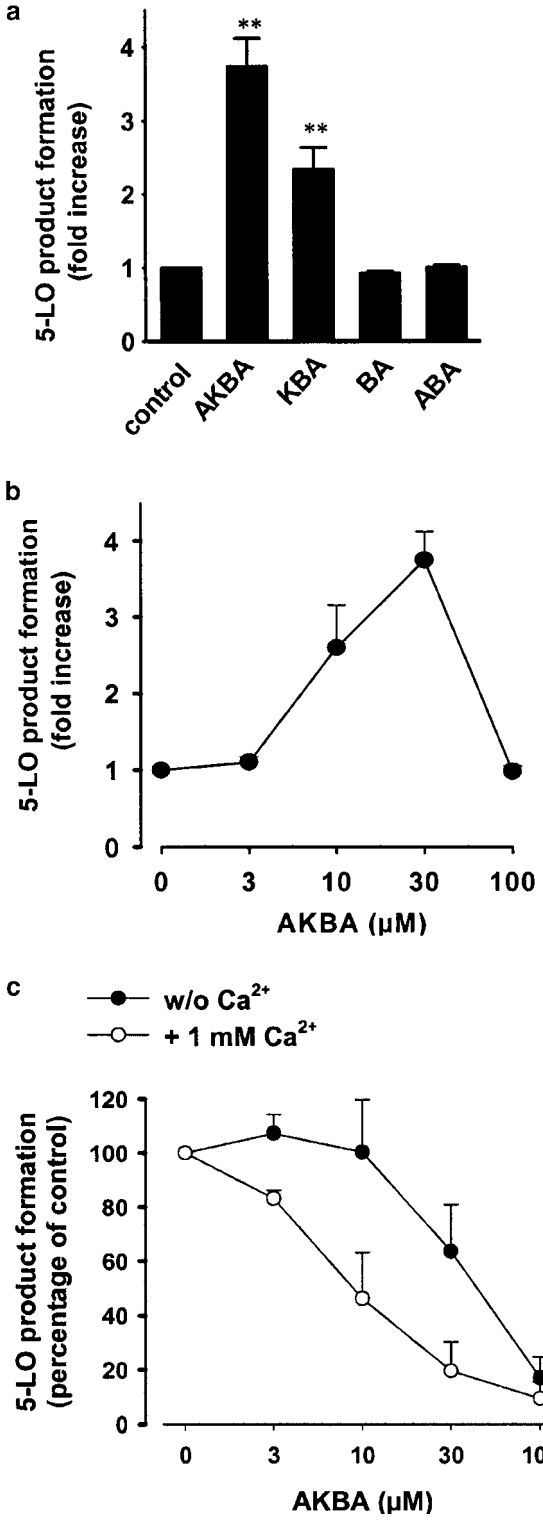

Figure 4 Effects of 11-keto-BAs on the formation of 5-LO metabolites. (a) Freshly isolated PMNL (5×10^6) were resuspended in 1 ml PG buffer containing 1 mM EDTA and preincubated with 30 μM BAPTA/AM for 15 min. Then, cells were stimulated with 20 μM AA alone or together with the indicated BAs (30 μM, 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

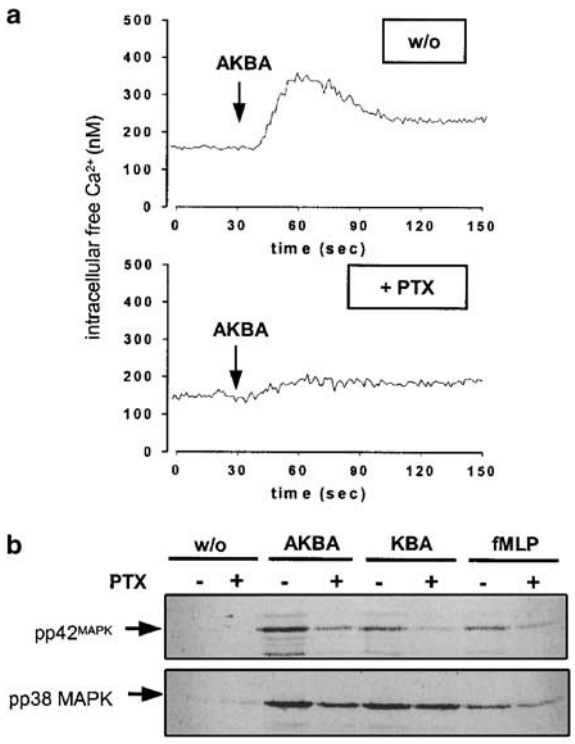

Figure 5 PTX suppresses Ca^{2+} mobilisation and activation of MAPK in PMNL induced by boswellic acids. (a) Ca^{2+} mobilisation. Freshly isolated PMNL (1 ± 10^7 ml^{-1} PGC buffer) were loaded with 2 μM Fura-2 for 30 min at 37°C. Then, cells were preincubated with or without 2 μg ml^{-1} PTX for 60 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

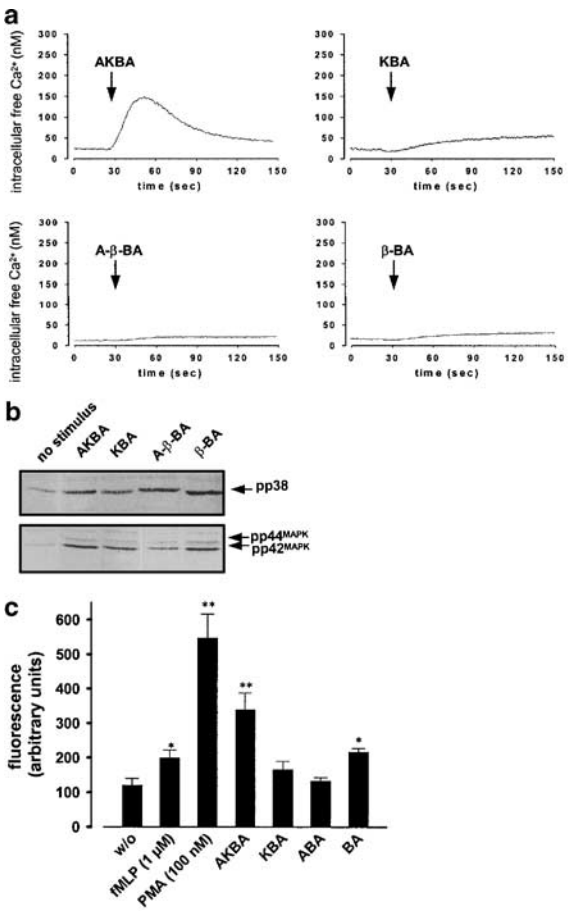

Figure 6 Effects of BAs on Ca^{2+} release, MAPK activation, and ROS production in granulocytic HL60 cells. HL60 cells were differentiated towards granulocytic cells in the presence of 1.5% DMSO, 2 ng ml^{-1} TGFβ, and 500 pM calcitriol for 4 days. (a) Mobilisation of Ca^{2+}. Cells (10^7 ml^{-1} PGC 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.

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Paper II

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Induction of central signalling pathways and select functional effects in human platelets by β-boswellic acid

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1 We have recently shown that in polymorphonuclear leukocytes, 11-keto boswellic acids (KBAs) induce Ca^{2+} mobilisation and activation of mitogen-activated protein kinases (MAPK). Here we addressed the effects of BAs on central signalling pathways in human platelets and on various platelet functions.

2 We found that β-BA (10 μM), the 11-methylene analogue of KBA, caused a pronounced mobilisation of Ca^{2+} from internal stores and induced the phosphorylation of p38 MAPK, extracellular signal-regulated kinase (ERK)2, and Akt. These effects of β-BA were concentration dependent, and the magnitude of the responses was comparable to those obtained after platelet stimulation with thrombin or collagen.

3 Based on inhibitor studies, β-BA triggers Ca^{2+} mobilisation *via* the phospholipase (PL)C/inositol-1,4,5-trisphosphate pathway, and involves Src family kinase signalling.

4 Investigation of platelet functions revealed that β-BA (⩾10 μM) strongly stimulates the platelet-induced generation of thrombin in an *ex-vivo in-vitro* model, the liberation of arachidonic acid (AA), and induces platelet aggregation in a Ca^{2+}-dependent manner.

5 In contrast to β-BA, the 11-keto-BAs (KBA or AKBA) evoke only moderate Ca^{2+} mobilisation and activate p38 MAPK, but fail to induce phosphorylation of ERK2 or Akt, and do not cause aggregation or significant generation of thrombin.

6 In summary, β-BA potently induces Ca^{2+} mobilisation as well as the activation of pivotal protein kinases, and elicits functional platelet responses such as thrombin generation, liberation of AA, and aggregation.

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Abbreviations: AA, arachidonic acid; Aβ-BA, 3-*O*-acetyl-boswellic acid; ADP, adenosine diphosphate; AKBA, 3-*O*-acetyl-11-keto-boswellic acid; 2-APB, 2-aminoethoxydiphenylborate; AUC, area under the curve; BA, boswellic acid; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; ER, endoplasmatic reticulum; ERK, extracellular signal-regulated kinase; ETP, endogenous thrombin potential; GPCR, G protein-coupled receptor; IP_3, inositol-1,4,5-trisphosphate; KBA, 11-keto-boswellic acid; LO, lipoxygenase; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; PAF, platelet-activating factor; PG buffer, PBS plus 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; PLC, phospholipase C; PMNL, polymorphonuclear leukocytes; PRP, platelet rich plasma; RFU, relative fluorescence units; SDS-b, 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer; TG, thapsigargin; TRAP, thrombin receptor-activating peptide; TXA_2, thromboxane A_2

Introduction

Platelets play critical roles in vascular thrombosis and inflammation. Activation of platelets may lead to secretion of granular contents and release of arachidonic acid (AA), shape change, adhesion, and aggregation (Holmsen, 1994). Agonists of platelets can be subdivided into strong activators, such as thrombin or collagen, and weak agonists including platelet-activating factor (PAF), adenosine diphosphate (ADP), serotonin, or thromboxane (TX)A_2 that require autocrine stimulation for the entire platelet response (Holmsen, 1994). Furthermore, platelets are partially activated when brought in close contact with surfaces, for example by adhesion to leukocytes or by aggregation.

Soluble platelet agonists, such as thrombin, ADP, PAF, or TXA_2, typically bind to specific G protein-coupled receptors (GPCRs), leading to the activation of phospholipase (PL)C (Ruggeri, 2002). PLC isoenzymes in turn, produce diacylglycerols (DAGs) and inositol-1,4,5-trisphosphate (IP_3), the latter releases Ca^{2+} *via* IP_3 receptors from the endoplasmatic reticulum (ER) (Rhee, 2001). GPCR stimulation may also lead to activation of phosphatidylinositol 3-kinase (PI 3-K)

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isoforms, resulting in Akt phosphorylation. Moreover, mitogen-activated protein kinase (MAPK) cascades, signalling pathways distal of G proteins, are activated upon platelet stimulation (Papkoff *et al*., 1994; Kramer *et al*., 1995). Altogether, Ca^{2+}, MAPKs, and PI 3-K/Akt regulate important platelet functions, for example, activation of cytosolic phospholipase (cPL)A_2 that liberates AA from phospholipids. In fact, agonist-stimulated platelets generate abundant AA (Kroll & Schafer, 1989), that is mainly metabolised to biologically active prostanoids, including TXA_2, and to 12(*S*)-hydro(pero)xyeicosatetraenoic acid (Yoshimoto & Takahashi, 2002).

The pentacyclic triterpenes boswellic acids (BAs, Figure 1) are thought to be responsible for the pharmacological actions of *Boswellia serrata* (BS) extracts, observed in several models of inflammation (Safayhi & Sailer, 1997). 5-lipoxygenase (5-LO) (Safayhi *et al*., 1992), leukocyte elastase (Safayhi *et al*., 1997), IκB kinases (Syrovets *et al*., 2005), and topoisomerases (Syrovets *et al*., 2000) are molecular targets of BAs. The anti-inflammatory properties of BAs have been attributed to inhibition of 5-LO (Safayhi & Sailer, 1997) but also to suppressed lipopolysaccharide-mediated TNF-α induction in monocytes (Syrovets *et al*., 2005). Moreover, BAs induce apoptosis of tumor cells (Glaser *et al*., 1999; Liu *et al*., 2002), accompanied by decreased ERK phosphorylation (Park *et al*., 2002) and enhanced caspase activity (Liu *et al*., 2002).

The functional properties and the potencies of the BAs depend on their structure, in particular on the absence or presence of the 11-keto group (Safayhi *et al*., 1992; Altmann *et al*., 2002; Liu *et al*., 2002). Thus, 11-keto-BA (KBA) and 3-*O*-acetyl-11-keto-BA (AKBA, Figure 1), but not the 11-methylene (11-me) analogues β-BA and Aβ-BA, potently inhibit 5-LO (Safayhi *et al*., 1992), induce caspase activation (Liu *et al*., 2002), Ca^{2+} mobilisation, and MAPK activation in polymorphonuclear leukocytes (PMNL) (Altmann *et al*., 2002), whereas 11-me-BAs were more efficient in inhibition of topoisomerases (Syrovets *et al*., 2000) and induction of apoptosis (Glaser *et al*., 1999).

Recently, we demonstrated that 11-keto-BAs can activate PMNL by mobilisation of Ca^{2+} and stimulation of MAPKs (Altmann *et al*., 2002), coupled to functional PMNL responses (Altmann *et al*., 2004). In the present study we identified β-BA as an agonist for platelets inducing essential signal transduction pathways as well as functional platelet responses, for example release of endogenous AA, thrombin generation, and Ca^{2+}-dependent aggregation.

Methods

Materials

BAs were kindly provided by Dr J. Jauch (Saarbruecken, Germany). Argatroban was a gift from Mitsubishi Pharma (Tokio, Japan) and WEB 2086 was a gift from Boehringer Ingelheim (Ingelheim, Germany). Collagen was from Nykomed Pharma (Unterschleißheim, Germany). U-73122, Calbiochem (Bad Soden, Germany); BAPTA/AM, Fura-2/AM, and forskolin, Alexis (Grünberg, Germany); NF-449 and wortmannin, Biotrend (Köln, Germany); thrombin receptor-activating peptide (TRAP), Bachem (Weil am Rhein, Germany); MRS-2179 and all other chemicals were obtained from Sigma (Deisenhofen, Germany).

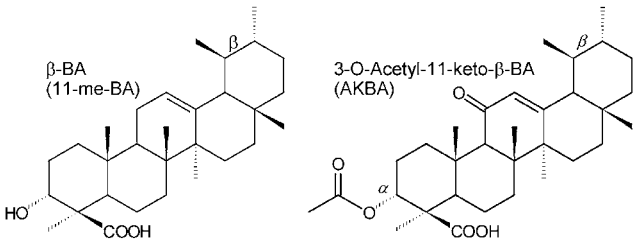

Figure 1 Chemical structures of β-BA and AKBA. AKBA lacking the 11-acetyl group yields KBA; 3-*O*-acetylation of β-BA yields Aβ-BA.

Cells

Platelets were freshly isolated from human venous blood of healthy adult donors (St Markus Hospital, Frankfurt, Germany) as described (Albert *et al*., 2002). Washed platelets were finally resuspended in PBS pH 7.4 and 1 mg ml^{-1} glucose (PG buffer) or in PBS pH 7.4 and 1 mg ml^{-1} glucose plus 1 mM $CaCl_2$ (PGC buffer). For incubations with solubilised compounds, ethanol or DMSO was used as vehicle, never exceeding 1% (vol by vol). For functional platelet test (AA release, thrombin generation, aggregation, flow-cytometry of platelet activation markers) platelet-rich plasma (PRP) was prepared from freshly drawn blood (in 3.13% citrate, designated 'citrate-chelated PRP') from healthy adult donors by centrifugation for 7 min at 750 × *g*. Depending on the experimental setup, PRP was recalcified to obtain a final [Ca^{2+}] of 1.5 mM, or diluted (14%, vol by vol) in a buffer containing 20 mM HEPES, 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. Alternatively, platelets were isolated from PRP to yield washed platelets. Some flow-cytometry experiments were also carried out using whole blood (in 3.13% citrate).

Viability assessment

Washed platelets (~3 × 10^8 ml^{-1} PG buffer) were prewarmed for 15 min at 37°C. Then, $CaCl_2$ (1 mM) and any agent (DMSO, β-BA, AKBA, thrombin, or Ca^{2+} ionophore A23187) were added, and samples were incubated for another 15 min at 37°C. The particle distribution pattern in each sample was then determined using a Sysmex Cell Counter (Norderstedt, Germany) and compared to the DMSO sample (negative control, viable cells) and the A23187 sample (cell fragmentation and lysis due to ionophore action).

Measurement of intracellular Ca^{2+} levels

Platelets (6 × 10^8 ml^{-1} PG buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, 10^8 cells ml^{-1} PG buffer were incubated 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. At 2 min prior stimulation, 1 mM $CaCl_2$ or 1 mM EDTA was added. The fluorescence was measured and [Ca^{2+}]$_i$ was calculated according to Grynkiewicz *et al*. (1985).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Platelets (10^9 ml^{-1} PG buffer containing 1 mM $CaCl_2$ or 1 mM EDTA plus 30 μM BAPTA/AM, respectively) were incubated with the indicated stimuli at 37°C. The reaction was stopped by addition of the same volume of ice-cold 2 × SDS–PAGE sample loading buffer (SDS-b). Samples for SDS–PAGE (aliquots corresponding to 10^6 cells in 20 μl volume) were prepared, and proteins were separated as described (Werz *et al*., 2002). Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. Western blotting using phospho-specific antibodies (New England Biolabs (Beverly, MA, U.S.A.), 1 : 1000 dilution, each) against pERK1/2 (Thr202/Tyr204), pp38 MAPK (Thr180/Tyr182), or pAkt (Ser473), was performed as described (Werz *et al*., 2002).

Determination of release of [^{3}H]-labelled AA from intact platelets

PRP was labelled with 19.2 nM. [^{3}H]AA (1 μCi ml^{-1}, specific activity 200 Ci mmol^{-1}) for 2 h at 37°C in the presence of 100 μM aspirin. Then, cells were washed twice with PBS pH 5.9 plus 1 mM $MgCl_2$, 11.5 mM $NaHCO_3$, 1 g l^{-1} glucose, and 1 mg ml^{-1} fatty acid-free BSA, and finally resuspended in PG buffer (10^8 ml^{-1}). Preparation of cells at pH 5.9 is thought to minimise temperature-induced activation (Maurer-Spurej *et al*., 2001). After 15 min at RT, 1 mM $CaCl_2$ was added 2.5 min prior stimulation with the indicated agents at 37°C. After 5 min, incubations were put on ice for 10 min, followed by centrifugation (5000 × *g*, 15 min). Aliquots (300 μl) of the supernatants were measured (Micro Beta Trilux, Perkin Elmer) to detect the amounts of [^{3}H]-labelled AA released into the medium.

Measurement of thrombin generation

Thrombin generation was assessed by using a fluorometric assay, based on the cleavage of a thrombin-specific fluorogenic substrate resulting from stimulation of recalcified or citrate-chelated PRP, yielding the so-called endogenous thrombin potential (ETP) (Hemker *et al*., 2000). In all, 80 μl of PRP and 20 μl of buffer containing the thrombin generation trigger were added to each well of a 96-well microtitre plate. The Fluoroskan Ascent Type 374 plate fluorometer (Labsystems; Finland) was used with excitation wavelength 390 nm, emission wavelength 460 nm, and a measurement integration time per well of 20 ms. The first derivative of the fluorescence–time curve reflects the course of thrombin activity in the sample. The parameter of interest in the samples using recalcified PRP was the maximal generation rate which is the peak of the first derivative (ETP peak velocity, relative fluorescence units (RFU) min^{-1}) of the thrombin generation curve, or, due to low peak values in Ca^{2+}-free samples, the ETP–area under the curve (AUC).

Measurement of platelet activation markers CD62 and PAC-1 by flow cytometry

Whole blood samples (containing 3.13% sodium citrate), recalcified PRP, or washed platelets resuspended in PGC were incubated with β-BA, AKBA, TRAP, or vehicle (DMSO, control) for 2 or 15 min at RT. To measure CD62 and PAC-1, samples were diluted 1 : 1 in 20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 g l^{-1} BSA, pH 7.4, and aliquots of 5 μl were incubated with saturating concentrations of CD41-PC7, CD62-PE, and PAC1-FITC at RT for 15 min in the dark. Samples were fixed with formaldehyde 1% (in PBS), washed twice (CellWash, Becton-Dickinson, Heidelberg, Germany), and resuspended in 300 μl PBS. Isotype-matched IgG and IgM antibodies were used to correct for the nonspecific binding of the specific antibodies. P-selectin (represented by CD62) and PAC-1 antigen expression were quantified using Cellquest software (Becton-Dickinson). Three-color flow cytometric analysis was used with logarithmic modes set for all channels. A gate was set around the platelet population (CD41), and 5000 events were acquired from each probe. The percentage of CD62-positive cells (%) as well as their mean channel fluorescence intensity (MFI) was determined at a level which yields a value of 1% for mouse IgG1-PE labelled sample. A histogram of PAC1-FITC against cell events was generated and MFI of the total platelet population was recorded.

Acquisition of data was carried out using a FACSCalibur flow cytometer with CELLQuest™ (Becton-Dickinson). The instrument calibration and compensation was assured daily with calibration beads (CaliBRITE™ Beads, Becton-Dickinson) and FACSComp™. Fluorescence-conjugated antibodies CD41-PC7, CD62-PE, and PE-labelled isotype IgG1 control were obtained from Beckman Coulter (Krefeld, Germany), PAC1-FITC and FITC-isotype IgM were from Becton-Dickinson.

Ex vivo *platelet aggregation (turbidimetric)*

Aggregation of platelets in pure or diluted PRP was determined using a turbidimetric light-transmittance device. For aggregation, the response to 30 μM β-BA, 30 μM AKBA, or (as positive controls) 2 U ml^{-1} thrombin, or 1 μg ml^{-1} collagen is given as per cent of the maximal light transmission A_{max}. In Ca^{2+}-containing samples, $CaCl_2$ was added right before the start of the measurement. Aggregation was recorded for 15 min.

Statistics

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD *post hoc* tests. Where appropriate, Student's *t*-test for paired observations was applied. A *P*-value of <0.05 (*) or <0.01 (**) was considered significant.

Results

BAs evoke Ca^{2+} mobilisation in washed human platelets

In the presence of extracellular Ca^{2+} (1 mM), BAs lacking the 11-keto moiety (Aβ-BA and β-BA, 10 μM each, chemical structure see Figure 1) induced a transient but robust elevation of $[Ca^{2+}]_i$ in washed platelets that peaked 18–30 s following exposure, whereas KBA was ineffective and AKBA caused only a weak and rather slow Ca^{2+} mobilisation (Figure 2a

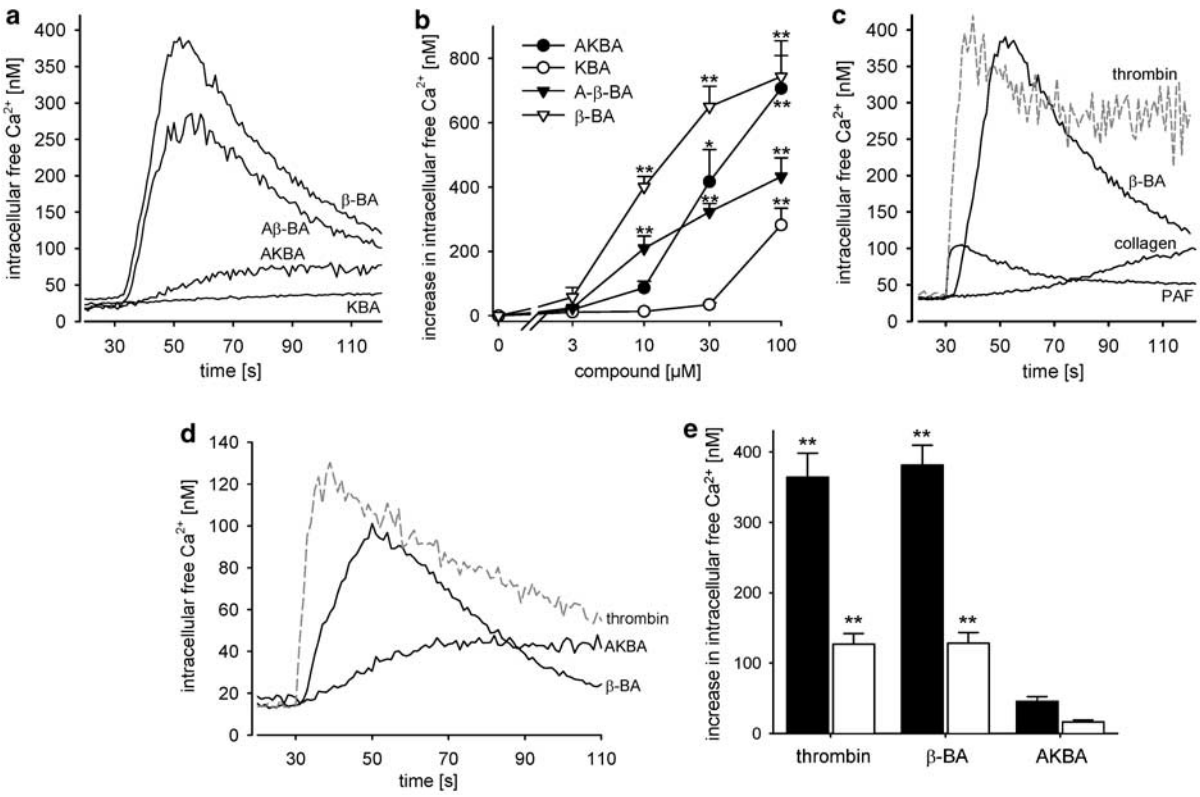

Figure 2 BAs induce intracellular Ca^{2+} mobilisation. To Fura-2-loaded platelets (10^8 ml^{-1} PG buffer), 1 mM $CaCl_2$ (a–c and e) or 1 mM EDTA (d and e) was added 2 min prior stimulation, and $[Ca^{2+}]_i$ was determined. (a) Ca^{2+} mobilisation in the presence of extracellular Ca^{2+}. BAs (10 μM, each) were added 30 s after the measurement was started. (b) Concentration–response curves of BAs in the presence of extracellular Ca^{2+}. The maximal increase in $[Ca^{2+}]_i$ obtained within 100 s of measurement is given. (c) Ca^{2+} mobilisation induced by various agonists. The following agonists were used: β-BA (10 μM), thrombin (0.5 U ml^{-1}), collagen (8 μg ml^{-1}), and PAF (100 nM). (d) Ca^{2+} mobilisation in the absence of extracellular Ca^{2+}. BAs (10 μM, each) or thrombin (0.5 U ml^{-1}) were added 30 s after the measurement was started. (e) Comparison of Ca^{2+} mobilisation in the presence (black bars) or absence (white bars) of 1 mM of extracellular Ca^{2+}. The maximal increase in $[Ca^{2+}]_i$ after stimulation with thrombin (0.5 U ml^{-1}) or BAs (10 μM, each) was determined within 100 s of measurement. Values are given as mean + s.e., $n = 5$; curves are representative for at least five experiments. One-way ANOVAs followed by Tukey HSD tests were applied to data related to unstimulated controls in (b) and (e) $^*P < 0.05$ or $^{**}P < 0.01$.

and b). β-BA was effective already at 3 μM, though not yet significant (Figure 2b). At 10 μM, the maximum increase in $[Ca^{2+}]_i$ (381 ± 28 nM) elicited was comparable to that obtained by thrombin (0.5 U ml^{-1}; 364 ± 34 nM), and exceeded the signal obtained by PAF (100 nM; 62 ± 5 nM, Figure 2c). However, thrombin- and PAF-induced Ca^{2+} mobilisation was more rapid, peaking 5–10 s after exposure and (for thrombin) was more sustained. Collagen (8 μg ml^{-1}) caused a slow and only moderate elevation of $[Ca^{2+}]_i$ (78 ± 7 nM, after 90 s). 11-Keto BAs caused significant Ca^{2+} mobilisation at higher concentrations ($\geqslant$20–30 μM) (Figure 2b), which again was rather slow. Thus, the potencies and the kinetics differ between 11-keto-BAs and their 11-me analogues.

$[Ca^{2+}]_i$ was also measured in the absence of extracellular Ca^{2+}. β-BA, Aβ-BA (not shown), and AKBA as well as thrombin evoked an internal Ca^{2+} release with similar kinetics observed for the total Ca^{2+} response in the presence of extracellular Ca^{2+}, respectively (Figure 2D, compare Figure 2a). Nevertheless, in the absence of extracellular Ca^{2+}, elevation of $[Ca^{2+}]_i$ was reduced to about 37 ± 14% for thrombin and 28 ± 17% for β-BA, as compared to the total Ca^{2+} response (Figure 2e).

β-*BA-, but not AKBA-induced* Ca^{2+} *mobilisation is PLC dependent*

U-73122 (3 μM) and 2-aminoethoxydiphenylborate (2-APB, 50 μM) were used to examine the participation of PLC in BA-induced Ca^{2+} mobilisation. Cells were stimulated with β-BA or AKBA (10 μM each), with thrombin (positive control), or thapsigargin (TG, negative control). Thrombin evokes Ca^{2+} elevations *via* a GPCR/PLC-dependent pathway (Coughlin, 2000), whereas TG induces Ca^{2+} mobilisation by inhibition of the ER Ca^{2+}-ATPase, thus circumventing PLC and GPCR signalling (Gouy *et al*., 1990). Thrombin- and β-BA-induced Ca^{2+} elevation was strongly suppressed by U-73122 (Figure 3a), both, in the presence and in the absence of extracellular Ca^{2+} (Table 1). In contrast, Ca^{2+} mobilisation induced by TG was not suppressed by U-73122, and the response to AKBA was even potentiated (Figure 3a, Table 1). Generally, U-73122 preincubation already caused a slight elevation of the resting Ca^{2+} levels (Figure 3a). U-73343, the inactive analogue of U-73122, had no effect (not shown). 2-APB (50 μM) (Maruyama *et al*., 1997), an inhibitor of IP_3-mediated elevations in cytosolic $[Ca^{2+}]$, also suppressed Ca^{2+}

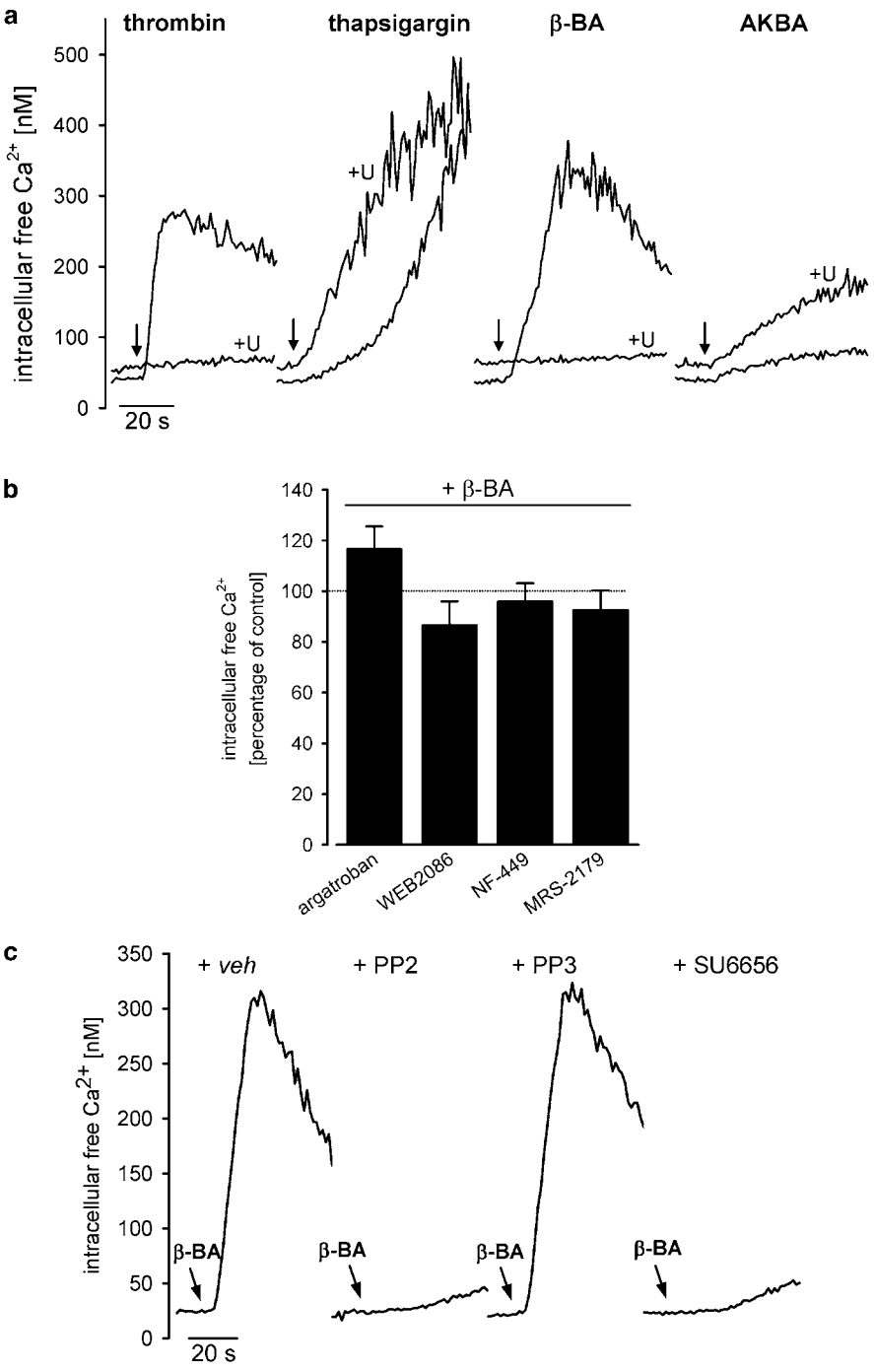

Figure 3 Modulation of β-BA-induced Ca^{2+} mobilisation by pharmacological inhibitors. (a) Effects of U-73122. Fura-2-loaded platelets (10^8 ml^{-1} PG buffer) were preincubated with U-73122 (3 μM, trace labelled '+U') or vehicle (DMSO) for 15 min. $CaCl_2$ (1 mM) was added 2 min prior stimulation with thrombin (0.5 U ml^{-1}), thapsigargin (0.1 μM), or BAs (10 μM, each), and $[Ca^{2+}]_i$ was determined. Curves are representative for at least five experiments. (b) Effects of argatroban (100 ng ml^{-1}), WEB 2086 (30 μM), NF 449 (1 μM), or MRS-2179 (10 μM). Fura-2-loaded platelets (10^8 ml^{-1} PG buffer) were preincubated with the indicated compounds or vehicle (DMSO) for 15 min. $CaCl_2$ (1 mM) was added 2 min prior stimulation with 10 μM β-BA. The maximal increase in $[Ca^{2+}]_i$ determined within 100 s of measurement is expressed as percentage of control (10 μM β-BA). Values are given as mean + s.e., $n = 5$. (c) Effects of Src family kinase inhibitors. Fura-2-loaded platelets (10^8 ml^{-1} PG buffer) were preincubated with PP2 (3 μM), PP3 (3 μM), SU6656 (10 μM), or vehicle (DMSO) for 15 min. $CaCl_2$ (1 mM) and β-BA (10 μM) were added, and $[Ca^{2+}]_i$ was determined. Curves are representative for at least four experiments.

mobilisation induced by β-BA in the presence (73 ± 7% inhibition, $n = 3$) and in the absence of Ca^{2+} (54 ± 11% inhibition, $n = 3$). An equal reduction was found for the thrombin response (not shown). Collectively, our data indicate that Ca^{2+} mobilisation induced by β-BA is mediated by the PLC/IP_3 signalling pathway.

Since β-BA could first induce the generation of an endogenous platelet agonist that in turn causes PLC/IP_3-coupled

Table 1 Effects of U-73122 on Ca^{2+} mobilisation in the absence and presence of Ca^{2+}

	+Ca^{2+} residual signal (percentage of control)	*−Ca^{2+} (+EDTA) residual signal (percentage of control)*
Thrombin	10±4 ($n=5$)**	12±3 ($n=5$)**
TG	90±12 ($n=5$)	116±19 ($n=4$)
β-BA	5±2 ($n=6$)**	23±8 ($n=6$)**
AKBA	170±68 ($n=5$)	202±41 ($n=5$)

Fura-2-loaded platelets (10^8 ml^{-1} PG buffer) were preincubated with U-73122 (3 μM) for 15 min. $CaCl_2$ (1 mM) or EDTA (1 mM) were added, and after 2 min, platelets were stimulated with thrombin (0.5 U ml^{-1}), TG (0.1 μM), or BAs (10 μM, each). Maximum amplitudes were compared to control measurements in the absence of U-73122. Data are expressed as mean+s.e., $n=4$–6 (see table). Statistical analysis (*t*-tests for correlated samples, inhibitor *versus* control samples for each stimulus) was performed prior to data normalisation, $^{**}P<0.01$.

elevation of $[Ca^{2+}]_i$, antagonists of typical platelet stimuli were utilised to unravel such an autocrine mode of action. The thrombin antagonist argatroban (100 ng ml^{-1}) failed to significantly suppress the effects of β-BA (Figure 3b), whereas it completely blocked thrombin-induced Ca^{2+} mobilisation (not shown). Similarly, the PAF receptor antagonist WEB 2086 (30 μM) as well as the purinergic receptor antagonists NF-449 (1 μM, targeting $P2X_1$) and MRS-2179 (10 μM, targeting $P2Y_1$, and $P2Y_{12}$) did not markedly affect β-BA-induced elevation of $[Ca^{2+}]_i$ (Figure 3b), although these compounds abolished the responses induced by their respective agonists (not shown).

Src family kinases are involved in β-BA-induced Ca^{2+} mobilisation

The role of Src family kinases in β-BA-induced Ca^{2+} mobilisation was assessed using the selective Src family kinase inhibitors PP2 (and its inactive analogue PP3) (Hanke *et al*., 1996) and SU6656 (Blake *et al*., 2000). PP2 (3 μM) blunted the Ca^{2+} response initiated by β-BA (92±2% inhibition, $n=7$, see Figure 3c) whereas the inactive analogue PP3 (3 μM) was hardly effective (89±8% residual activity, $n=4$, see Figure 3c). Also, the structurally unrelated Src kinase inhibitor SU6656 (10 μM) likewise abolished the β-BA signal (93±1% inhibition, $n=4$, Figure 3c). In sharp contrast, no such inhibitory effects of PP2 on Ca^{2+} signals induced by thrombin, PAF, or AKBA were apparent (not shown).

β-BA induces MAPK activation and Akt/PKB phosphorylation

All BAs tested led to a concentration-dependent activation of p38 MAPK in platelets (Figure 4a). Moreover, β-BA (and Aβ-BA, not shown) also concentration-dependently (10–100 μM) activated ERK2 (Figure 4b), which was maximal 30–60 s upon stimulation, slightly preceding thrombin-induced ERK2 activation (maximum after 2 min) (Figure 4c). The EC_{50} for thrombin to activate p38 MAPK and ERK2 was determined at ≈1 U ml^{-1} (not shown). AKBA (and KBA, not shown) were virtually ineffective to activate ERK2. U-73122 (not shown) as well as the Ca^{2+} chelators EDTA and BAPTA/AM, moderately reduced β-BA-induced ERK2 activation (Figure 4d and e),

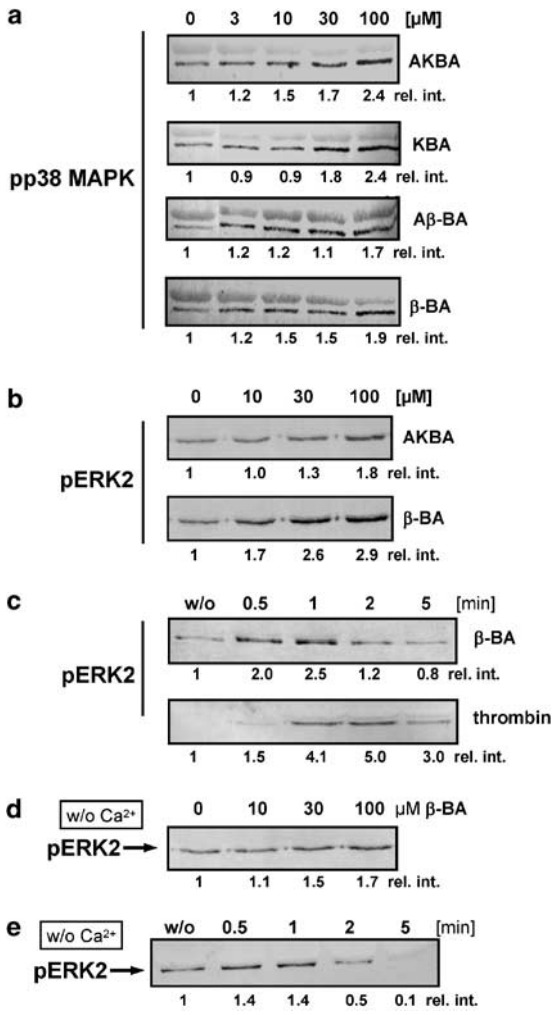

Figure 4 BAs induce the activation of MAPKs. Activation of p38 MAPK (a) and ERK2 (b). Platelets (10^9 ml^{-1} PGC buffer) were stimulated with the indicated concentrations of the BAs at 37°C. Reactions were terminated after 1 min to assess ERK2 phosphorylation, and after 1.5 min to assess p38 MAPK phosphorylation. Samples were subjected to SDS–PAGE and Western blotting using phospho-specific antibodies against the dually phosphorylated form of the MAPKs. (c) Time course of ERK2 activation in the presence of Ca^{2+}. Platelets (10^9 ml^{-1} PGC buffer) were stimulated with β-BA (30 μM) or thrombin (1 U ml^{-1}) at 37°C for the indicated times and phosphorylation of ERK2 was determined. (d) ERK2 activation by β-BA in the absence of Ca^{2+}. Platelets (10^9 ml^{-1} PG buffer containing 1 mM EDTA and 30 μM BAPTA/AM) were preincubated for 15 min at RT, stimulated with the indicated concentrations of β-BA for 1 min and phosphorylation of ERK2 was determined. (e) Time course of ERK2 activation by β-BA in the absence of Ca^{2+}. Platelets (10^9 ml^{-1} PG buffer containing 1 mM EDTA and 30 μM BAPTA/AM) were preincubated for 15 min at RT, stimulated with 30 μM β-BA, and phosphorylation of ERK2 was determined after the times indicated. The relative intensities (rel. int.) of blot bands were determined by densitometry using the BioRad Quantitate One software. The results shown are representative of at least three independent experiments.

indicating that PLC and Ca^{2+} may contribute, but are not absolutely required.

β-BA concentration-dependently increased the phosphorylation of Akt at Ser473, whereas AKBA had no significant effect (Figure 5a). Again, the effects of β-BA were comparable to thrombin. Thus, Akt phosphorylation induced by β-BA was

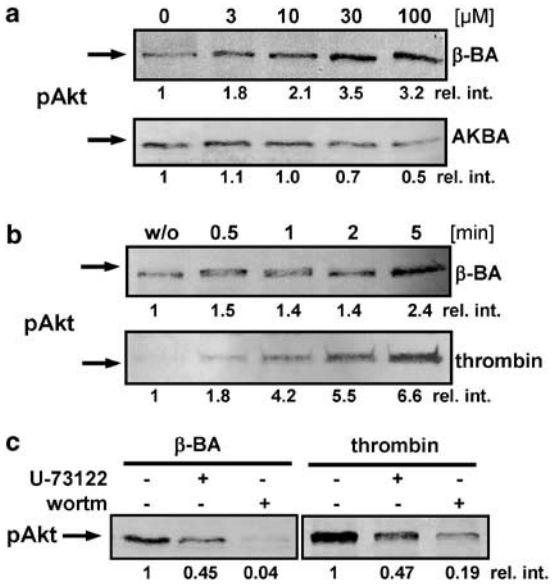

Figure 5 β-BA induces phosphorylation of Akt. (a) Concentration–response experiments. Platelets (10^9 ml^{-1} PGC buffer) were stimulated with the indicated concentrations of BAs for 4 min at 37°C and Akt phosphorylated at Ser473 was assessed. (b) Time course of Akt phosphorylation. Platelets (10^9 ml^{-1} PGC buffer) were incubated with β-BA (30 μM) or thrombin (1 U ml^{-1}) at 37°C for the indicated times and Akt phosphorylation was assessed. (c) Effects of U-73122 and wortmannin (wortm) on Akt phosphorylation. Platelets (10^9 ml^{-1} PGC buffer) were preincubated with 3 μM U-73122 or 200 nM wortmannin as indicated and then stimulated with β-BA (30 μM) or thrombin (1 U ml^{-1}), respectively, for 4 min at 37°C and Akt phosphorylation was assessed. The relative intensities (rel. int.) of blot bands were determined by densitometry using the BioRad Quantitate One software. Results are representative of at least three independent experiments.

most pronounced after 2–5 min (Figure 5b), and was completely blocked by the PI 3-K inhibitor wortmannin (wortm., 200 nM) and strongly blunted by U-73122 (Figure 5c). Moreover, removal of total Ca^{2+} with EDTA and BAPTA/AM abolished the effect of β-BA (not shown). Similar inhibitory effects on Akt phosphorylation by wortmannin and U-73122 (Figure 5c) as well as by Ca^{2+} removal (not shown) were seen when thrombin was used as stimulus.

Induction of cell viability assessment and AA release

The effects of BAs on platelet viability were determined by analysis of the particle distribution pattern of washed platelet samples treated with β-BA, AKBA (30 μM, each), DMSO, or Ca^{2+} ionophore A23187 (5 μM) for 15 min at 37°C. Particle size spreading (given by the particle volume, unit: fl, horizontal axis) and numbers were similar for β-BA, AKBA, and DMSO, whereas ionophore-induced platelet lysis caused a heterogeneous dispersion (Figure 6a). Thus, exposition of platelets to β-BA for 15 min does not seem to affect cell viability.

An elevation of $[Ca^{2+}]_i$ and/or activation of members of the MAPK family are considered important for the liberation of AA by the $cPLA_2$ (Gijon & Leslie, 1999). Incubation of [^{3}H]AA-labelled platelets with β-BA caused a concentration-dependent increase in the amounts of [^{3}H]AA released into the medium. At 30 μM, β-BA was equipotent to 2 U ml^{-1} thrombin (Figure 6b).

Effects of β-BA on aggregation

In recalcified (1.5 mM $CaCl_2$, free Ca^{2+} was calculated as approx. 1 mM) PRP, β-BA (10 or 30 μM) stimulated aggregation

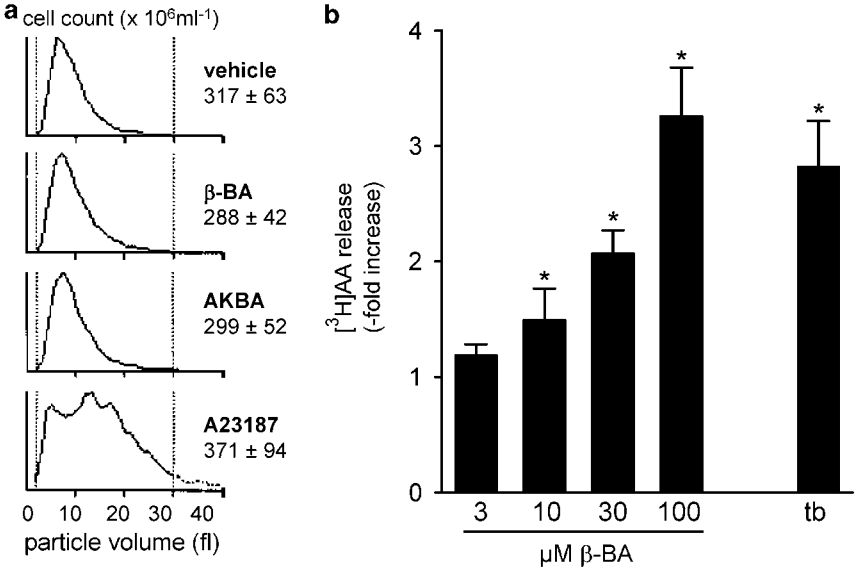

Figure 6 β-BA induces the liberation of AA; effects on cell viability. (a) Analysis of cell viability. Washed platelets were resuspended in PGC buffer and exposed to the indicated stimuli (vehicle (DMSO), 30 μM β-BA, 30 μM AKBA, 5 μM A23187, from top) for 15 min at 37°C. The particle size distribution pattern and particle number in each sample was determined. DMSO (negative control, viable cells) and A23187 samples (positive control, lysed cells) were used as reference. Curves are representative for at least three independent determinations. Cell count values are given as mean + s.e., $n = 3$–4. (b) β-BA induces the release of AA. Platelets were labelled with [^{3}H]AA for 2 h. $CaCl_2$ (1 mM) was added to the cells (10^8 in 1 ml PG buffer), and after 2.5 min, cells were stimulated with the indicated concentrations of β-BA or 2 U ml^{-1} thrombin (tb). [^{3}H]AA released into the medium was measured after 5 min at 37°C. Data are expressed as increase over unstimulated cells, values are given as mean + s.e., $n = 5$. Statistical analysis (directed t-tests for correlated samples) was applied to original data prior to normalisation, $^*P < 0.05$.

6–8 min after addition (Figure 7a, middle left trace). In contrast, aggregation induced by collagen (1 μg ml^{-1}) was much more rapid (Figure 7a, middle right trace). Spontaneous aggregation due to unspecific platelet activation (e.g. stirring) occurred after >12–15 min. Similarly, in samples that received AKBA (30 μM), aggregation was first evident after approx. 12 min (Figure 7a, lower trace). In contrast, in citrate-chelated PRP (no Ca^{2+}), β-BA (30 μM) caused no aggregation, whereas collagen remained a full agonist (Figure 7b). Also, no unspecific aggregation was observed in the absence of extracellular Ca^{2+}. The Ca^{2+} dependency of the β-BA effect was confirmed using PRP diluted in a Ca^{2+}-containing HEPES buffer (14% PRP, final concentration). Under these conditions, aggregation induced by β-BA (30 μM) was more rapid (after 4–6 min) than in pure PRP (Figure 7c). DMSO and AKBA (30 μM) were inactive, and collagen again acted as an immediate and full agonist (Figure 7c). It should be noted that in diluted PRP without Ca^{2+}, none of the stimuli induced aggregation (not shown). Together, β-BA-stimulated aggregation strictly depended on the presence of extracellular Ca^{2+}.

Thrombin generation and expression of activation markers

β-BA was tested for its ability to generate thrombin from PRP, expressed as the ETP. β-BA (10 μM) potently stimulated thrombin generation, whereas AKBA (10 μM) was inactive (Figure 8a, left panel). Although collagen was only moderately superior to β-BA in the peak thrombin generation velocity, there was again a delayed onset of the β-BA effect, visualised by the kinetic progression of the ETP (Figure 8b). In the absence of Ca^{2+}, neither stimulus induced a marked increase in the ETP over time although analysis of the ETP-AUCs revealed a slight stimulatory effect of β-BA (10 μM) as compared to DMSO and collagen that both were inactive (Figure 8a, right panel).

Finally, the expression of the activation markers PAC-1 (the activated GPIIb/IIIa-receptor for fibrinogen) and CD62, which indicates the release of platelet alpha-granules, were assessed. Incubations were carried out in (I) whole blood (containing 3.13% citrate), (II) recalcified PRP, and (III) washed platelets in Ca^{2+}-containing PGC buffer, for 2 or 15 min. Neither β-BA (30 or 100 μM) nor AKBA (30 μM) led to a significant expression of CD62 and PAC-1 under all experimental settings (I–III), whereas TRAP (used as positive control) was a strong activator (Figure 8c).

Discussion

We identified 11-me-BAs (i.e. β-BA) as naturally occurring compounds that induce central signalling pathways and that elicit select functions in human platelets. Depending on the structure of the BAs, the effectiveness and the routes, utilised to activate downstream signalling pathways and functional responses, are highly distinct. For β-BA, Src family kinases and the PLC/IP_3 pathway seem to be involved in Ca^{2+} mobilisation, and β-BA causes activation of ERK2 and the PI 3-K/Akt route. Moreover, β-BA induces the release of AA, a pronounced generation of thrombin, and Ca^{2+}-dependent platelet aggregation. In contrast, AKBA-induced Ca^{2+} mobilisation is not connected to Src family kinases and PLC/IP_3

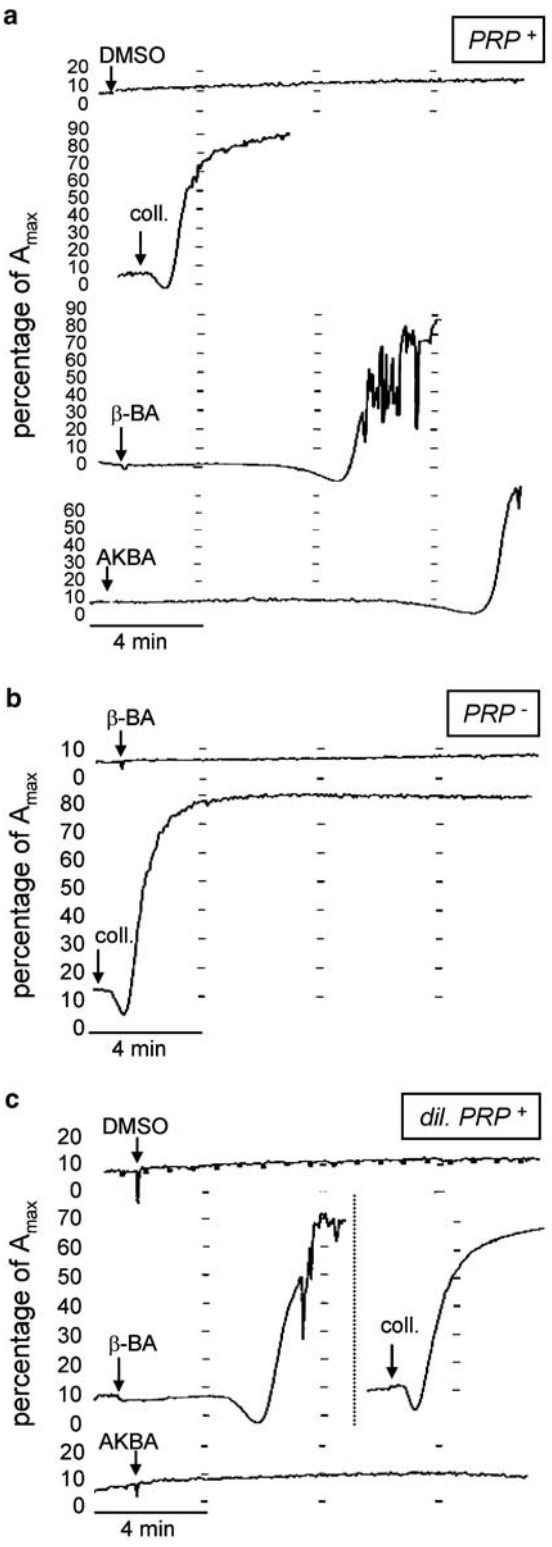

Figure 7 Effects of β-BA on platelet aggregation. (a) Aggregation in recalcified PRP (PRP^+). Samples were stimulated with vehicle (DMSO, 0.3%), β-BA (30 μM), collagen (1 μg ml^{-1}), or AKBA (30 μM) as indicated and aggregation curves were recorded (maximum 15 min). The aggregation response is given as per cent of the maximal light transmission A_{max}. Curves are representative for at least five independent determinations. (b) Aggregation in citrate-chelated PRP. The experimental conditions were the same as above, $n=4$. (c) Aggregation in PRP diluted in Ca^{2+}-containing HEPES buffer, $n=5$.

signalling, and AKBA failed to induce phosphorylation of Akt and ERK2, as well as functional platelet responses.

Among the BAs tested for Ca^{2+} mobilisation, β-BA is the most potent analogue. At 10 μM, the effectiveness of β-BA exceeded that of PAF or collagen, and was comparable with that of the potent platelet agonist thrombin. Such β-BA concentrations are in the range of β-BA levels in human plasma (10.1 μM), determined after oral application of 4 × 786 mg BS extract/day within 10 days (Buchele & Simmet, 2003). The 3-*O*-acetyl group slightly hampers (receptor-)activation and the 11-keto moiety significantly decreases the potency and also alters the signalling routes in platelets. In sharp contrast to platelets, only 11-keto BAs, but not 11-me-BAs, caused stimulation of PMNL (Altmann *et al*., 2002; 2004). Possibly, PMNL and platelets selectively express closely related but not identical receptors specific for AKBA or 11-me-BAs, respectively. Important receptors for soluble agonists known to regulate $[Ca^{2+}]_i$ in platelets are the purinergic $P2X_1$ and $P2Y_{1/12}$ receptors, the TXA_2 receptor, the PAF receptor, the $5\text{-}HT_{2A}$ receptor, and the PAR-1 and -4 (Jackson *et al*., 2003). Whether β-BA acts at one (or more) of these receptors is unknown. However, antagonists of thrombin (argatroban), PAF (WEB 2086), and ADP (NF449 and MRS2179) did not affect β-BA-induced Ca^{2+} mobilisation.

Thrombin is the most potent platelet agonist acting *via* PAR-1 and -4. PARs are coupled to trimeric $G_q/G_i/G_{12/13}$ proteins enabling the Gα and Gβγ subunits to stimulate PLC-β subtypes (Lee *et al*., 1996; Coughlin, 2000), resulting in IP_3-dependent Ca^{2+} mobilisation from intracellular storage sites with concomitant store-operated Ca^{2+} entry (Rosado & Sage, 2001). In analogy to thrombin, β-BA caused Ca^{2+} mobilisation from internal stores, which was sensitive to U-73122 and to 2-APB, confirming the involvement of PLC/IP_3. However, it should be noted that PLC/IP_3-independent effects of U-73122 (Broad *et al*., 1999) and 2-APB on cellular Ca^{2+} influx systems have been reported (Dobrydneva & Blackmore, 2001). Of interest, the proximal routes mediating PLC/IP_3-dependent Ca^{2+} mobilisation appear to be different for β-BA and thrombin (or PAF). Thus, Src family kinase inhibitors abolished the β-BA-induced response, but not the responses elicited by thrombin or PAF. PLC-γ is the most abundant PLC isoform in platelets (Lee *et al*., 1996) and is an operative element in Ca^{2+} mobilisation mediated by adhesion receptors (Rhee, 2001). Whereas, soluble ligands such as thrombin, ADP, PAF, or TXA_2 act *via* GPCRs to stimulate PLC-β isoenzymes, the PLC-γ isoforms are regulated through phosphorylation by Src family kinases (Rhee, 2001). In analogy to agonists that act *via* adhesion receptors but unlike thrombin, β-BA may utilise the Src family kinases/PLC-γ pathway to induce Ca^{2+} mobilisation. Another difference between β-BA- and thrombin-mediated Ca^{2+} mobilisation is

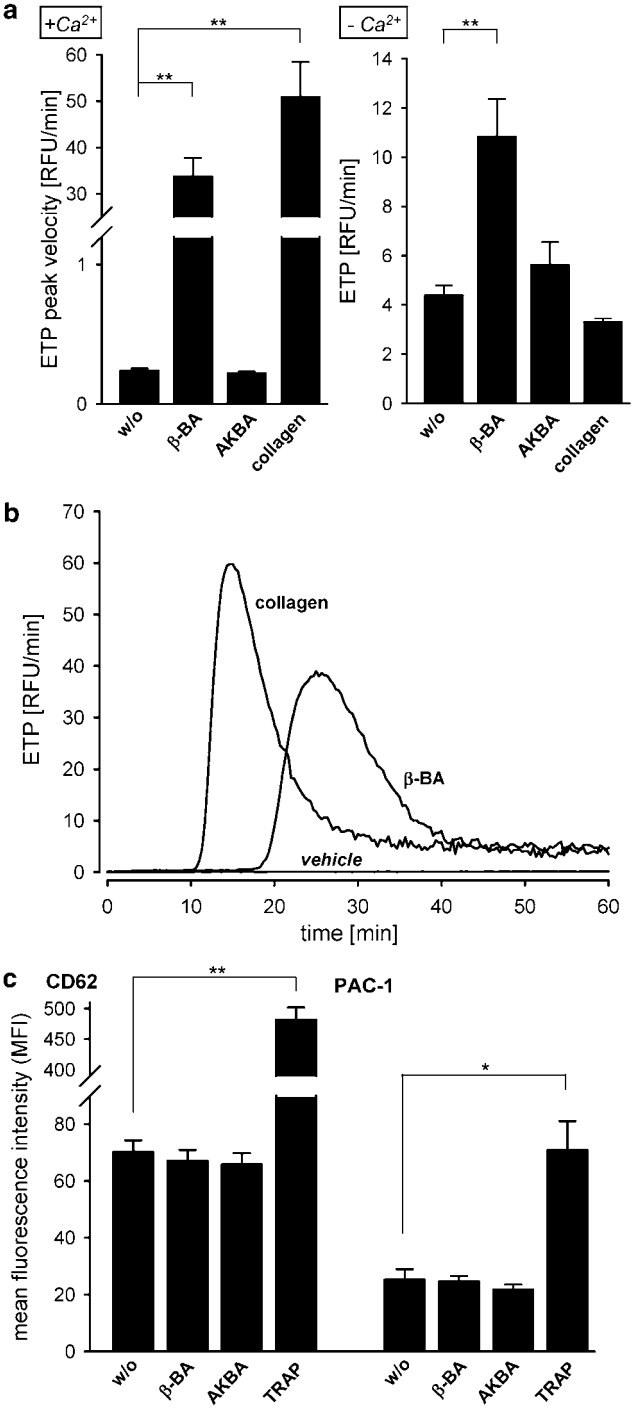

Figure 8 Thrombin generation and activation marker expression. (a) Thrombin generation was assessed in recalcified PRP (given as ETP peak velocity, left bar chart), or citrate-chelated PRP (given as ETP-AUC, right bar chart). PRP and buffer containing the indicated stimuli were added to each well of a 96-well microtitre plate. β-BA (10 μM), AKBA (10 μM), collagen (2 μg ml^{-1}, final concentrations each), and vehicle (DMSO) were tested for their ability to induce thrombin generation. Data are expressed as mean + s.e., $n = 4$ (β-BA, AKBA, collagen) or $n = 8$ (vehicle). One-way ANOVA and Tukey HSD tests were performed, $^{**}P < 0.01$. (b) Representative original traces of the ETP kinetic progression. Cells in recalcified PRP were stimulated as described above. (c) Expression of the platelet activation markers CD62 and PAC-1. Flow cytometry in recalcified PRP was performed as described in the Methods section. Expression of CD62 (left bar chart) and PAC-1 (right bar chart) after stimulation with vehicle (DMSO), β-BA (30 μM), AKBA (30 μM), or TRAP (10 μM) is given. The percentage of CD62-positive cells (%) as well as their mean channel fluorescence intensity (MFI) was determined (left diagram). Right, a histogram of PAC1-FITC against cell events was generated and MFI of total platelet population was recorded., $n = 4$. One-way ANOVA and Tukey HSD tests were performed, $^{*}P < 0.05$ or $^{**}P < 0.01$.

the significant delay of the response to β-BA as compared to the rapid effect of thrombin. Possibly, aside of acting as a direct ligand at a certain (adhesion) receptor, β-BA may first induce the generation of an endogenous agonist that in turn causes PLC-γ/IP_3-coupled Ca^{2+} mobilisation *via* (adhesion) receptors. Attempts to unravel a putative autocrine mode of action are in progress in our laboratory.

Typical platelet agonists such as thrombin, collagen, or TXA_2 activate PI 3-K and its downstream effector Akt, important mediators of agonist-induced platelet activation (Kim *et al*., 2004), as well as p38 MAPK and ERKs (Papkoff *et al*., 1994; Kramer *et al*., 1995; Saklatvala *et al*., 1996). The MAPK are a point of convergence of complex signalling networks, regulating cell proliferation and differentiation (Papkoff *et al*., 1994). In platelets, the functions of MAPK are mainly uncharacterised and the signal transduction steps are poorly understood. All BAs tested activated p38 MAPK with similar efficacy, but only β-BA (and Aβ-BA) rapidly and significantly activated ERK2. Also, β-BA, but not AKBA, evoked Akt phosphorylation, and in analogy to thrombin, the PI 3-K and/or the PLC/Ca^{2+} pathway is involved. Therefore, the receptor for BAs mediating p38 MAPK activation might be different from that transmitting signals to activate ERK2 and Akt. The latter (11-me-BA specific) receptor may also mediate increases in $[Ca^{2+}]_i$, generation of thrombin, release of AA and aggregation, since AKBA and KBA failed to elicit these events.

Investigation of the platelet functions elicited by β-BA provided controversial results. As a rule, the distinct responses of activated platelets depend on the strength (potency) of the agonist, and these responses can be ordered in an activation sequence: (1) aggregation, (2) granule secretion, (3) AA liberation, and (4) acid hydrolase secretion (Steen & Holmsen, 1987). For the induction of these responses, the magnitude of Ca^{2+} mobilisation is an important parameter. In fact, β-BA (10–30 μM) substantially elevated $[Ca^{2+}]_i$ and potently induced thrombin generation, being equipotent in this respect with collagen at 2 μg ml^{-1} in a model utilising native platelets. Also, β-BA potently evoked the liberation of free AA from washed platelets, although at concentrations slightly higher than those required for Ca^{2+} mobilisation, probably due to the presence of fatty acid-free albumin that may bind BAs. In general, liberation of free AA is a response distal of aggregation and degranulation, and its induction normally requires a potent agonist-activating platelets with high strength. Surprisingly, however, the efficacy of β-BA was much reduced for the induction of aggregation. In contrast to collagen, the response of β-BA was strictly dependent on the presence of extracellular Ca^{2+} and was characterised by a prolonged lag phase (4–8 min), a rather slow initial decrease in light transmission, and a submaximal slope of the aggregation curve. This response in some way resembles the 'unspecific' aggregation induced by shear stress (stirring), normally occurring after 12–15 min, in contrast to the rapid (<1 min) signal evoked by a strong agonist (i.e. collagen). Therefore, β-BA may rather facilitate aggregation by other factors than being a full agonist. Moreover, β-BA failed to induce degranulation and fibrinogen receptor activation (CD62, PAC-1 expression). Together, despite the pronounced elevation of $[Ca^{2+}]_i$, only select functional platelet responses were observed after stimulation with β-BA. Along these lines it was found that platelets in polycythaemia vera exhibit decreased aggregation after stimulation with PAF, although an equal increase in $[Ca^{2+}]_i$ was seen as compared to platelets from healthy donors (Le Blanc *et al*., 2000). Also, a patient was described with defective platelet aggregation in response to ionophore A23187, despite normal increases in $[Ca^{2+}]_i$ (Fuse *et al*., 1999). Hence, elevation of $[Ca^{2+}]_i$ in platelets is one important signalling step for eliciting various platelet responses, but must not necessarily lead to the induction of all Ca^{2+}-dependent platelet functions. It is conceivable that β-BA on one hand is a platelet agonist that potently induces central signalling pathways (Ca^{2+} mobilisation, MAPK/Akt phosphorylation) and select responses such as thrombin generation and AA release, but on the other hand lacks the stimulation of certain signalling components or executing molecules particularly important for a rapid aggregation, degranulation, and fibrinogen receptor activation.

At present, our findings cannot be readily related to the anti-inflammatory properties of BS extracts, observed in animal models or in studies with human subjects (Safayhi & Sailer, 1997). Nevertheless, due to its high effectiveness and the importance of the signalling molecules and the select platelet functions induced, the receptor(s) mediating the actions of β-BA in platelets warrant further elucidation. Since the effective concentrations of β-BA (10 μM) are in range of β-BA levels in human plasma (see above), one should be aware of its pharmacological actions on platelets when administering BS extracts to patients.

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Paper III

Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase

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ABSTRACT

Boswellic acids (BAs) inhibit the transformation of arachidonic acid (AA) to leukotrienes via 5-lipoxygenase but can also enhance the liberation of AA in human leukocytes and platelets. Utilizing human platelets, we explored the molecular mechanisms underlying the BA-induced release of AA and the subsequent metabolism by platelet-type 12-lipoxygenase (p12-LO). Both, β-BA as well as 3-*O*-acetyl-11-keto-BA (AKBA) markedly enhanced the release of AA via cytosolic phospholipase (PL)A_2, whereas for generation of 12-hydro(pero)xy-eicosatetraenoic acid (12-H(P)ETE), AKBA was less potent than β-BA and was without effect at higher concentrations (≥ 30 μM). In contrast to thrombin, β-BA-induced release of AA and formation of 12-H(P)ETE was more rapid and occurred also in the absence of Ca^{2+}. The Ca^{2+}-independent release of AA and 12-H(P)ETE production elicited by β-BA was not affected by pharmacological inhibitors of signaling molecules relevant for agonist-induced AA liberation and metabolism. Notably, in cell-free assays, β-BA increased p12-LO catalysis about two-fold in the absence, but not in the presence of Ca^{2+}, whereas AKBA inhibited p12-LO activity. No direct modulatory effects of BAs on $cPLA_2$ activity in cell-free assays were evident. Accordingly, immobilized KBA (linked to sepharose beads) selectively precipitated p12-LO from platelet lysates but failed to bind $cPLA_2$. Taken together, we show that BAs induce the release of AA and the synthesis of 12-H(P)ETE in human platelets by unique, Ca^{2+}-independent routes, and we identified p12-LO as a selective molecular target of BAs.

The pentacyclic triterpenes boswellic acids (BAs) are regarded as the active pharmacological principles of ethanolic extracts of *Boswellia serrata* (B.S.), and there is accumulating evidence for anti-inflammatory and anti-tumorigenic effects of BAs based on experimental cellular and animal models (Anthoni et al., 2006; Poeckel et al., 2006; Safayhi et al., 1992; Syrovets et al., 2005a; Syrovets et al., 2005b; Winking et al., 2000). Attempts to identify the responsible molecular mechanisms and/or receptors revealed a number of proteins that may be targeted by BAs including 5-lipoxygenase (5-LO), human leukocyte elastase, topoisomerases and IκB kinases (Safayhi et al., 1997; Safayhi et al., 1995; Syrovets et al., 2000; Syrovets et al., 2005b). Interaction with these targets may indeed provide a molecular basis for the pharmacological effects observed in animals and human subjects. In particular suppression of leukotriene biosynthesis from arachidonic acid (AA) by inhi-

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ABBREVIATIONS: AA, arachidonic acid; Aβ-BA, 3-O-acetyl-boswellic acid; AKBA, 3-O-acetyl-11-keto-boswellic acid; BA, boswellic acid; BEL, bromoenol lactone; B.S., Boswellia serrata; cPLA2, cytosolic phospholipase A2; 12-H(P)ETE, 12-hydro(pero)xyeicosatetraenoic acid; KBA, 11-keto-boswellic acid; LO, lipoxygenase; MAPK, mitogen-activated protein kinase; p12-LO, platelet-type 12-lipoxygenase; PG buffer, PBS plus 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM CaCl2; PI 3-K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphat; PMNL, polymorphonuclear leukocytes; PLC, phospholipase C; WB, Western-blotting.

bition of 5-lipoxygenase is generally regarded as the most important pharmacological action of BAs accounting for their anti-inflammatory properties (Safayhi et al., 1997; Safayhi et al., 1995).

Many cell types are able to release AA from phospholipids within cellular membranes by the action of specific phospholipases (PL)A_2. AA is an important precursor for a number of highly bioactive metabolites formed by various oxygenases including cyclooxygenases (COX), LOs, and monooxygenases of the CYP family. The 85-kDa cytosolic PLA_2 ($cPLA_2$) has been accounted as responsible enzyme providing free AA as substrate for COX and LOs in leukocytes and platelets (Leslie, 2004). This soluble enzyme is distributed within the cytosol of resting cells and associates upon elevation of intracellular Ca^{2+} and/or phosphorylations of serine residues by members of the MAPK family (Gijon and Leslie, 1999), occurring in response to a number of GPCR agonists. In addition, binding to phosphytidylinositol-4,5-bisphosphate (PIP2) (Balsinde et al., 2000) or ceramide(1-phosphate) (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005) via specific binding-site(s) may promote $cPLA_2$ catalysis.

Exposure of leukocytes or platelets to BAs differentially affects certain signaling pathways and functional responses including Ca^{2+} mobilization, MAPK activation, formation of reactive oxygen species, release of AA and stimulation of 5-LO product formation. Thus, stimulating properties (Altmann et al., 2002; Altmann et al., 2004; Poeckel et al., 2005; Sailer et al., 1998) as well as inhibitory effects (Poeckel et al., 2006; Safayhi et al., 1992; Safayhi et al., 1995; Werz et al., 1998) of BAs have been reported for these functions, depending on the cell type investigated and the respective experimental settings. For example, inhibition of 5-LO by AKBA, IC_{50} values in the range of 1.5 μM (Safayhi et al., 1995) up to 50 μM (Werz et al., 1997; Werz et al., 1998) were determined, but also 5-LO stimulatory effects in this concentration range were described (Altmann et al., 2004; Sailer et al., 1998).

We recently observed that BAs are capable of elevating the release of AA in human isolated polymorphonuclear leukocytes (Altmann et al., 2004) and platelets (Poeckel et al., 2005). Platelets do not express 5-LO, but contain the closely related p12-LO that converts AA to 12-hydro(pero)xyeicosatetraenoic acid (12-H(P)ETE) (Yoshimoto and Takahashi, 2002). Unlike 5-LO, the activity of p12-LO is hardly dependent on co-factors such as ATP, FLAP etc., and in fact upon supply of AA, substantial 12-H(P)ETE formation occurs in the platelet without the absolute requirement of induction of additional signaling pathways. Here we investigated the effects of BAs on the liberation of AA and its subsequent conversion by p12-LO, and we attempted to elucidate the underlying mechanisms and to identify molecular targets of BAs.

Materials and Methods

Materials.

BAs were synthesized and prepared as described (Jauch and Bergmann, 2003). Antibodies against human p12-LO was kindly provided by Dr Colin D. Funk, Kingston, Canada. SB203580, PP2, PP3, SU6656, methyl-arachidonyl-fluorophosphonate (MAFP), bromoenol lactone (BEL), the $cPLA_2\alpha$ inhibitor, and U0126, Calbiochem (Bad Soden, Germany); BAPTA/AM and Fura-2/AM, Alexis (Grünberg, Germany); wortmannin, Biotrend (Köln, Germany); cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), Biomol (Plymouth Meeting, PA); EAH-Sepharose 4B, GE Healthcare Bio-Sciences (Freiburg, Germany); all other chemicals were obtained from Sigma (Deisenhofen, Germany).

Cells.

Platelets were freshly isolated from human venous blood of healthy adult donors (St. Markus Hospital, Frankfurt, Germany) as described (Poeckel et al., 2005). Washed platelets were finally resuspended in PBS pH 7.4 and 1 mg/ml glucose (PG buffer) or in PBS pH 7.4 and 1 mg/ml glucose plus 1 mM $CaCl_2$ (PGC buffer). For incubations with solubilized compounds, ethanol or DMSO was used as vehicle, never exceeding 1 % (vol/vol). For AA release, platelet rich plasma (PRP) was prepared from freshly drawn blood (in 3.13% citrate) from healthy adult donors by centrifugation for 10 min at 750 × g.

Determination of release of [^{3}H]-labeled arachidonic acid from intact platelets.

PRP was labelled with 19.2 nM [^{3}H]AA (1 μCi/ml, specific activity 200 Ci/mmol) for 2 hours at 37°C in the presence of 100 μM aspirin to avoid clotting. Then, cells were washed twice with PBS pH 5.9 plus 1 mM $MgCl_2$, 11.5 mM $NaHCO_3$, 1 g/l glucose, and 1 mg/ml fatty acid-free BSA, and finally resuspended in PG buffer (10^8/ml). Preparation of cells at this pH is thought to minimize temperature-induced activation. Platelets were incubated at 37°C with 1 mM EDTA plus 30 μM BAPTA/AM for 15 min or incubated with $CaCl_2$ (1 mM) for 2.5 min prior stimulation with the indicated agents. After the indicated times, incubations were put on ice for 10 min, followed by centrifugation (5,000 × g, 15 min). Aliquots (300 μl) of the supernatants were measured (Micro Beta Trilux, Perkin Elmer) to detect the amounts of [^{3}H]-labeled AA released into the medium.

Determination of 12-lipoxygenase product formation.

To determine 12-LO product formation in intact cells, freshly isolated platelets (10^8/ml PG buffer) were supplemented with either 1 mM $CaCl_2$, 1 mM EDTA, or 1 mM EDTA plus 30 μM BAPTA/AM. Platelets were preincubated with the indicated agents for 15 min at 37°C. After addition of stimuli and further incubation at 37°C for the times indicated, 12-LO products (12(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (12-H(P)ETE) were extracted and then analyzed by HPLC as described (Albert et al., 2002). 12-HETE plus 12-HPETE elute as one major peak, integration of this peak represents p12-LO product formation, expressed as ng metabolites per 10^8 cells.

For determination of 12-LO product formation in broken cell preparations, platelets (10^8/ml PG buffer) were sonicated (3 × 10 sec) and centrifuged (100,000×g/70 min/4°C). To the resulting 100,000×g supernatant (S100), BAs were added and samples were pre-warmed at 37°C for 30 sec. $CaCl_2$ (2 mM) was added as indicated and 12-LO product formation was started by addition of AA (10 μM). After 10 min at 37°C, the formation of 12-H(P)ETE was determined as described for intact cells.

Immobilization of boswellic acids and protein pull-down assays.

For immobilization of BAs at EAH Sepharose 4B beads, the free 3-OH group of the BAs was used (manuscript in preparation: Kather, N., Tausch, L., Poeckel, D., Werz, O., and Jauch, J. (2006)). In brief, β-BA and KBA were treated with glutaric anhydride to form the half-esters Glut-BA and Glut-KBA, respectively, and analyzed by ^{1}H- and ^{13}C-NMR as well as by MS. These substances were ready for immobilization at EAH Sepharose 4B by standard amide coupling procedures. The carboxylic acid of the BA-core was unlikely to react under standard conditions due to steric crowding. The success of the coupling reaction was determined by two methods: a) Glut-BAs were used in defined excess (2 μmol of the Glut-BAS per 1 μmol NH_2-groups of the EAH Sepharose 4B). After the coupling reaction, the hypothetical excess of Glut-BAs (1

μmol) could be indeed recovered. b) Treatment of Glut-BAs with KOH in *iso*-propanol under reflux for ca. 3 h cleaved the ester bond and gave BA and KBA respectively, analyzed by thin layer chromatography.

For protein pull-down experiments, 10^9 platelets were lysed in 1 ml lysis buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM PMSF, 2 μg/ml leupeptin, 120 μg/ml soybean trypsin inhibitor). After sonication, 3 × 8 s and centrifugation for 10 min at 12.000×g, 50 μl of the sepharose slurries (50 %, vol/vol) were added to supernatants and incubated at 4°C over night under continuous rotation. The Seph-beads were washed 3 times with binding buffer (HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA) and precipitated proteins were finally separated and denatured by addition of SDS-b. After boiling (95°C, 6 min), Seph-beads were removed by centrifugation and the supernatant containing proteins were separated by SDS PAGE and proteins were visualized by Western Blotting (WB) or Coomassie staining, respectively.

Statistics.

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-hoc tests. Where appropriate, Student´s *t* test for paired and correlated samples was applied. A p value of <0.05 (*) or <0.01 (**) was considered significant.

Results

Boswellic acids induce AA release independent of Ca^{2+}

In the presence of extracellular Ca^{2+} (1 mM), β-BA and its 11-keto counterpart AKBA (**fig. 1**) concentration-dependently increased the liberation of AA with significant effects at 10 μM, each (**fig. 2A**). No marked differences in the potencies between β-BA and AKBA were obvious and the efficacy of BAs was comparable to thrombin (2 U/ml) or Ca^{2+}-ionophore A23187 (5 μM) (**fig. 2A**). Intriguingly, when cells were depleted from intracellular (chelation with BAPTA/AM) and extracellular (chelation with EDTA) Ca^{2+}, BAs still exhibited a strong stimulatory effect on AA release with similar efficacies for β-BA and AKBA (**fig. 2B**). Although the absolute levels of AA released into the medium in response to β-BA or AKBA (30 μM, each) were higher in the presence of Ca^{2+}, the relative increases in the absence of Ca^{2+} were more pronounced (4.6- to 5.4-fold), as when Ca^{2+} was present (2.1- and 2.4-fold), which apparently is due to reduced basal AA levels in unstimulated cells where Ca^{2+} has been depleted. However, the release of AA evoked by BAs in the absence of Ca^{2+} was much slower as compared to conditions where Ca^{2+} was present. Note that in Ca^{2+}-depleted cells, thrombin and A23187 were much less active as compared to BAs, but still an about two-fold stimulation over untreated cells was evident (**fig. 2B**). This effect of A23187 is surprising and a plausible explanation can not be provided, but possibly could be caused by Ca^{2+}-independent, unspecific actions on phospholipid membranes. Taken together, BAs are capable to substantially release AA from intact platelets, also in the absence of Ca^{2+}.

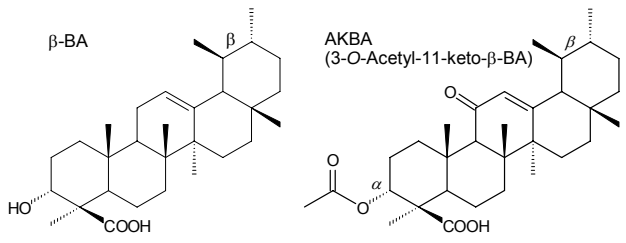

Fig. 1 Chemical structures of β-BA and AKBA

AKBA lacking the 3-O-acetyl group yields KBA; 3-O-acetylation of β-BA results in Aβ-BA.

Effect of BAs on 12-H(P)ETE formation

12-H(P)ETE is a major metabolite of AA in platelets produced by p12-LO (Hamberg and Samuelsson, 1974) that can be easily monitored by RP-HPLC representing a sensitive read out for evaluation of platelet AA metabolism. Washed platelets were incubated with vehicle (DMSO), β-BA and AKBA (30 μM, each), thrombin (2 U/ml), or exogenous AA (10 μM, positive control), either in Ca^{2+}-containing medium or under Ca^{2+}-free conditions (pre-treatment with BAPTA/AM plus EDTA). As shown in **fig. 3A,** β-BA strongly stimulated the formation of 12-H(P)ETE to a comparable level as thrombin. AKBA exerted a much weaker effect than β-BA. In Ca^{2+}-depleted cells, stimulation with thrombin is virtually ineffective, whereas β-BA clearly stimulated 12-H(P)ETE formation, and a minor stimulation was also seen for AKBA (**fig. 3A**). BAs lacking the 11-keto group (β-BA and Aβ-BA) caused a concentration-dependent increase in 12-H(P)ETE formation, whereas BAs containing the 11-keto moiety (KBA and AKBA) were hardly effective, and for AKBA the formation of 12-H(P)ETE was even lower at higher concentrations (**fig. 3B**). Thus, the 11-keto group seemingly hampers the formation of 12-H(P)ETE. A similar pattern was found in Ca^{2+}-depleted cells (not shown). Selective inhibitors of $cPLA_2$ ($cPLA_2\alpha$ inhibitor, 1 μM; MAFP, 10 μM) and 12-LO (CDC, 10 μM) strongly suppressed 12-H(P)ETE formation under all experimental conditions, whereas an inhibitor of the Ca^{2+}-independent $iPLA_2$ (BEL, 5 μM) caused no suppression (**fig. 3C**). In conclusion, both β-BA and AKBA induce the release of AA equally well, but only β-BA but AKBA potently stimulates 12-H(P)ETE formation, which in part is Ca^{2+}-independent.

Kinetic analysis of 12-H(P)ETE formation

The kinetics of 12-H(P)ETE formation in platelets was studied. The time necessary for half maximal 12-H(P)ETE synthesis ($t_{max1/2}$) was determined by regression analysis using a 3-parameter Hill equation ($f(t) = a \times t^b/(c^b + t^b)$). In the

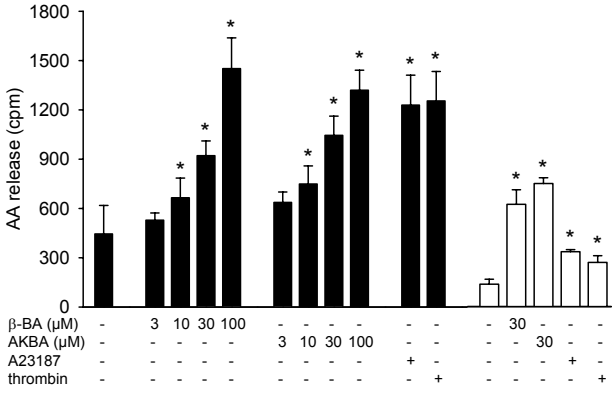

Fig. 2 BAs elevate the liberation of AA in platelets

Platelets (10^8) were incubated at 37°C with 1 mM EDTA plus 30 μM BAPTA/AM for 15 min (white bars) or incubated with $CaCl_2$ (1 mM) for 2.5 min (black bars), and then stimulated with the indicated concentrations of β-BA or AKBA, thrombin (2 U/ml), or ionophore A23187 (5 μM). [^{3}H]AA released into the medium was measured after 5 min in the presence of Ca^{2+} (black bars) or after 15 min in the absence of Ca^{2+} (white bars). Data are given as cpm, mean + S.E., n = 5, $p<0.05$ (*).

presence of Ca^{2+}, β-BA (30 μM) induced a rapid 12-H(P)ETE generation ($t_{max1/2}$ = 37 s) entering a plateau phase after 3 min (**fig. 4A**). A similarly rapid 12-H(P)ETE production was recorded when AA (2 μM, $t_{max1/2}$ = 41 s, **fig. 4B**) or ionophore A23187 (2.5 μM, $t_{max1/2}$ = 44 s, **fig. 4E**), were added to platelets. Importantly, the kinetic profile of thrombin was different and was considerably delayed ($t_{max1/2}$ = 157 s, **fig. 4C**). AKBA (30 μM) gave a less consistent kinetic profile with a $t_{max1/2}$ of ≥ 100 s (**fig. 4D**).

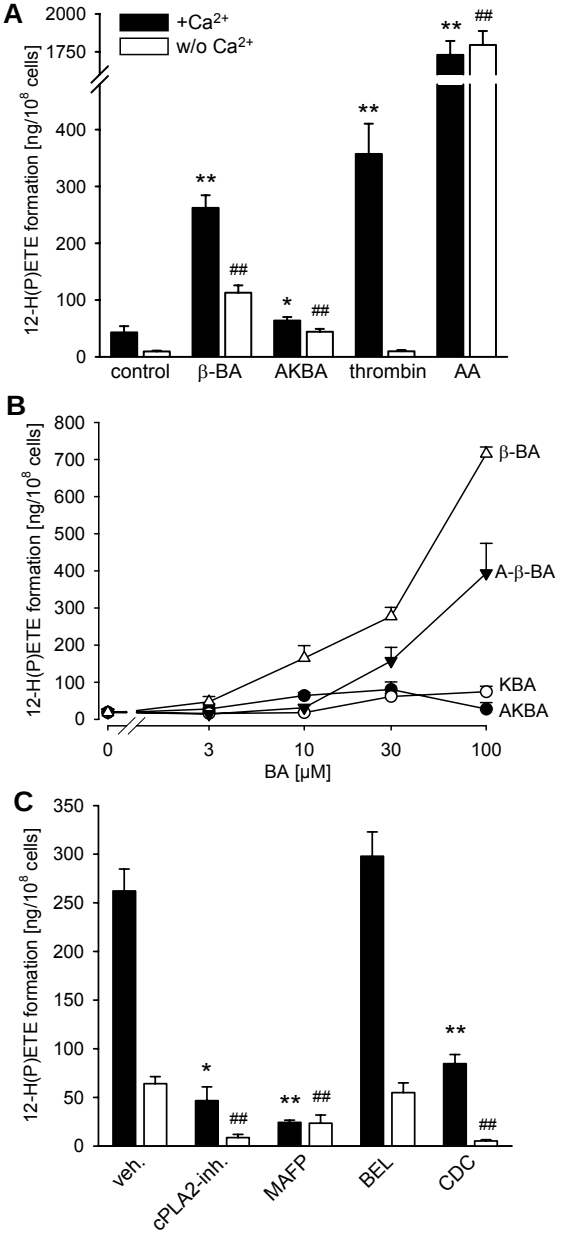

Fig. 3 BAs stimulate the formation of p12-LO and COX-1 products in intact platelets

(**A**) 12-H(P)ETE formation. Platelets (10^8/ml PG buffer) were supplemented with either 1 mM $CaCl_2$ or 1 mM EDTA plus 30 μM BAPTA/AM as indicated. After, 15 min at 37°C, β-BA (30 μM), AKBA (30 μM) thrombin (2 U/ml), AA (10 μM) or vehicle (DMSO) were added and after another 10 min, the reaction was terminated and 12-H(P)ETE formation was determined. (**B**) Concentration-response experiments for 12-H(P)ETE formation. Platelets (10^8/ml PGC buffer) were treated with β-BA, Aβ-BA, KBA or AKBA at the indicated concentrations and after 10 min at 37°C, the reaction was terminated and 12-H(P)ETE formation was determined. (**C**) Effects of p12-LO and PLA_2 inhibitors. Platelets were supplemented with either 1 mM $CaCl_2$ or 1 mM EDTA plus 30 μM BAPTA/AM as indicated and preincubated with CDC (10 μM), MAFP (10 μM), cPLA2α inhibitor (1 μM), and BEL (5 μM). After 10 min β-BA (30 μM) was added and 12-H(P)ETE formation was determined. Data are given as mean + S.E., n = 3-5. *p< 0.05; **, ##p< 0.01.

Lack of extracellular Ca^{2+} (1 mM EDTA) did not strongly alter the kinetic progression of 12-H(P)ETE formation induced by β-BA ($t_{max1/2}$ = 28 s, **fig. 4A**) or by exogenously added AA ($t_{max1/2}$ = 33 s, **fig. 4B**). However, when also intracellular Ca^{2+} was removed by BAPTA/AM, β-BA-, but not AA-induced 12-H(P)ETE formation was remarkably delayed but continuously increased. Long-term kinetic recordings show that whereas the 12-H(P)ETE level in Ca^{2+}-containing buffer gradually decreases after approx. 60 min, it continuously increases up to a plateau after 150 min in Ca^{2+}-depleted cells (**fig. 4F**). When Ca^{2+}-depleted cells were stimulated with thrombin, no detectable increase in 12-H(P)ETE formation was observed (**fig. 4C**). Together, β-BA induces AA liberation/12-H(P)ETE formation in platelets by a rapid Ca^{2+}-mediated pathway as well as by a Ca^{2+}-independent route(s).

Pharmacological dissection of signalling pathways activated by β-BA

The signalling pathways underlying the Ca^{2+}-dependent and Ca^{2+}-independent mechanisms of AA liberation and generation of 12-H(P)ETE were investigated using a pharmacological inhibitor approach. In the presence of Ca^{2+}, increased AA liberation due to β-BA was suppressed by the PI 3-K inhibitor wortmannin (**fig. 5A**). Also, the Src family kinase inhibitors SU6656 and PP2 (but not its inactive variant PP3) reduced the effects of β-BA. In contrast, inhibitors of MEK/ERK (U0126) and p38 MAPK (SB203580) failed in this respect. Note that in Ca^{2+}-depleted cells, no significant suppression of β-BA-evoked AA release by any of the above inhibitors was observed (**fig. 5A**).

In analogy to AA release, also the β-BA-evoked generation of 12-H(P)ETE (**fig. 5B**, left panel) was blocked by wortmannin, SU6656 and PP2 (but not by PP3), whereas U0126 or SB203580 were hardly active. Again, as observed for the release of AA, in the absence of Ca^{2+}, β-BA-evoked 12-LO product formation was not sensitive to any of these inhibitors (**fig. 5B**, left panel). Moreover, in control experiments, where 12-H(P)ETE was elicited by addition of exogenous AA in order to circumvent supply of endogenous substrate, no or only minor effects of the inhibitors were observed, regardless of the presence of Ca^{2+} (**fig. 5B**, right panel). Thus, the inhibitory effects of wortmannin, PP2 and SU6656 on β-BA-induced 12-H(P)ETE generation appear to primarily affect release of AA, rather than p12-LO activity. In conclusion, β-BA-evoked AA release/12-H(P)ETE formation in the presence of Ca^{2+} seemingly involves PI 3-K and Src family kinases, whereas in Ca^{2+}-depleted cells none of these signalling molecules apparently contribute.

Effects of BAs on $cPLA_2$ and p12-LO activity in cell-free assays

To test stimulation of $cPLA_2$ by BAs *in vitro*, we determined the effects of β-BA or AKBA on AA release from platelet membrane lipids in the absence (inclusion of 1 mM EDTA) as well as in the presence of 2 mM Ca^{2+}. AA release was increased by Ca^{2+} by about 2.4-fold, and was suppressed by the $cPLA_2\alpha$ inhibitor, assuring that $cPLA_2$ is the AA-releasing enzyme in this assay. No significant and concentration-dependent modulation of the AA release was observed by 1 to 100 μM BA (not shown), regardless of the

presence of Ca^{2+}, implying that BAs do not stimulate $cPLA_2$ activity *in vitro*. Also, there was no increased association of $cPLA_2$ with platelet membranes after either incubation of platelet homogenates with BAs or after exposure of intact platelets to BAs (assessed by WB, not shown), independent of the presence of Ca^{2+}, suggesting that BAs do not promote binding of $cPLA_2$ to membrane phospholipids *in vitro*.

The effects of BAs on p12-LO activity in the platelet S100 were investigated. Platelet S100 was incubated with AKBA plus 2 μM AA in the presence of either 1 mM EDTA or 1 mM Ca^{2+}. 12-H(P)ETE formation was about 3-fold higher in the presence of Ca^{2+} (**fig 6A**). AKBA caused a concentration-dependent inhibition of p12-LO activity. In the presence of Ca^{2+}, the IC_{50} was about 15 μM, whereas without Ca^{2+} the IC_{50} was approx. 50 μM. In contrast to AKBA, 12-H(P)ETE formation was differentially modulated by β-BA. Thus, only a weak inhibition of p12-LO activity by β-BA (IC_{50} > 100 μM) was detectable in the presence of Ca^{2+} (**fig 6B**). However, in the absence of Ca^{2+}, β-BA elevated 12-H(P)ETE up to approx. 2-fold at a threshold concentration of 10 μM (**fig 6B**), which was sensitive to the p12-LO inhibitor CDC (not shown). Together, β-BA stimulates p12-LO catalysis in the absence of Ca^{2+}, whereas AKBA generally suppresses the catalytic activity of p12-LO, and no direct modulation of $cPLA_2$ is apparent for either BA.

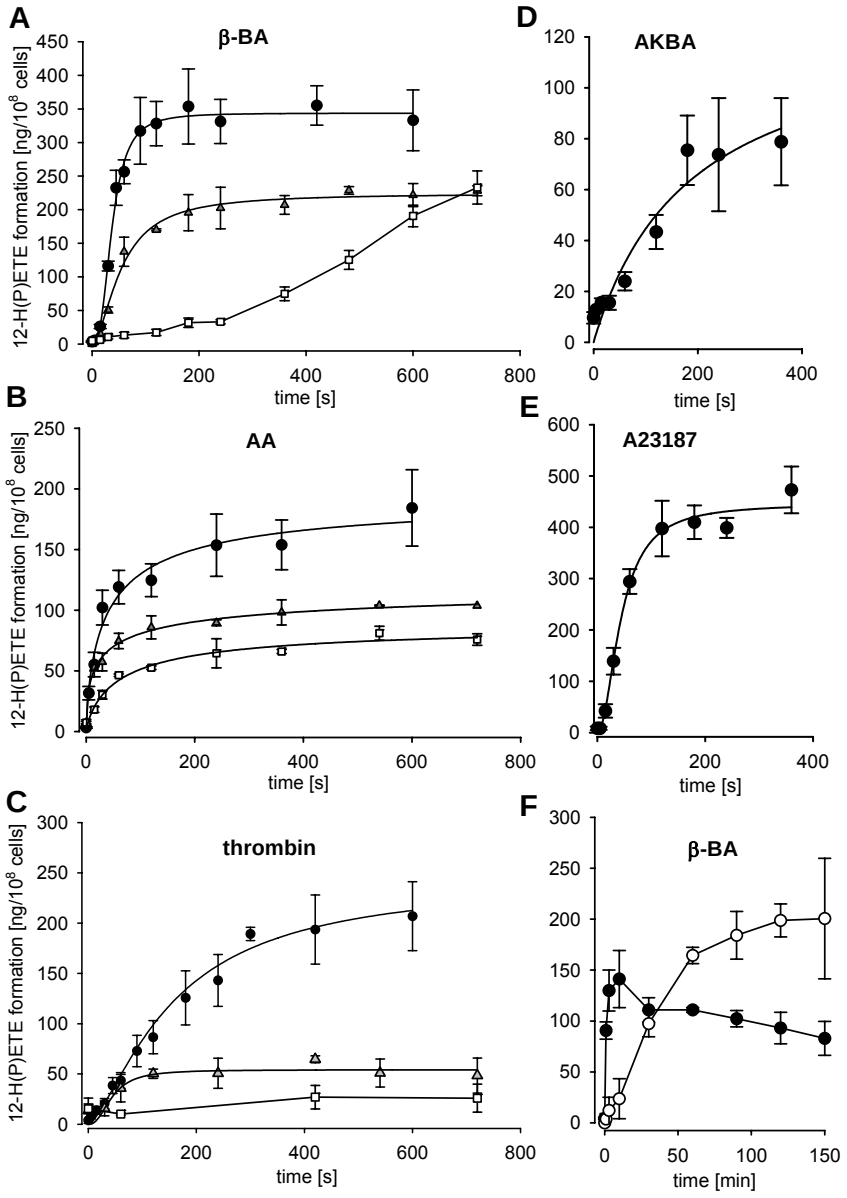

Fig. 4 Kinetics of 12-H(P)ETE formation in intact platelets. Platelets (10^9) were resuspended in 10 ml PG buffer containing either 1 mM $CaCl_2$ (filled circles), 1 mM EDTA (triangles), or 1 mM EDTA plus 30 μM BAPTA/AM (open symbols). After 15 min at 37°C, cells were stimulated with either β-BA (30 μM **A,** and 10 μM **F**), 2 μM AA (**B**), 1 U/ml thrombin (**C**), 30 μM AKBA (**D**), or 5 μM ionophore (**E**). Aliquots of 1 ml corresponding to 10^8 cells were mixed with 1 ml of ice-cold methanol to stop the 12-LO reaction after the indicated times, and 12-H(P)ETE formation was determined. Data are given as mean + S.E., n = 3-5.

Interaction of BAs with $cPLA_2$ and 12-LO

To assess direct interaction of BAs with $cPLA_2$ or p12-LO, a protein fishing assay was performed using KBA as bait that was covalently linked to EAH Sepharose 4B beads via a glutaric acid linker (KBA-Seph). EAH-Sepharose beads without ligand (Seph) were used as control, platelet 12,000×g supernatants served as protein source. Coomassie-staining of gels after SDS-PAGE or Ponceau S staining of membranes after blotting assured comparable unspecific protein-binding by Seph and KBA-Seph (not shown). As shown in **fig. 7**, no $cPLA_2$ protein was detectable (by WB analysis) in precipitates using Seph or KBA-Seph. $cPLA_2$ was abundantly present in the corresponding platelet lysates and clearly detectable. However, substantial amounts of 12-LO were present in KBA-Seph pull-downs, but not in precipitates using Seph as negative control. Since 5-LO was postulated as AKBA-binding protein (Sailer et al., 1998), we attempted to confirm 5-LO binding by our protein fishing strategy using 12,000×g supernatants of PMNL as source for 5-LO. Both Seph and KBA-Seph moderately bound 5-LO, without significant quantitative differences (**fig. 7**). In summary, p12-LO could be selectively precipitated by KBA immobilized to sepharose beads.

Discussion

Activation of platelets by adequate stimuli may lead to substantial release of AA by $cPLA_2$, connected to subsequent conversion by COX-1 and p12-LO, depending on the strength of the stimuli and the nature of the signalling molecules involved (Coffey et al., 2004; Hamberg and Samuelsson, 1974; Holmsen, 1994). Ca^{2+} is a determinant for these processes, as it stimulates cellular activation and catalysis of both $cPLA_2$ (Gijon and Leslie, 1999; Leslie, 2004) and p12-LO (Baba et al., 1989). Besides Ca^{2+}, serine phosphorylations by MAPK (Borsch-Haubold et al., 1999) and interaction with PIP2 (Balsinde et al., 2000) or sphingolipids (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005) activate $cPLA_2$. In contrast, for p12-LO,

there is only limited information regarding cellular activation (Coffey et al., 2004), and except the redox-tone (Bryant et al., 1982), which is of general importance for LO activation, only Ca^{2+} is known as (moderate) stimulatory cofactor (Baba et al., 1989). It is assumed that the capacity of platelets to form 12-H(P)ETE is essentially linked to the supply of AA. Since BAs induce massive mobilization of Ca^{2+} and activate MAPK in platelets (Poeckel et al., 2005), it was reasonable that BAs as a result may elicit release of AA and concomitantly also 12-H(P)ETE synthesis.

β-BA and AKBA evoked AA release with comparable potencies, similar as the strong platelet agonists thrombin or ionophore that act by recruiting $cPLA_2$ via phosphorylation and/or elevation of $[Ca^{2+}]_i$ (Borsch-Haubold et al., 1995; Kramer et al., 1996). The liberation of AA was rapid and sensitive to selective inhibitors of the Ca^{2+}-dependent $cPLA_2$, suggesting that in analogy to thrombin and ionophore, $cPLA_2$ is the responsible PLA_2 isoform. However, in contrast to thrombin and ionophore, BAs may induce $cPLA_2$ activation, at least in part, independent of Ca^{2+}. Note that BEL did not compromise AA release in the absence of Ca^{2+}, which excludes $iPLA_2$ (Hazen et al., 1991) as responsible enzyme. Also, determination of 12-H(P)ETE shows that BAs but not thrombin partially act in a Ca^{2+}-independent manner. AKBA failed to substantially induce 12-H(P)ETE, probably related to its inhibitory action on p12-LO (discussed below). Moreover, our kinetic analysis of cellular 12-H(P)ETE production favours an additional, Ca^{2+}-independent $cPLA_2$/12-LO activation pathway. Thus, 12-H(P)ETE formation induced by β-BA was much more rapid than by thrombin, although increases in $[Ca^{2+}]_i$ by β-BA are delayed as compared to thrombin (Poeckel et al., 2005). Therefore, it is unlikely that the rapid and robust 12-H(P)ETE synthesis induced by β-BA is mediated solely by elevation of $[Ca^{2+}]_i$.

The Ca^{2+}-dependency of $cPLA_2$ in platelets is well established, but alternate signaling routes such as phosphorylation by MAPKs contribute (Borsch-Haubold et al., 1999; Borsch-Haubold et al., 1995; Kramer et al., 1996). In fact, BAs activate MAPK in platelets (Poeckel et al., 2005), however, MAPK inhibitors failed to suppress β-BA-induced AA release and 12-H(P)ETE synthesis. Nevertheless, our inhibitor approach indicate that PI 3-K and Src family kinases may be integrated in β-BA-evoked responses, at least under conditions where Ca^{2+} is present. Since Src family kinases and PI 3-K are also involved in β-

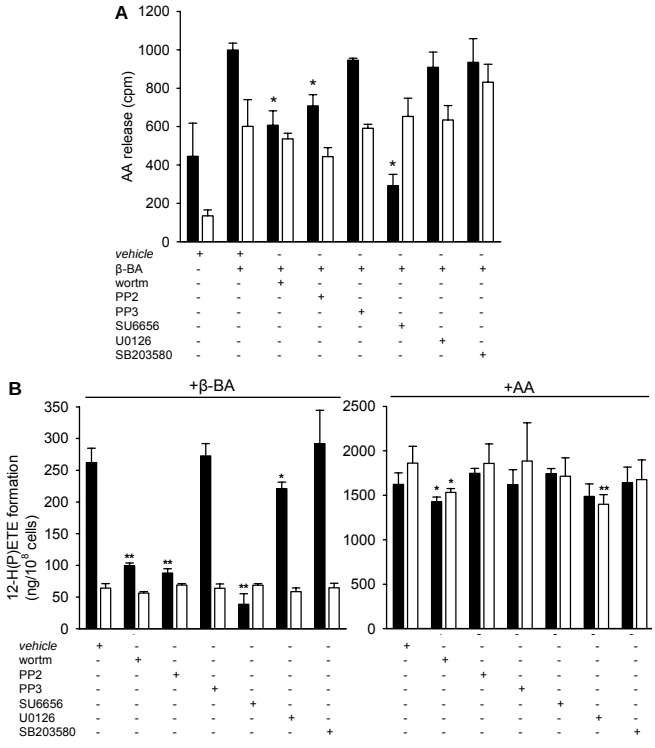

Fig. 5 Effects of relevant pharmacological inhibitors on β-BA-induced AA release and 12-H(P)ETE formation.

(**A**) AA release. Platelets were labelled with [^{3}H]AA as described in the legend of fig. 2. After washing, cells were either left untreated (black bars) or 1 mM EDTA plus 30 μM BAPTA/AM (white bars). Then, cells were preincubated with 200 nM wortmannin (wort), 3 μM PP2 or PP3, 5 μM SU6656, 3 μM U-0126, 10 μM SB203580, or vehicle (DMSO, negative/positive) as indicated for 15 min at 37°C. $CaCl_2$ (1 mM) was added to the cells as indicated and after 2.5 min, cells were stimulated with 30 μM β-BA. [^{3}H]AA released into the medium was measured after 5 min (black bars) or 15 min (white bars), respectively. (**B**) 12-H(P)ETE formation. Platelets were resuspended in 1 ml PG buffer plus 1 mM $CaCl_2$ (black bars) or in PG buffer containing 1 mM EDTA plus 30 μM BAPTA/AM (white bars) and preincubated with 200 nM wortmannin (wort), 3 μM PP2 or PP3, 5 μM SU6656, 3 μM U-0126, 10 μM SB203580 or vehicle (DMSO) as indicated. Then, 30 μM β-BA (left panel) or 2 μM AA (right panel) was added and 12-H(P)ETE formation was determined. Data are expressed as percentage of control (100 %, vehicle) and values are given as mean + S.E., n = 3-4. *p< 0.05; **p< 0.01.

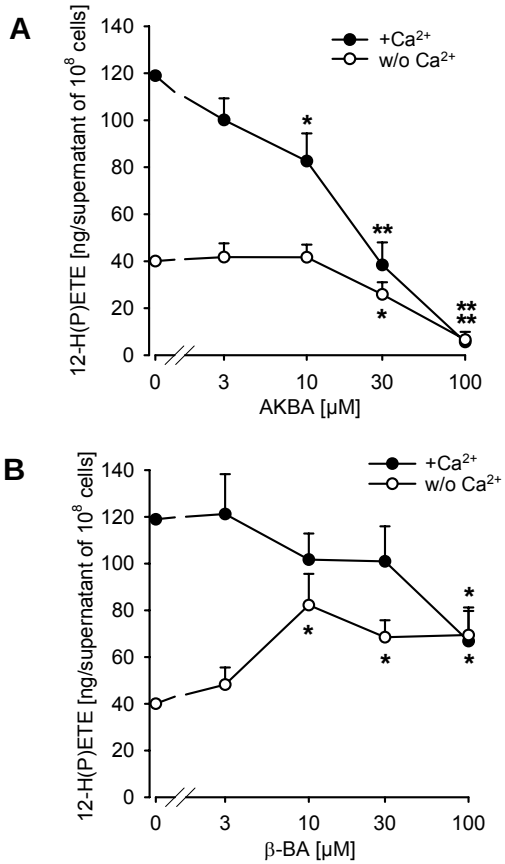

Fig. 6 Effects of BAs on the activity of p12-LO in cell free assays

Platelets were sonicated and S100 was prepared. AKBA (**A**) or β-BA (**B**) were added to the S100 at the indicated concentrations, and the synthesis of 12-H(P)ETE was started by addition of AA (2 μM) with or without 2 mM $CaCl_2$, as indicated. 12-H(P)ETE was determined by HPLC. Data are given as mean + S.E., n = 3-5, *p< 0.05; **p< 0.01.

BA-induced Ca^{2+} mobilisation (Poeckel et al., 2005), the suppressive effects of the respective inhibitors are likely to be due to inhibition of Ca^{2+} mobilisation, rather than uncoupling Ca^{2+}-independent signals to $cPLA_2$. This is supported by the fact that the inhibitors completely failed to suppress β-BA-induced responses in the absence of Ca^{2+}. Moreover, no inhibition of 12-H(P)ETE formation was evident after stimulation with exogenous AA, implying that AA release rather than p12-LO activation is primarily affected by the inhibitors. PI 3-K and Src family kinases have also been implicated in the formation of 12-H(P)ETE from endogenous AA in platelets stimulated by collagen and collagen-related peptide (Coffey et al., 2004).

Apart from Ca^{2+} and phosphorylation, $cPLA_2$ is activated by direct interaction with PIP2 or ceramide and ceramide 1-phosphate (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005), and it appeared possible that also BAs could activate $cPLA_2$ by direct interactions. However, $cPLA_2$ failed to bind KBA-Seph and BAs did not stimulate $cPLA_2$ activity in cell-free assays, excluding such interrelations. Collectively, we conclude that BAs activate $cPLA_2$ independent of Ca^{2+} and phosphorylation by a yet unrecognized mechanism.

Initially, BAs were identified as inhibitors of 5-LO (Safayhi et al., 1992; Safayhi et al., 1995) that may interfere with a regulatory AA-binding site in a Ca^{2+}-dependent manner (Sailer et al., 1998). Among the BAs, AKBA is the most potent 5-LO inhibitor with high selectivity for 5-LO whereas inhibition of p12-LO in intact platelets was excluded (Safayhi et al., 1992). We found that AKBA inhibits p12-LO in cell-free assays with an IC_{50} value (15 μM), significantly below the values determined for 5-LO under comparable assay conditions (50 μM) (Werz et al., 1997; Werz et al., 1998). Of interest, p12-LO bound to KBA-Seph, but was absent in pull-downs using Seph, implying a rather selective interaction between KBA-Seph and p12-LO. Note that the amounts of 5-LO in KBA-Seph and Seph precipitates from 12,000×g supernatants of PMNL lysates were about the same, implying unspecific binding of 5-LO to KBA-Seph.

Direct suppression of p12-LO activity by AKBA may explain why despite induction of marked AA release in intact platelets, no subsequent conversion to 12-H(P)ETE was evident, whereas β-BA (or Aβ-BA) concentration-dependently induced 12-H(P)ETE formation. In agreement with others (Baba et al., 1989), Ca^{2+} increased p12-LO activity in platelet S100 about 3-fold, and β-BA mimicked this effect as it stimulated p12-LO activity without Ca^{2+}. In contrast, in the presence of Ca^{2+}, β-BA did not further stimulate p12-LO. Apparently, the 11-keto moiety determines the quality of p12-LO modulation by BAs, and contrasting effects of BAs depending on the 11-keto moiety were observed before also in other experimental settings (Altmann et al., 2004; Poeckel et al., 2005; Poeckel et al., 2006).

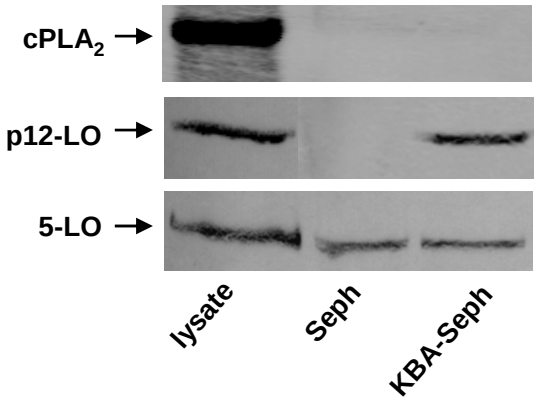

Fig. 7 AKBA selectively binds p12-LO

12,000×g supernatants of platelet lysates (for precipitation of $cPLA_2$ and 12-LO) or PMNL lysates (for 5-LO), were incubated over night at 4°C with either KBA-Seph or with crude Seph. Beads were intensively washed, solubilized by addition of SDS-b and separated by SDS-PAGE. Proteins were visualized by WB using specific antibodies against $cPLA_2$, 12-LO or 5-LO. Aliquots of the corresponding lysates were used as positive controls. Similar results were obtained in three additional experiments.

The conclusions from our results deviate from the long established view of BAs as negative modulatory agents of the AA cascade, as we demonstrate strong induction of AA release and formation of 12-H(P)ETE by BAs in platelets. Also, we suggest p12-LO as a definite target of BAs with superior susceptibility as compared to 5-LO. The question of the pharmacological consequence resulting from the divergent effects of β-BA and AKBA on 12-H(P)ETE biosynthesis *in vivo* remains to be answered. After oral intake of 4 × 786 mg B.S. extracts (containing about 3.7% AKBA, 10.5% Aβ-BA, 6.1% KBA and 18.2% β-BA) per day, the plasma levels of AKBA (0.1 μM) (Buchele and Simmet, 2003) are far below the concentrations required to efficiently suppress p12-LO (IC_{50} = 15 μM). On the other hand, β-BA reached plasma levels (10.1 μM), virtually sufficient to induce 12-H(P)ETE formation. In our *in vitro* assays, relevant amounts (approx. 5 μg/ml) of B.S. extracts, containing diverse BAs, strongly induced AA release as well as 12-H(P)ETE synthesis (not shown). 12-H(P)ETE may act as chemoattractant for leukocytes (Goetzl, 1980), mediates angiogenesis and tumour metastasis (Honn et al., 1994), possesses inhibitory neuromodulatory effects (Piomelli et al., 1987) and is involved in cardiovascular diseases (Gonzalez-Nunez et al., 2001), which should be taken into account when administering BA-containing medicine. Besides the dissection of the influences of BAs on 12-H(P)ETE as mediator in (patho-)physiology applied as complex composed extracts of *B.S.*, it also remains a future challenge to fully elucidate the Ca^{2+}/phosphorylation-independent signalling routes leading to $cPLA_2$ activation and increased release of AA by BAs.

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Paper IV

Manuscript

Immobilization of Boswellic acids at EAH Sepharose™ for "target fishing"

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Key words

Natural products / Terpenoids / Immobilization / Sepharose™ / X-Ray Analysis

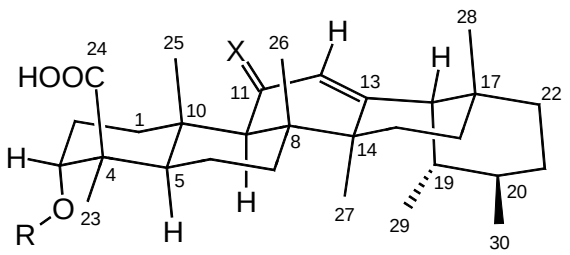
Abstract

Boswellic acids are known as potent anti-inflammatory drugs. In order to identify their molecular target proteins, a "target fishing" approach is suitable where boswellic acids are immobilized at Sepharose™ as bait. Here, we present the chemical part of the project: synthesis of glutaroyl-boswellic acids and immobilization to EAH Sepharose™.

Introduction

Chronic inflammatory diseases[1] like rheumatoid arthritis, chronic bronchitis, asthma, chronic inflammatory bowel diseases (ulcerative colitis and Crohn's Disease) as well as chronic inflammatory skin diseases (e.g. neurodermitis) are a major challenge for medical science. The Indian folk medicine *Ayurveda* treats these diseases with extracts of frankincense from *Boswellia serrata*, which is also used in Western medicine with great success.[2]

Ethanolic extracts of *Boswellia serrata* contain –among others– the pentacyclic triterpenes boswellic acids (BAs),[3] which are regarded as pharmacological principles of the resins of *Boswellia serrata* and other *Boswellia* species. The chemical structure of the *β*-boswellic acids are shown in Fig. 1.



R = H;	X = H, H;	BA	**1a**
R = Ac;	X = H, H;	ABA	**1b**
R = H;	X = O;	KBA	**1c**
R = Ac;	X = O;	AKBA	**1d**

Figure 1. Structures of the four *β*-boswellic acids found in incense (major components); Abbreviations: BA: boswellic acid; ABA: 3-Acetoxy-boswellic acid; KBA: 11-Keto-boswellic acid; AKBA: 3-Acetoxy-11-keto-boswellic acid

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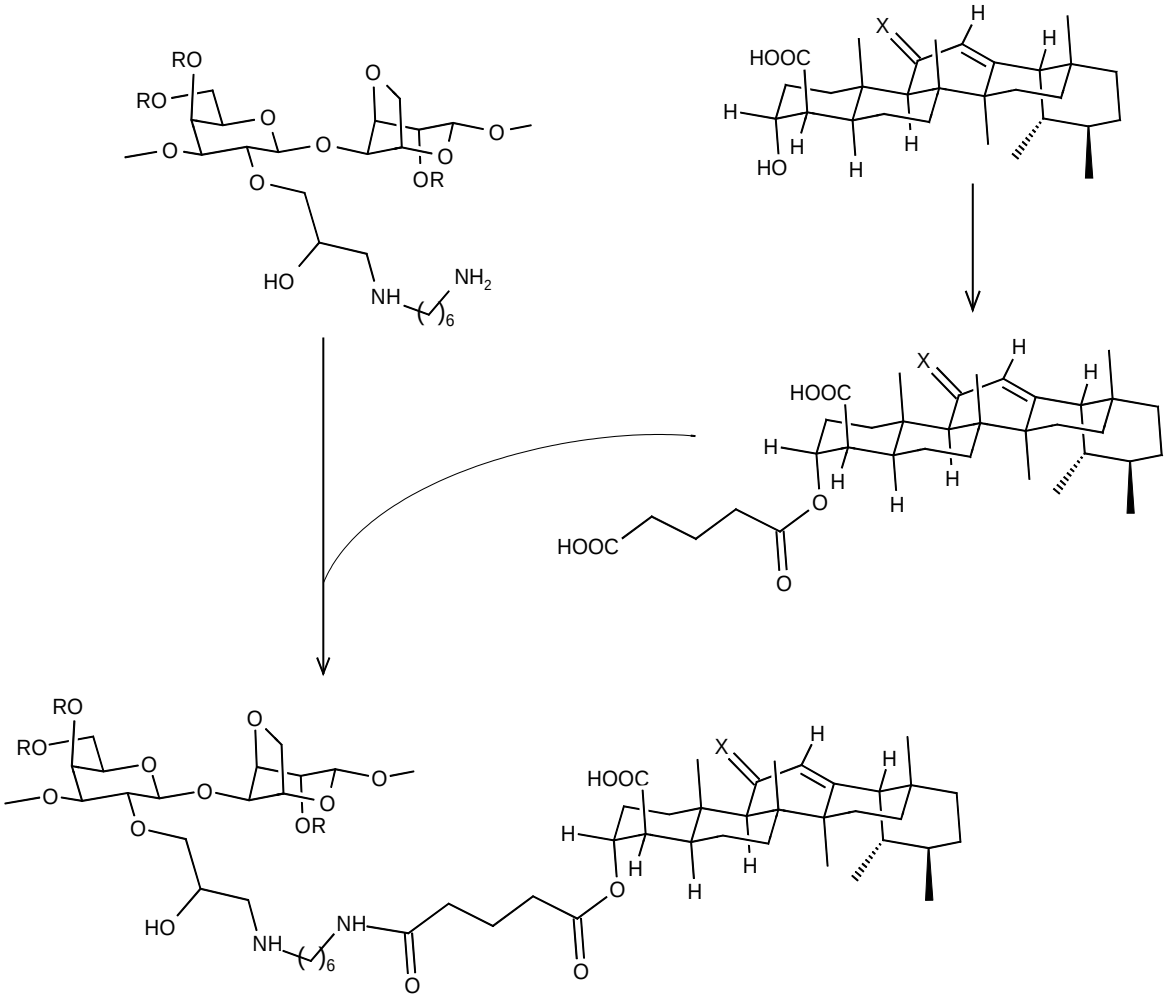
Although anti-inflammatory and anti-tumorigenic effects of BAs have been stated in several reports,[4] the underlying molecular mechanisms and in particular the molecular targets of BAs are still elusive. Nevertheless, a number of proteins related to inflammation and/or cancer including 5-lipoxygenase (5-LO), human leukocyte elastase, topoisomerases and IκB kinases have been postulated as targets of BAs.[4c, 4d, 5] However, there is only limited and indirect proof for direct binding of BA to these proteins. One approach that can be utilized for fishing of target proteins using small molecular weight compounds (ligands) as bait is the immobilization of the ligand to an insoluble biocompatible resin via a linker molecule.[6] So, in order to identify target proteins of BAs, we had to immobilize BAs connected to an appropriate linker to a suitable resin.

Here, we wish to report our synthetic results towards this end.



Scheme 1. Plan of immobilizing boswellic acids to EAH Sepharose for target fishing.

Results and Discussion

As biocompatible resin we chose EAH Sepharose 4B™ (Scheme 1), since this agarose-based resin is quite often used in gel filtration and affinity chromatography and does not denaturate any proteins and enzymes.[7] For the immobilization of BAs we decided to couple the free 3-OH group of BA and KBA with glutaric anhydride (Scheme 1) to obtain the glutaric acid half esters of BA and KBA respectively, which still have one primary carboxyl group useful to build an amide with the amino group of EAH Sepharose 4B™ (Scheme 1).

First, we had to prepare glutaroyl-KBA **2** and glutaroyl-BA **3**, respectively. Neither of these substances is known. However, some derivatives of glycyrrhetinic acid and steroids with succinyl linker are known[8]. Treatment of KBA **1c** with glutaric anhydride in pyridine as solvent was very sluggish at reflux temperature using catalytic amounts of DMAP[9]. Switching to 4-pyrrolidino-pyridine[10] as catalyst accelerated the reaction, but reaction times still were in the range of approx. 20 hours (h). Using 1.5 equivalents of 4-pyrrolidino-pyridine lowered the reaction time to acceptable 7 h with 75% yield of **3**. Glutaroyl-KBA **3** fortunately crystallized from acetone and yielded suitable crystals for X-ray analysis. The molecular structure is shown in Fig. 2.

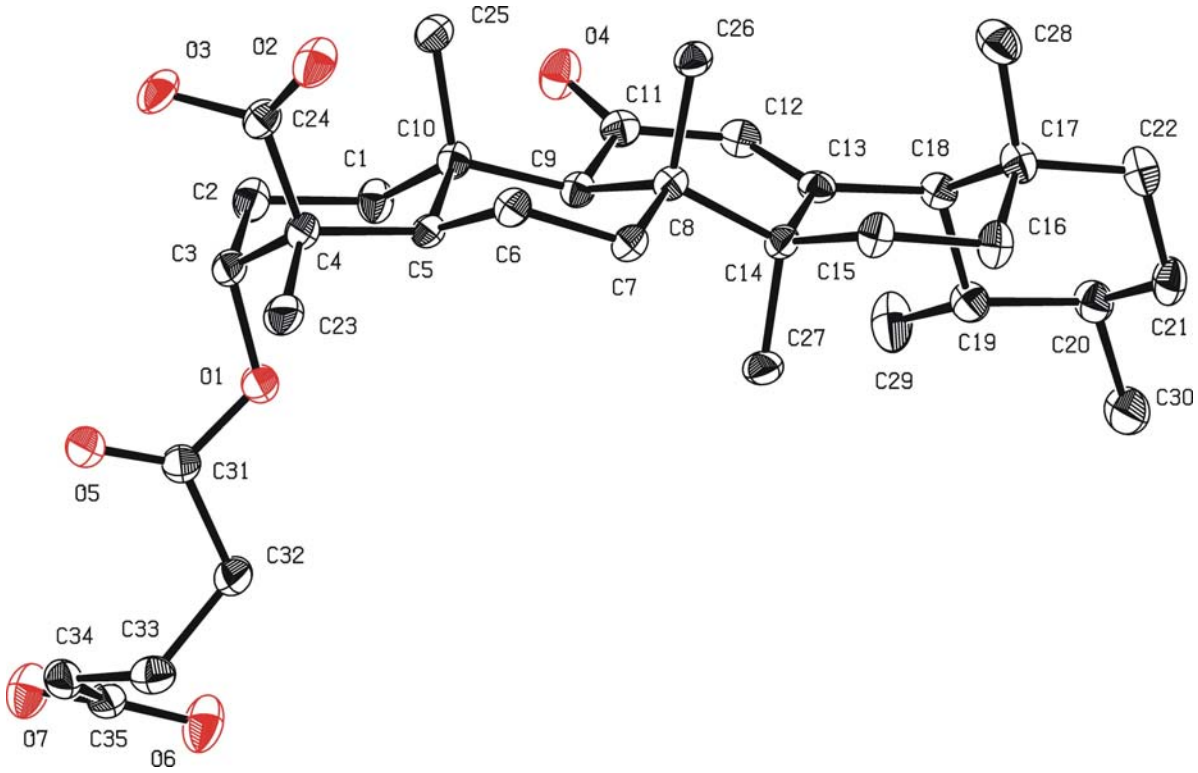


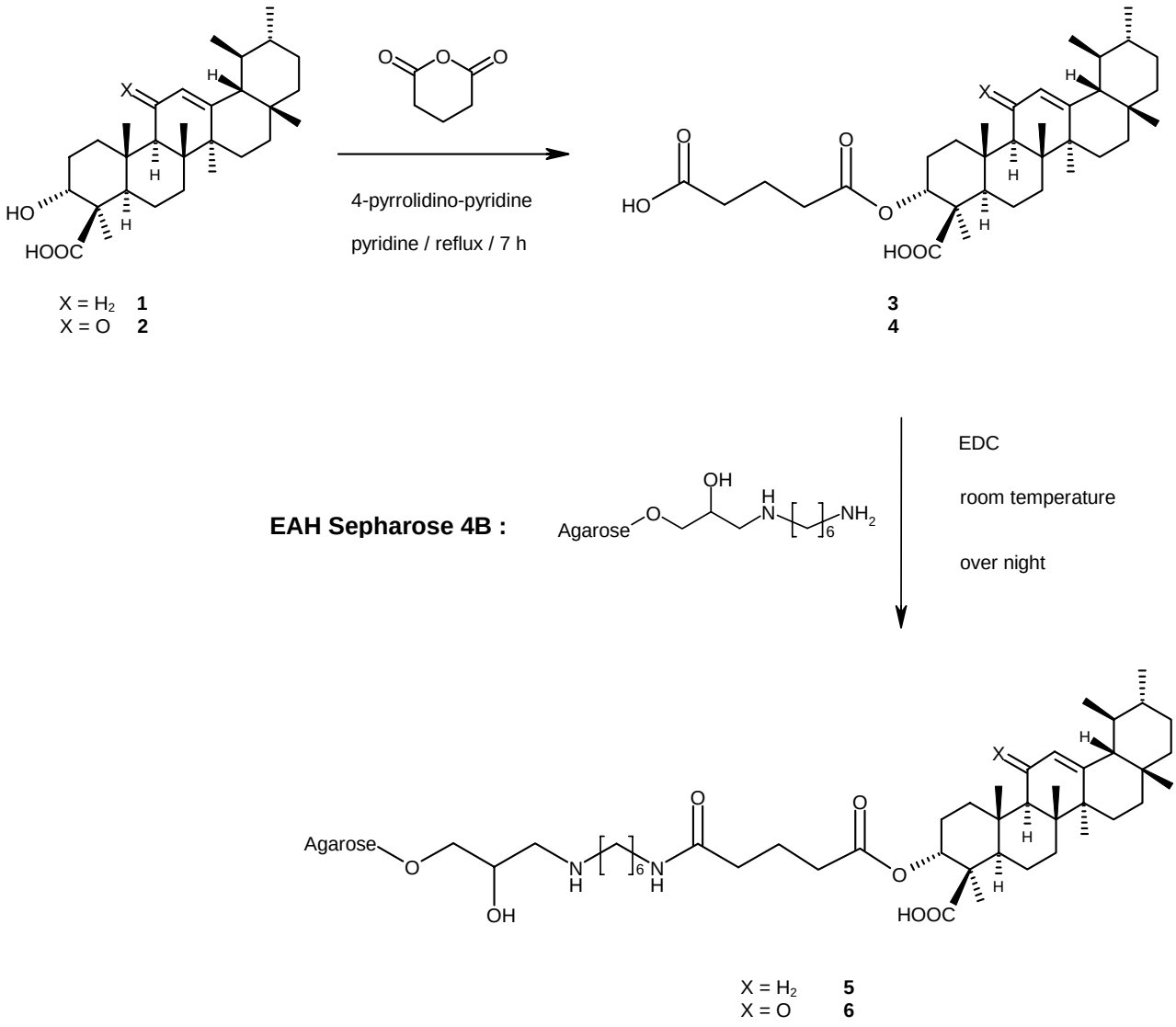
Figure 2. ORTEP style plot of glutaroyl-KBA **3** in the solid state. Thermal ellipsoids are drawn at the 50% probability level. Crystal acetone and hydrogen atoms are omitted for clarity.

Treatment of BA **1a** under the conditions developed for the reaction of KBA with glutaric anhydride yielded glutaroyl-BA **2** in 76% yield.

With glutaroyl-BA **2** and glutaroyl-KBA **3** in hand, we tried to couple these substances to EAH sepharose™. Usually, this coupling is done with EDC in aqueous solution, acidified to pH 4.5, as recommended by the supplier of EAH sepharose™. Unfortunately, glutaroyl-BA as well as glutaroyl-KBA are poorly soluble in water. In cases where the substance to be coupled to sepharose is hardly soluble in water, dioxane can be used as cosolvent up to a final concentration of 50% (v/v). Even in water/dioxane 50/50 (v/v), the glutaroyl-BA **2** and glutaroyl-KBA **3** are not completely soluble in the concentration required for efficient coupling. Using a larger amount of dioxane gave a homogenous solution with **2** and **3**, but coupling did not occur and the glutaroyl-BAs could be recovered quantitatively. So, we had to run the coupling of glutaroyl-BA **2** and

glutaroyl-KBA **3** under heterogeneous conditions, where the glutaroyl-BAs dissolve only partially at the beginning of the reaction and the remaining residue dissolves during the reaction as glutaroyl-BAs are coupled to the sepharose beads.

The complete synthesis is shown in scheme 2.



Scheme 2. Synthesis of glutaric acid half esters of boswellic acids and immobilization thereof at EAH Sepharose 4B™.

Characterization of the immobilized BAs by spectroscopic methods is difficult and not informative. Therefore, we decided to determine the turnover of the coupling reaction as follows: as we used two equivalents of glutaroyl-BAs for coupling to one equivalent of EAH sepharose, the not consumed excess of glutaroyl-BAs should be recovered. This is indeed the case (see experimental part). Additionally, cleavage of the immobilized BAs with KOH/*i*PrOH/reflux was also successful.

In biochemical studies ([11] and ongoing investigations) it is attempted to pull down proteins that specifically bind to the immobilized BAs. Such protein pull-downs can be separated by gel electrophoresis and after comparison with pull-downs utilizing crude EAH sepharose beads (lacking BAs as bait, negative control), the respective proteins are then selected and identified by MALDI mass spectrometry.

Experimental Section

General Remarks:

Peroxides in dioxane were removed by filtration through basic aluminium oxide. Reactions with dioxane as solvent were run under N_2 to prevent peroxide formation. Flash chromatography is performed with silica gel from Merck, Darmstadt, Germany, with particle size 40-63 µm (normal phase) and with RP18 silica gel prepared according to the literature. For the complete assignment of the NMR-signals, ^{1}H-spectra, ^{13}C-spectra, DEPT90- and DEPT135-spectra, H,H-COSY, HMQC, HMBC, HMQC-COSY and NOESY-spectra (Bruker AV 500) were processed with the program MestReC.[12] Mass spectra were recorded on a Bruker MAT 95S.

EAH Sepharose 4B™ was purchased from Amersham Biosciences, Freiburg, Germany, and EDC was purchased from Fluka, Taufkirchen, Germany. Boswellic acids were synthesized as described previously.[13]

Glutaroyl-β-boswellic acid 2: *β*-boswellic acid (742.3 mg, 1.6 mmol) was dissolved in dry pyridine (16 ml). To this solution glutaric anhydride (1.826 g, 16 mmol) and 4-pyrrolidino-pyridine (336.0 mg, 2.3 mmol) were added successively. The reaction mixture was heated to reflux for 7 h. After cooling to room temperature, the brown mixture was diluted with diethyl ether (ca. 100-200 ml) and washed with 1 N HCl (3 x 100 ml). The combined HCl-solution was re-extracted once with diethyl ether (ca. 100 ml). The combined organic phases were washed with water to remove traces of HCl and with brine (ca. 50 ml) and finally dried ($MgSO_4$). After evaporating the solvent, the orange-brown residue (ca. 800 mg) was chromatographed on silica gel and eluted with pentane / diethyl ether 2:1 (v/v) + 1% HOAc. Yield: 694.0 mg (75%) as a white powder. Crystals could be obtained from chloroform by slowly evaporating the solvent.

^{1}H-NMR ($(CD_3)_2CO$, 500.13 MHz, δ in ppm): δ = 5.30 (t, *J* = 2.4 Hz, 1H, H-3); 5.20 (t, *J* = 3.4 Hz, 1H, H-12); 2.46 (t, *J* = 7.4 Hz, 2H, H-34); 2.40 (t, *J* = 7.4 Hz, 2H, H-32); 2.21-2.14 (m, 1H, H-2*β*); 2.11-2.07 (m, 1H, H-16*α*); 2.00-1.86 (m, 6H, H-6*β*, H-11*α*, H-11*β*, H-15β, H-33); 1.78-1.75 (m, 1H, H-6*α*); 1.70-1.67 (m, 1H, H-9); 1.64-1.58 (m, 2H, H-2*α*, H-7*α*); 1.55-1.49 (m, 2H, H-1*β*, H-5*α*); 1.47-1.26 (m, 8H, H-1*α*, H-7*β*, H-18, H-19, H-21*α*, H-21*β*, H-22*α*, H-22*β*); 1.23 (s, 3H, H-23); 1.15 (s, 3H, H-27); 1.09 (s, 3H, H-26); 1.07-1.02 (m, 1H, H-15*α*); 0.98 (s, 3H, H-25); 0.94-0.89 (m, 5H, H-16*β*, H-20, H-30); 0.84 (s, 3H, H-28); 0.84 (d, *J* = 5.8 Hz, 3H, H-29).

^{13}C-NMR ($(CD_3)_2CO$, 125.76 MHz): δ = 179.0 (C-24, COOH); 175.1 (C-35, COOH (glutaroyl)); 173.6 (C-31, >C=O (glutaroyl)); 141.4 (C-13, H>C=C<); 126.6 (C-12, -HC=C<); 75.0 (C-3, HO-CH-); 61.1 (C-18, >CH-); 52.3 (C-5, >CH-); 48.7 (C-9, >CH-); 48.2 (C-4, >C<); 44.0 (C-8, >C<); 43.3 (C-22, -CH_2-); 41.9 (C-14, >C<); 41.5 (C-19, >CH-); 41.4 (C-20, >CH-); 39.1 (C-10, >C<); 36.5 (C-1, -CH_2-); 35.5 (C-17, >C<); 35.1 (C-34, -CH_2- (glutaroyl)); 34.9 (C-7, -CH_2-); 34.3 (C-32, -CH_2- (glutaroyl)); 32.9 (C-21, -CH_2-); 30.3 (C-28, -CH_3); 29.8 (C-16, -CH_2-); 28.3 (C-15, -CH_2-); 25.2 (C-23, -CH_3); 25.4 (C-2, -CH_2-); 25.1 (C-11, -CH_2-); 24.7 (C-27, -CH_3); 22.7 (C-30, -CH_3, 22.1 (C-33, -CH_2 (glutaroyl)); 21.6 (C-6, -CH_2); 18.9 (C-29, -CH_3); 18.4 (C-26, -CH_3); 14.9 (C-25, -CH_3).

MS (EI, 70 eV): m/z = 570 (8, [M^+]); 471 (8); 454 (12); 438 (28); 423 (19); 394 (25); 379 (15); 218 (100); 203 (33); 189 (22).

HRMS (EI, 70 eV): calc.: 570,3982 for $C_{35}H_{54}O_6$; obs.: 570,3951

Glutaroyl-11-keto-β-boswellic acid 3: same procedure as for **2**.

^{1}H-NMR ($(CD_3)_2CO$, 500.13 MHz, δ in ppm): δ = 5.49 (s, 1H, H-12); 5.29 (t, *J* = 2.6 Hz, 1H, H-3); 2.53-2.48 (m, 2H, H-1*β*, H-9); 2.44 (t, *J* = 7.4 Hz, 2H, H-34); 2.39 (t, *J* = 7.4 Hz, 2H, H-32); 2.27-2.16 (m, 2H, H-2*β*, H-16*α*); 1.97-1.88 (m, 4H, H-6*β*, H-15*β*, H-33); 1.80-1.74 (m, 2H, H-6*α*, H-7*α*); 1.61-1.43 (m, 7H, H-2*α*, H-5*α*, H-7*β*, H-18, H-19, H-21*β*, H-22*α*); 1.40-1.36 (m, 5H, H-21*β*, H-22*α*, H-27); 1.33-1.25 (m, 2H, H-1*α*,

H-15α); 1.23 (s, 3H, H-23); 1.21 (s, 3H, H-26); 1.18 (s, 3H, H-25); 1.07-1.02 (m, 1H, H-16β); 0.98-0.94 (m, 4H, H-20, H-30); 0.86 (s, 3H, H-28); 0.83 (d, J = 6.4 Hz, 3H, H-29).
^{13}C-NMR ($(CD_3)_2CO$, 125.76 MHz, δ in ppm): δ = 199.8 (C-11, >C=O); 178.8 (C-24, COOH); 175.1 (C-35, COOH (glutaroyl)); 173.7 (C-31, >C=O (glutaroyl)); 165.8 (C-13, H>C=C<); 132.2 (C-12, H>C=C<); 74.9 (C-3, HO>CH-); 62.0 (C-9, >CH-); 60.8 (C-18, >CH-); 52.0 (C-5, >CH-); 48.1 (C-4, >C<); 46.7 (C-8, >C<); 45.5 (C-14, >C<); 42.7 (C-22, -CH_2-); 41.1 (C-19, >CH-); 40.9 (C-20, >CH-); 39.2 (C-10, >C<); 36.4 (C-1, -CH_2-); 35.7 (C-17, >C<); 35.1 (C-34, -CH_2- (glutaroyl)); 34.5 (C-7, -CH_2-); 34.3 (C-32, -CH_2- (glutaroyl)); 32.6 (C-21, -CH_2-); 30.2 (C-28, -CH_3); 29.2 (C-16, -CH_2-); 28.9 (C-15, -CH_2-); 25.3 (C-23, -CH_3); 25.2 (C-2, -CH_2-); 22.4 (C-30, -CH_3); 22.1 (C-33, -CH_2- (glutaroyl)); 21.9 (C-27, -CH_3); 21.9 (C-6, -CH_2-); 18.7 (C-29, -CH_3); 19.9 (C-26, -CH_3); 14.8 (C-25, -CH_3).
MS (EI, 70 eV): m/z = 584 (4; [M+]); 452 (15); 408 (18); 273 (19); 232 (39); 228 (60); 182 (100)
HRMS (EI, 70 eV): calc.: 584,3713 for $C_{35}H_{52}O_7$; obs.: 584,3713

Immobilization of glutaroyl-β-boswellic acid at EAH Sepharose 4B: First, EAH Sepharose 4B (10 ml) was washed with 0.5 M aqueous NaCl (800 ml) in a sintered glass filter and drained. Second, distilled water (100 ml) was acidified to pH 4 by dropwise addition of 1 N HCl. Additionally, dioxane was added (100 ml) with stirring. This dioxane/water-mixture was used as reaction medium and as solvent to wash the product. Glutaroyl-β-boswellic acid (102.7 mg, 0.18 mmol) was dissolved in the dioxane/water mixture (15 ml) by vigorous shaking or stirring, giving a slightly turbid emulsion. This is added to the drained EAH-Sepharose 4B with shaking (stirring with a magnetic stirring bar will destroy the Sepharose beads). Next, EDC (354.1 mg, 1.85 mmol) was dissolved in the dioxane/water mixture (5 ml) and this solution was added dropwise to the reaction mixture. Now, the complete reaction mixture was shaken over night. The solvent was stripped off * (sintered glass filter) and the drained glutaroyl-BA-Sepharose was washed with dioxane/water * (3 x 80 ml), with water (3 x 80 ml) and finally with 1M aqueous NaCl, which contained 20 vol.% ethanol (3 x 80 ml). The product was stored as suspension in the above mentioned NaCl solution (ca. 5-10 ml).

*These combined filtrates were evaporated to remove most of the dioxane and then re-extracted with diethyl ether. The etheral phase was dried with $MgSO_4$ and evaporated to leave 49.8 mg of glutaroyl-β-boswellic acid. So, approx. 53 mg of glutaroyl-β-boswellic acid (0.093 mmol) were immobilized at Sepharose. Sepharose contains 7-10 μmol NH_2 groups per ml, i.e. immobilization is almost quantitative.

Immobilization of glutaroyl-11-keto-β-boswellic acid at EAH Sepharose 4B: same procedure as above.

Single crystal X-ray structure determination of glutaroyl-KBA 3: Crystal data and details of the structure determination are presented in Table 3. Suitable single crystals for the X-ray diffraction study were grown from acetone. A clear colourless fragment was stored under perfluorinated ether, transferred in a Lindemann capillary, fixed, and sealed. Preliminary examination and data collection were carried out on an area detecting system (NONIUS, MACH3, κ–CCD) at the window of a rotating anode (NONIUS, FR591) and graphite monochromated MoK_α radiation (λ = 0.71073 Å). The unit cell parameters were obtained by full-matrix least-squares refinement of 3285 reflections. Data collection were performed at 123 K (OXFORD CRYOSYSTEMS) within a Θ-range of 1.77°< Θ < 25.28. Nine data sets were measured in rotation scan modus with $\Delta\varphi$ /$\Delta\Omega$ = 1.0. A total number of 40471 intensities were integrated. Raw data were corrected for Lorentz, polarization, and, arising from the scaling procedure, for latent decay and absorption effects. After merging (R_{int} = 0.035) a sum of 6340 (all data) and 6155 [$I>2\sigma(I)$], respectively, remained and all data were used. The structure was solved by a combination of direct methods and difference Fourier syntheses. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atom positions were found in the difference Fourier map calculated from the model containing all non-hydrogen atoms. The

hydrogen positions were refined with individual isotropic displacement parameters. Full-matrix least-squares refinements with 647 parameters were carried out by minimizing $\Sigma w(F_o^2-F_c^2)^2$ with SHELXL-97 weighting scheme and stopped at shift/err < 0.001. The final residual electron density maps showed no remarkable features. The correct enantiomere is given by synthesis. Neutral atom scattering factors for all atoms and anomalous dispersion corrections for the non-hydrogen atoms were taken from *International Tables for Crystallography*. All calculations were performed on an Intel Pentium II PC, with the STRUX-V system, including the programs PLATON, SIR92, and SHELXL-97[14].

Table 3. Summary of the crystal data and details of data collection and refinement for glutaroyl-KBA.

Empirical formula	$C_{35}H_{52}O_7$, C_3H_6O
Formula mass	642.84
Crystal system	monoclinic
Space group	$P2_1$ (no. 4)
a [Å]	8.0205(1)
b [Å]	18.8900(1)
c [Å]	11.8491(1)
β [°]	103.2313(3)
V [Å^3]	1747.57(3)
Z	2
ρ_{calcd} [g cm^{-3}]	1.222
μ [mm^{-1}]	0.084
T [K]	123
F(000)	700
Crystal size [mm]	0.51×0.53×0.56
Θ-range [°]	1.77/25.28
Index ranges	h:±9/k:±22/l:±14
Reflections collected	40471
Independent reflections [I_o>2σ(I_o)/all data/R_{int}]	6155/6340/0.035
Data / restraints / parameters	6340/1/647
$R1$ [I_o>2σ(I_o)/all data]	0.0270/0.0282
$wR2$ [I_o>2σ(I_o)/all data]	0.0665/0.0675
GOF	1.041
Weights a/b	0.0373/0.2932
$\Delta\rho_{max/min}$ [e·Å^{-3}]	0.17/-0.16

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3-*O*-Acetyl-11-keto-boswellic Acid Decreases Basal Intracellular Ca^{2+} Levels and Inhibits Agonist-Induced Ca^{2+} Mobilization and Mitogen-Activated Protein Kinase Activation in Human Monocytic Cells

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ABSTRACT

Previously, we showed that 11-keto-boswellic acid and 3-*O*-acetyl-11-keto-BA (AKBA) stimulate Ca^{2+} mobilization and activate mitogen-activated protein kinases (MAPKs) in human polymorphonuclear leukocytes (PMNLs). Here, we addressed the effects of boswellic acids on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and on the activation of $p38^{MAPK}$ and extracellular signal-regulated kinase (ERK) in the human monocytic cell line Mono Mac (MM) 6. In contrast to PMNLs, AKBA concentration dependently (1–30 μM) decreased the basal $[Ca^{2+}]_i$ in resting MM6 cells but also in cells where $[Ca^{2+}]_i$ had been elevated by stimulation with platelet-activating factor (PAF). AKBA also strongly suppressed the subsequent elevation of $[Ca^{2+}]_i$ induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), PAF, or by the direct phospholipase C activator 2,4,6-trimethyl-*N*-(*meta*-3-trifluoromethyl-phenyl)-benzenesulfonamide, but AKBA failed to prevent Ca^{2+} signals induced by thapsigargin or ionomycin. Suppression of Ca^{2+} homeostasis by AKBA was also observed in primary monocytes, isolated from human blood. Moreover, AKBA inhibited the activation of $p38^{MAPK}$ and ERKs in fMLP-stimulated MM6 cells. Although the effects of AKBA could be mimicked by the putative phospholipase C (PLC) inhibitor U-73122 (1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), AKBA appears to operate independent of PLC activity since the release of intracellular inositol-1,4,5-trisphosphate evoked by 2,4,6-trimethyl-*N*-(*meta*-3-trifluoromethyl-phenyl)-benzenesulfonamide was hardly diminished by AKBA. Inhibitor studies indicate that AKBA may decrease $[Ca^{2+}]_i$ by blocking store-operated Ca^{2+} and/or nonselective cation channels. Together, AKBA interferes with pivotal signaling events in monocytic cells that are usually required for monocyte activation by proinflammatory stimuli. Interruption of these events may represent a possible mechanism underlying the reported anti-inflammatory properties of AKBA.

Boswellia serrata gum resin extracts have been traditionally applied in folk medicine for centuries to treat various chronic inflammatory diseases, and experimental data from animal models and clinical studies on humans confirmed an anti-inflammatory potential of *B. serrata* extracts (for review, see Safayhi and Sailer, 1997; Ammon, 2002). Detailed analysis of the ingredients of these extracts revealed that the pentacyclic triterpenes boswellic acids (BAs) possess pharmacological activities and may be responsible for the respective anti-inflammatory properties (Safayhi and Sailer, 1997). Approaches to elucidate the cellular and molecular mecha-

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ABBREVIATIONS: BA, boswellic acid; 5-LO, 5-lipoxygenase; NF, nuclear factor; TNF, tumor necrosis factor; PAF, platelet-activating factor; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; MAPK, mitogen-activated protein kinase; PMNL, polymorphonuclear leukocyte; MM, Mono Mac; AKBA, 3-*O*-acetyl-11-keto-boswellic acid; ERK, extracellular signal-regulated kinase; *m*-3M3FBS, 2,4,6-trimethyl-*N*-(*meta*-3-trifluoromethyl-phenyl)-benzenesulfonamide; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; 2-APB, 2-aminoethoxydiphenylborate; PBS, phosphate-buffered saline; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PG, PBS plus 1 mg/ml glucose; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; SDS-b, 2× SDS-PAGE sample loading buffer; IP_3, inositol-1,4,5-trisphosphate; KBA, 11-keto-boswellic acid; AβBA, 3-*O*-acetyl-boswellic acid; PLC, phospholipase C; TG, thapsigargin; SOCC, store-operated Ca^{2+} channel; LOE908, (*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-*N*,*N*-di-[2-(2,3,4-trimethoxyphenyl) ethyl]-acetamide; SK&F96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride; NSCC, nonselective cation channel; SEA0400, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy-5-ethoxyaniline; KB-R7943, 2-[2-[4-(4-dinitrobenzyoxy)phenyl]ethyl]isothiourea.

nisms underlying the clinical effects of BAs identified 5-lipoxygenase (5-LO) (Safayhi et al., 1992), human leukocyte elastase (Safayhi et al., 1997), topoisomerase I and II (Syrovets et al., 2000), and IκB kinases (Syrovets et al., 2005) as molecular targets. Accordingly, it is speculated that BAs may exert their anti-inflammatory effect mainly by inhibiting the release of proinflammatory leukotrienes from leukocytes (Safayhi et al., 1992) and/or by inhibition of NF-κB and subsequent down-regulation of TNFα expression in activated monocytes (Syrovets et al., 2005).

Stimulation of inflammatory cells by an adequate agonist may evoke a number of functional responses including chemotaxis, phagocytosis, degranulation, formation of reactive oxygen species, release of cytokines and chemokines, and liberation of lipid mediators. The transduction and mediation of such agonist-induced responses requires appropriate intracellular signaling systems that operate at multiple levels. Elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is one central signaling event for cell activation (Li et al., 2002), being involved in the regulation of functional responses such as degranulation or the generation of reactive oxygen species in agonist-challenged leukocytes (Bernardo et al., 1988). Extracellular stimuli, including the platelet-activating factor (PAF) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), increase the $[Ca^{2+}]_i$ in monocytes/macrophages, which is composed of a rapid release of Ca^{2+} from intracellular stores and a Ca^{2+} influx through plasma membrane Ca^{2+} channels (Randriamampita and Trautmann, 1989). Besides Ca^{2+}, protein phosphorylation is a common signal transduction mechanism integrating extracellular inflammatory signals into leukocyte functions; in particular, MAPK pathways have been shown to play important roles in this respect (Herlaar and Brown, 1999; Johnson and Druey, 2002). Accordingly, inhibitors of these kinases have been developed to intervene with inflammatory disorders.

We have recently shown that 11-keto-BAs can activate MAPK and induce Ca^{2+} mobilization in human isolated polymorphonuclear leukocytes (PMNLs) and granulocytic HL-60 cells, which could be linked to various functional responses, including release of arachidonic acid, increased formation of leukotrienes, and generation of reactive oxygen species (Altmann et al., 2002, 2004). Since monocytes play key roles in the course of inflammatory processes, we examined the effect of BAs on the Ca^{2+} homeostasis and MAPK pathways in human monocytic Mono Mac (MM) 6 cells. Interestingly, in contrast to PMNLs or HL-60 cells, AKBA exerted opposite effects in MM6 cells, inasmuch as it decreased basal $[Ca^{2+}]_i$, inhibited agonist-induced Ca^{2+} mobilization, and blocked agonist-induced activation of $p38^{MAPK}$ and ERKs. These findings support an anti-inflammatory potential of AKBA.

Materials and Methods

Materials. BAs were prepared as described previously (Jauch and Bergmann, 2003). 2,4,6-Trimethyl-*N*-(*meta*-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS) was a generous gift from Dr. T.G. Lee (SIGMOL, Pohang, Korea). U-73122 and SK&F96365 were purchased from Calbiochem (San Diego, CA), Fura-2/AM was from Alexis Corporation (Läufelfingen, Switzerland), PAF was from Cayman Chemical (Ann Arbor, MI), 2-aminoethoxydiphenylborate (2-APB) was from Tocris Cookson Inc. (Bristol, UK), and all other chemicals were obtained from Sigma Chemie (Deisenhofen, Germany).

Cells. MM6 cells were maintained in RPMI 1640 medium with glutamine supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 1× nonessential amino acids, 1 mM oxalacetic acid, and 10 μg/ml bovine insulin. All cultures were seeded at a density of 2×10^5 cells/ml. MM6 cells were treated with 2 ng/ml transforming growth factor β and 50 nM calcitriol for 4 days. Cells were harvested by centrifugation (200*g*, 10 min at room temperature) and washed once in phosphate-buffered saline (PBS), pH 7.4. To exclude toxic effects of BAs during various incubation periods, the viability of MM6 cells was analyzed by trypan blue exclusion. Incubation with 30 μM AKBA or 3 μM U-73122 at 37°C for up to 30 min caused no significant change in the number of viable cells.

Human PMNLs were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany) as described (Werz et al., 2002). In brief, venous blood was taken from healthy adult donors, and leukocyte concentrates were prepared by centrifugation at 4000*g*/20 min/20°C. PMNLs were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories GmbH, Linz, Austria), and hypotonic lysis of erythrocytes. Monocytes were obtained from the same leukocyte concentrates after dextran sedimentation and centrifugation on Nycoprep cushions. The mononuclear cells including lymphocytes and monocytes appear as a layer on Nycoprep cushion after centrifugation. The cells were washed twice with PBS, pH 5.9, containing 2 mM EDTA, resuspended in RPMI-1640 supplemented with 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 20% human plasma, and spread in cell culture flasks at 37°C and 5% CO_2. After 3 h, lymphocytes in suspension were removed, and adhered monocytes were gently detached and resuspended in PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

Measurement of Intracellular Ca^{2+} Levels. MM6 cells or blood monocytes (3×10^7/ml PG buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, 3×10^6 cells/ml PG buffer were incubated in a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Aminco-Bowman series 2; Thermo Electron Corporation, Waltham, MA) with continuous stirring. Two min prior to stimulation, 1 mM $CaCl_2$ or 1 mM EDTA was added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively, and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985). 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.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Prewarmed (37°C) MM6 cells were preincubated with the indicated concentrations of AKBA or vehicle [DMSO, final concentration ≤1% (v/v)] for 5 min prior to stimulation with fMLP (1 μM) for 1 min at 37°C. The reaction was stopped by addition of the same volume of ice-cold 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (SDS-b), samples for SDS-PAGE (aliquots corresponding to 2×10^6 cells in 20 μl SDS-b) were prepared, and proteins were separated as described (Werz et al., 2002). Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. Western blotting using phosphospecific antibodies (1:1000 dilution, each; New England Biolabs, Beverly, MA) against pERK1/2 (Thr202/Tyr204) and $pp38^{MAPK}$ (Thr180/Tyr182) was performed using a Li-Cor Odyssey two-color Western detection system (Li-Cor, Lincoln, NE), according to the instructions of the manufacturer. Alternatively, detection of immunoreactive proteins was performed as described previously using alkaline phosphatase-conjugated secondary antibody (Werz et al., 2002).

Determination of IP_3 Formation. Prewarmed (37°C) MM6 cells (1.2×10^7/ml PGC buffer) were either preincubated with the indicated compounds [or vehicle, DMSO ≤ 1% (v/v)] for 20 s and then subsequently stimulated with *m*-3M3FBS (100 μM) for 15 s or directly stimulated with the indicated compounds for 15 s at 37°C. Incubations were stopped by the addition of 0.2 volumes of ice-cold

$HClO_4$ [20% (v/v)] and kept on ice for 20 min. Further extraction and evaluation of IP_3 released into the medium was determined using an [^{3}H]IP_3 Biotrak Assay System (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Data are expressed as percentage of vehicle-treated controls ± S.E., $n = 3$ to 4. Statistical analysis was performed prior to normalization, $p < 0.05$ (*) or <0.01 (**).

Statistics. Statistical evaluation of the data were performed by one-way analysis of variance for independent or correlated samples followed by Tukey honestly significant difference post hoc tests. Where appropriate, Student's t test for paired observations was applied. $p < 0.05$ (*) or <0.01 (**) was considered significant.

Results

AKBA Decreases Basal $[Ca^{2+}]_i$ of Resting MM6 Cells. In our previous reports, we showed that AKBA and KBA, but not AβBA and βBA (lacking the 11-keto group), cause a marked mobilization of Ca^{2+} in human isolated PMNLs or in the granulocytic cell line HL-60 (Altmann et al., 2002, 2004). Accordingly, 30 μM AKBA induced a rapid and pronounced elevation of $[Ca^{2+}]_i$ in PMNLs, whereas AβBA was hardly effective (Fig. 1A, left panel). In contrast, exposure of differentiated MM6 cells to AKBA (30 μM) resulted in a sudden drop of resting $[Ca^{2+}]_i$ from 155 ± 8 to 73 ± 7 nM (Fig. 1A, right panel). This decrease in $[Ca^{2+}]_i$ was sustained, and markedly reduced levels of basal $[Ca^{2+}]_i$ were still detectable 20 min after exposure to AKBA (see below). In a previous study using MM6 cells (Feisst and Werz, 2004), we observed a similar drop of $[Ca^{2+}]_i$, when the PLC inhibitor U-73122 (3 μM) was added to the cells, which was confirmed in the present experiments (Fig. 1A, right panel). Of interest, in PMNLs, U-73122 (3 μM) caused no decrease in $[Ca^{2+}]_i$ (Fig. 1A, left panel). The effect of AKBA on resting $[Ca^{2+}]_i$ was concentration-dependent and was clearly detectable already at 1 μM (Fig. 1B). Combined addition of 30 μM AKBA plus 3 μM U-73122 gave no additive effects versus AKBA or U-73122 alone (data not shown).

In analogy to PMNLs, the effectiveness of the BAs to affect $[Ca^{2+}]_i$ in MM6 cells depended on the presence of the 11-keto group and the 3-*O*-acetyl moiety. Thus, the 11-keto-free counterpart of AKBA, namely AβBA (30 μM), hardly decreased $[Ca^{2+}]_i$, and KBA, lacking the 3-*O*-acetyl moiety, was less efficient than AKBA with respect to this response (Fig. 1, A and C). Finally, no effect was detectable for βBA (30 μM).

AKBA Decreases Elevated $[Ca^{2+}]_i$ in PAF-Activated MM6 Cells. To evaluate whether AKBA also affects elevated $[Ca^{2+}]_i$ induced by an agonist, MM6 cells were first treated with 100 nM PAF that raises $[Ca^{2+}]_i$, and AKBA, AβBA, or U-73122 was added 50 s later. Addition of AKBA or U-73122 evoked an immediate drop of $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 2). Notably, the minimum $[Ca^{2+}]_i$ attained after AKBA or U-73122 addition (78 ± 10 and 99 ± 9 nM, respectively) was lower than the basal Ca^{2+} levels

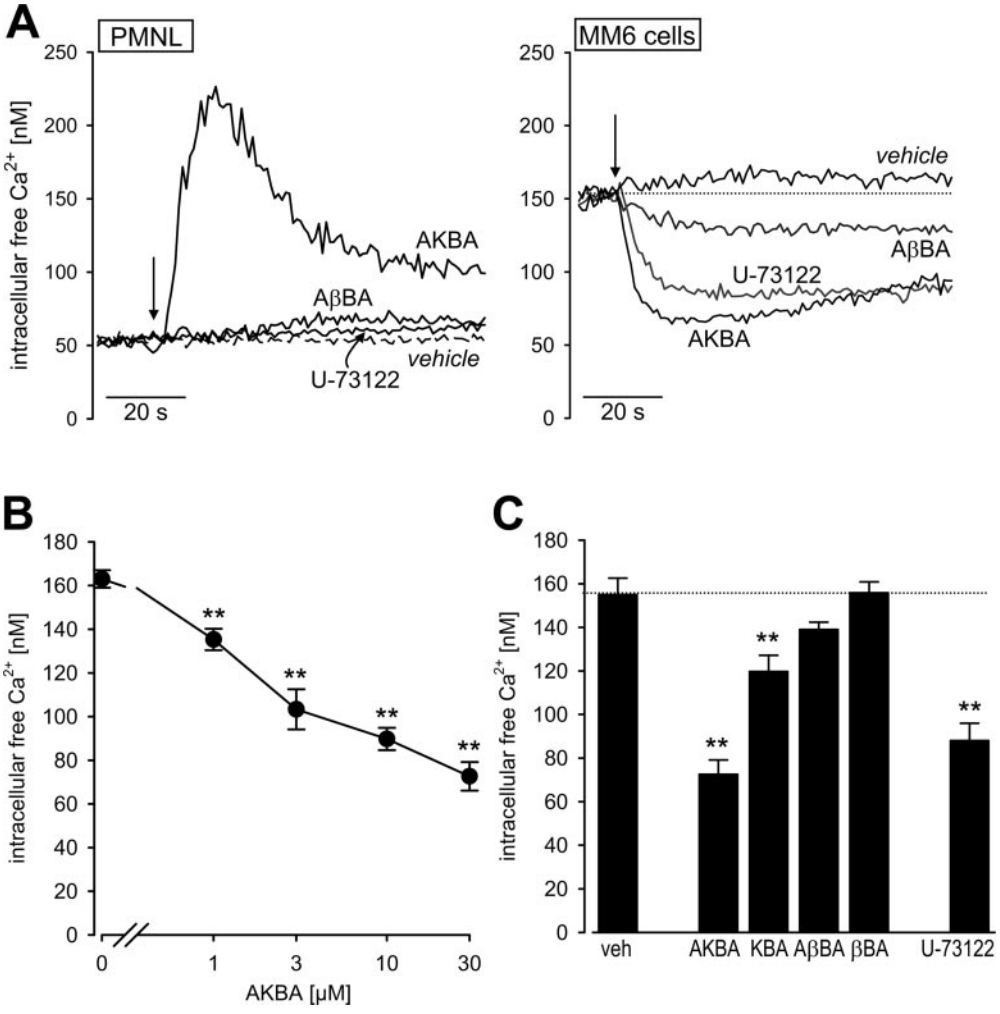

Fig. 1. Effects of BAs and U-73122 on basal $[Ca^{2+}]_i$ in resting PMNLs and MM6 cells. Fura-2-loaded PMNLs (10^7/ml PG buffer, A, left panel) or MM6 cells (3×10^6/ml PG buffer, A, right panel, B, and C), were supplemented with 1 mM $CaCl_2$, 2 min prior to stimulation, and $[Ca^{2+}]_i$ was determined. A, addition of AKBA, AβBA (30 μM, each), U-73122 (3 μM), or vehicle (DMSO) to resting PMNLs (left panel) or MM6 cells (right panel) is indicated by the arrow. Traces are representative for three to six independent recordings. B, concentration-response curves of AKBA on $[Ca^{2+}]_i$ in resting MM6 cells. The minimum $[Ca^{2+}]_i$ within 30 s after addition was determined. C, structure-activity relationship of BAs on $[Ca^{2+}]_i$ of resting MM6 cells. Vehicle (veh, DMSO), AKBA, KBA, AβBA, βBA (30 μM, each), or U-73122 (3 μM) was added as described above. The amplitude or an average value of the Ca^{2+} decrease (in nanomolar) was determined. Values in (B and C) are given as mean ± S.E., $n = 4$ to 5. Statistically different values compared with vehicle-treated controls are marked (**, $p < 0.01$).

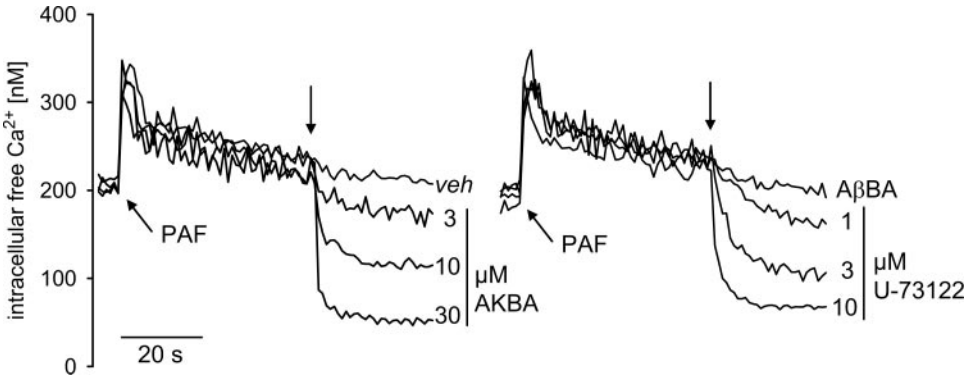

Fig. 2. Effects of BAs and U-73122 on elevated $[Ca^{2+}]_i$ in stimulated MM6 cells. MM6 cells were prepared as described in Fig. 1. PAF (0.1 μM) was added as indicated, followed by vehicle (veh) or AKBA (3–30 μM, left panel); AβBA (30 μM) or U-73122 (1–10 μM, right panel) after another 50 s. Curves are representative for three to five independent determinations.

prior to stimulation with PAF (157 ± 14 nM) and approached similar levels as found for cells exposed only to AKBA (73 ± 7 nM) or U-73122 (88 ± 8 nM), respectively (Fig. 2, compare with Fig. 1C). Thus, AKBA decreases $[Ca^{2+}]_i$ in MM6 cells to a comparable extent as U-73122, apparently regardless of the activation state of the cell.

AKBA Attenuates Agonist-Induced Elevation of $[Ca^{2+}]_i$. We sought to investigate whether AKBA could also prevent agonist-induced elevations of $[Ca^{2+}]_i$. Agents that elevate $[Ca^{2+}]_i$ involving PLC/IP_3 signaling (e.g., PAF, fMLP, and *m*-3M3FBS) but also stimuli that raise $[Ca^{2+}]_i$ independent of the PLC/IP_3 pathway like ionomycin or thapsigargin (TG) were added to MM6 cells that received BAs or U-73122, 20 s prior to agonist addition. As shown in Fig. 3A, AKBA and U-73122, but not AβBA, potently inhibited the subsequent Ca^{2+} mobilization induced by the physiological agonists PAF or fMLP as well as by the direct PLC activator *m*-3M3FBS (Bae et al., 2003). The IC_{50} value for AKBA was in the range of 10 to 30 μM, depending on the stimulus. Representative $[Ca^{2+}]_i$ traces of PAF-stimulated samples are displayed in Fig. 3B, left panel. In contrast, initial elevation of $[Ca^{2+}]_i$ induced by the ER/SR-Ca^{2+}-ATPase inhibitor TG or by the Ca^{2+}-ionophore ionomycin were not affected (Fig. 3A). Closer examination revealed that AKBA transforms the sustained elevation of $[Ca^{2+}]_i$ evoked by TG to a transient signal (Fig. 3B, right panel).

The amplitudes of the Ca^{2+} transients of the physiological stimuli PAF and fMLP were still strongly attenuated by AKBA, when the preincubation period was expanded from 20 s to 20 min (Fig. 3C). In contrast, the suppressive effects of U-73122 were seemingly transient and markedly declined within 20 min for PAF-stimulated cells, but not so for cells challenged with fMLP (Fig. 3C). Similarly, the decrease in basal $[Ca^{2+}]_i$ of (unstimulated) MM6 cells after prolonged (20 min) exposure to 30 μM AKBA was sustained, whereas for U-73122, the strong initial reduction of basal $[Ca^{2+}]_i$ detected right after exposure appeared to be transient and again was almost reversed after 20 min (Table 1). Therefore, AKBA exerts a sustained suppression on Ca^{2+} homeostasis by decreasing basal $[Ca^{2+}]_i$ as well as by attenuating (PLC/IP_3-mediated) Ca^{2+} mobilization.

It appeared possible that the suppressive effects of AKBA observed in MM6 could be related to the fact that MM6 is a human leukemia cell line. Therefore, we used primary monocytes isolated from human blood to investigate effects of AKBA on $[Ca^{2+}]_i$. As shown in Fig. 3D, AKBA (3 or 10 μM) rapidly decreased basal $[Ca^{2+}]_i$ and prevented PAF-induced Ca^{2+} mobilization in the same manner as observed for MM6 cells. AβBA was without effect, and higher AKBA concentrations (≥ 30 μM) caused a rather slow but continuous increase in $[Ca^{2+}]_i$ seemingly related to cell lysis or unspecific toxic effects of the compound (data not shown).

AKBA Attenuates Ca^{2+} Mobilization from Intracellular Stores. Next, we investigated if AKBA may also affect the PAF-induced release of Ca^{2+} from intracellular stores, a process that is typically PLC/IP_3-dependent. MM6 cells were resuspended in Ca^{2+}-free buffer containing 1 mM EDTA and treated with AKBA (AβBA or U-73122), followed by the addition of PAF after another 20 s. Neither AKBA (or AβBA) nor U-73122 exhibited an effect on basal $[Ca^{2+}]_i$ in resting cells under these conditions. However, AKBA or U-73122 reduced the release of Ca^{2+} from internal stores elicited by PAF (Fig. 4, A and B, left panel), although slightly higher concentrations of AKBA and U-73122 were required as compared with those needed to suppress total Ca^{2+} mobilization in the presence of extracellular Ca^{2+}. Surprisingly, also, Ca^{2+} mobilization from internal storage sites induced by TG (Fig. 4B, right panel) was partly antagonized by AKBA, implying that PLC inhibition may not be the sole mechanism by which AKBA affects $[Ca^{2+}]_i$ since TG-mediated Ca^{2+} mobilization circumvents the PLC/IP_3 route.

AKBA Attenuates fMLP-Evoked MAPK Activation. Cell activation, reflected by elevations in $[Ca^{2+}]_i$, is often accompanied by induction of signaling pathways leading to enhanced levels of phosphorylated members of the MAPK family (Belcheva and Coscia, 2002). The $p38^{MAPK}$ and ERKs are key effectors in the cellular signaling network of leukocytes. Addition of AKBA to resting MM6 cells caused a reduction of the basal levels of phosphorylated ERK-2, whereas phosphorylation of $p38^{MAPK}$ was not affected (Fig. 5A). Note that the phosphorylation state of MAPK solely indicates the activation of the kinases but might not exactly reflect the protein kinase activity toward its substrate(s).

Next, we examined whether AKBA prevents the activation of $p38^{MAPK}$ and ERKs induced by fMLP that, in contrast to PAF, causes prominent phosphorylation of the MAPK in MM6 cells (D. Poeckel and O. Werz, unpublished data). Indeed, activation of ERKs was potently prevented by AKBA; again, the amounts of phosphorylated ERK-2 were lower after preincubation with AKBA (10 and 30 μM) as compared with vehicle-treated control cells (Fig. 5B). AKBA also attenuated fMLP-induced phosphorylation of $p38^{MAPK}$ (Fig. 5B). It was shown that U-73122 substantially reduces the activation of $p38^{MAPK}$ in monocytic cells stimulated by lysophosphatidylcholine (Jing et al., 2000), and we found significant inhibition of fMLP-induced ERK2 activation by U-73122 in MM6

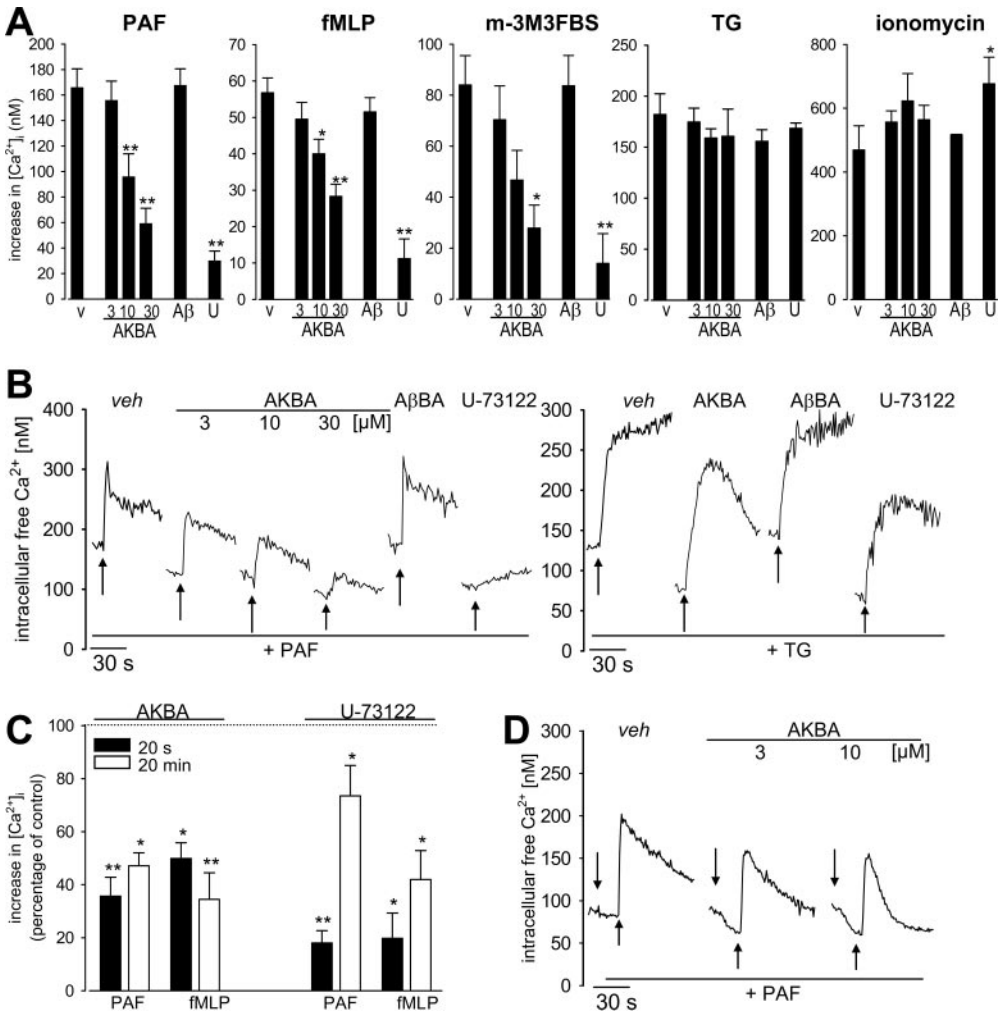

Fig. 3. AKBA and U-73122 antagonize agonist-induced Ca^{2+} mobilization. MM6 cells were prepared as described in Fig. 1. A, cells were treated with vehicle (v), AKBA (3, 10, and 30 μM), AβBA (Aβ, 30 μM), or U-73122 (U, 3 μM) followed by the addition of PAF (0.1 μM), fMLP (0.1 μM), *m*-3M3FBS (50 μM), thapsigargin (TG, 0.1 μM), or ionomycin (0.2 μM) after 20 s as indicated. The amplitude of the agonist-induced elevation of $[Ca^{2+}]_i$ was determined. Values are given as mean ± S.E., $n = 4$ to 5, and compared with the positive controls, $p < 0.05$ (*) or <0.01 (**). B, original Ca^{2+} recordings of measurements conducted for Fig. 3A. Left, cells were pretreated with vehicle (veh), AKBA (3, 10, and 30 μM), AβBA (30 μM), or U-73122 (3 μM) for 20 s, and PAF (0.1 μM) was added as indicated by the arrows. Right, cells were pretreated with vehicle (veh), AKBA (30 μM), AβBA (30 μM), or U-73122 (3 μM) for 20 s, and thapsigargin (TG, 0.1 μM) was added as indicated by the arrows. Curves are representative for three to five independent determinations. C, efficacy of AKBA and U-73122 to inhibit agonist-induced Ca^{2+} mobilization depends on the preincubation period. Cells were incubated with AKBA (30 μM) or U-73122 (3 μM). Then, PAF or fMLP (0.1 μM, each) was added as indicated either 20 s (black bars) or 20 min (white bars) after AKBA or U-73122. The resulting maximum increase in $[Ca^{2+}]_i$ was determined and compared with vehicle-treated controls, given as percentage of control ± S.E., $n = 3$ to 5. Statistical analysis was performed prior to normalization, $p < 0.05$ (*) or $p < 0.01$ (**). D, Fura-2-loaded primary monocytes (3×10^6/ml PGC buffer), freshly isolated from human blood, were treated with vehicle (v) or AKBA (3 or 10 μM), followed by the addition of PAF (0.1 μM) after 20 s, as indicated by the arrows. Curves are representative for three independent determinations.

TABLE 1

AKBA but not U-73122 causes sustained depression of $[Ca^{2+}]_i$

MM6 cells in PGC buffer were preincubated with vehicle (DMSO), AKBA, AβBA, or U-73122 for 20 s or 20 min, and $[Ca^{2+}]_i$ was determined, given as mean ± S.E.. $n = 4$ to 6, $p < 0.01$ (**).

Agonist	$[Ca^{2+}]_i$ after Addition of Agonist	
	20 s	20 min
	nM	
Vehicle (DMSO)	162 ± 4	157 ± 14
AKBA (30 μM)	73 ± 8**	83 ± 5**
AβBA (30 μM)	139 ± 4	146 ± 16
U-73122 (3 μM)	87 ± 10**	132 ± 13

cells (data not shown). AβBA, however, was inactive (data not shown).

Effects of Boswellic Acids on Cellular PLC Activity. To test whether AKBA (in analogy to U-73122) inhibits cellular PLC activity, we assayed the effects of AKBA on the IP_3 formation in intact MM6 cells. Cellular PLC was directly activated using 100 μM *m*-3M3FBS to obtain a prominent increase in IP_3 production (7.9-fold elevation, Fig. 6B). In agreement with its ability to block total Ca^{2+} mobilization, U-73122 (5 μM) inhibited *m*-3M3FBS-induced IP_3 formation (~80%, Fig. 6A). In contrast to its ability to decrease $[Ca^{2+}]_i$, AKBA failed to significantly suppress IP_3 formation. Intriguingly, AβBA, which hardly affected Ca^{2+} homeostasis, inhibited *m*-3M3FBS-induced IP_3 generation (~50%, Fig. 6A).

We then examined whether AKBA, AβBA, or U-73122 could also affect the basal IP_3 turnover in resting cells. In contrast to *m*-3M3FBS-activated MM6 cells, no significant stimulatory effect was observed for U-73122 (5 μM) or AβBA. However, AKBA caused a concentration-dependent increase in IP_3 formation as compared with vehicle-treated control

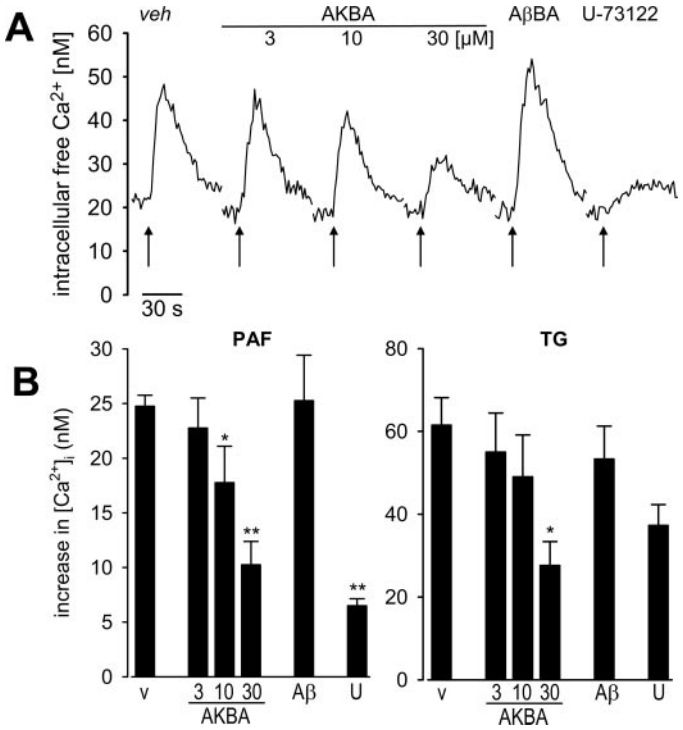

Fig. 4. Effects of BAs on Ca^{2+} release from internal stores. MM6 cells were prepared as described in Fig. 1, except that 1 mM EDTA was added instead of 1 mM Ca^{2+}. A, original Ca^{2+} recordings of samples stimulated by PAF (0.1 μM) after preincubation with vehicle (veh), AKBA (3, 10, and 30 μM), AβBA (30 μM), or U-73122 (3 μM) for 20 s. Curves are representative for three to four independent determinations. B, cells were treated with vehicle (v), AKBA (3, 10, and 30 μM), AβBA (Aβ, 30 μM), or U-73122 (U, 3 μM) followed by the addition of PAF (0.1 μM, left panel), or thapsigargin (TG, 0.1 μM, right panel). The amplitude of the stimulus-induced elevation of $[Ca^{2+}]_i$ was determined. Values are given as mean ± S.E., $n = 4$, $p < 0.05$ (*) or <0.01 (**).

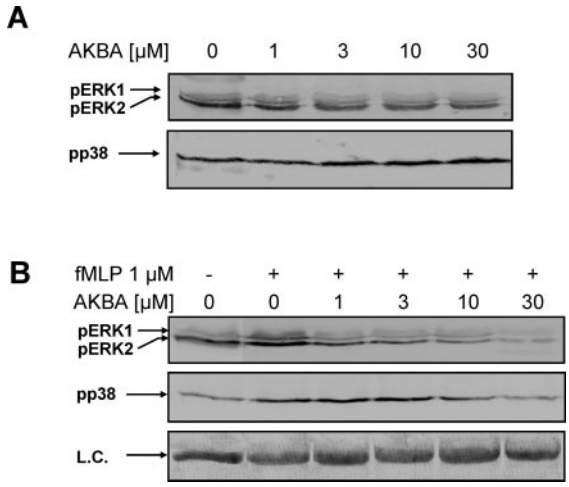

Fig. 5. AKBA attenuates the activation of ERK1/2 and $p38^{MAPK}$. A, prewarmed MM6 cells (2×10^6/100 μl PGC buffer) were incubated with the indicated concentrations of AKBA or vehicle (DMSO) for 5 min. B, prewarmed MM6 cells were first incubated with the indicated concentrations of AKBA or vehicle (DMSO) for 15 min prior to stimulation with fMLP (1 μM) at 37°C for 1 min. Reactions were terminated by addition of equal volumes of SDS-b. Samples were subjected to SDS-PAGE and Western blotting using phosphospecific antibodies against the dually phosphorylated form of the MAPKs. Samples were concurrently analyzed for $p38^{MAPK}$ and ERK1/2 activation using the 2-color Western detection system of Li-Cor (Odyssey), hence, loading control (L.C.) bands refer to both antibodies. The results shown are representative of at least three independent experiments.

cells (Fig. 6B). Possibly, this elevation of IP_3 might compromise the inhibitory effect of AKBA on the *m*-3M3FBS signal as shown in Fig. 6A, leading to an apparent weaker inhibition due to inherent AKBA-induced IP_3 production. Thus, a correlation between the suppression of Ca^{2+} homeostasis and inhibition of PLC by AKBA is not readily apparent, suggesting that AKBA operates at alternate targets than PLC to impair $[Ca^{2+}]_i$.

Inhibitors of Plasma Membrane Ca^{2+} Channels Mimic the Effects of AKBA and Abolish AKBA-Induced Decrease of $[Ca^{2+}]_i$. The fact that AKBA attenuates the secondary phase of TG-induced Ca^{2+} mobilization, which represents Ca^{2+} influx from the extracellular space, prompted us to elucidate if the AKBA-induced loss of intracellular (cytoplasmic) Ca^{2+} may be due to inhibition of plasma membrane Ca^{2+} influx channels such as store-operated Ca^{2+} channels (SOCCs), nonselective cation channels (NSCCs), or voltage-gated Ca^{2+} channels. SK&F96365, an inhibitor of NSCC and SOCC, reduced basal $[Ca^{2+}]_i$ in MM6 cells and was able to prevent the subsequent decrease of $[Ca^{2+}]_i$ induced by AKBA (Fig. 7). To distinguish between NSCC and SOCC, we applied 2-APB (50 μM), which blocks SOCCs and, on the other hand, LOE908 (10 μM), which selectively inhibits NSCC. Both 2-APB and LOE908 decreased basal $[Ca^{2+}]_i$ and prevented the effects of AKBA (Fig. 7). In contrast, inhibitors of voltage-gated Ca^{2+} channels (300 nM ω-conotoxin MVIIA or 1 μM verapamil, which block L- or N-type channels, respectively), SR-Ca^{2+} release channels (10 μM neomycin), or blockers of the Na^+-Ca^{2+} exchanger (SEA0400 or KB-R7943, 10 μM each) failed to significantly decrease basal $[Ca^{2+}]_i$ and to prevent effects of AKBA (data not shown). Together, these results indicate that AKBA may mediate the decrease of $[Ca^{2+}]_i$ by blocking Ca^{2+} influx from the extracellular space via inhibition of SOCC/NSCC.

Discussion

AKBA and KBA were shown to induce Ca^{2+} mobilization and activation of MAPK in primary PMNLs and granulocytic HL-60 cells, involving pertussis toxin-sensitive proximal signaling pathways (Altmann et al., 2002, 2004). Activation of these central signaling events were linked to typical functional responses of granulocytes, including peroxide formation and enhanced metabolism of arachidonic acid; in particular, an increased activity of 5-LO was evident (Altmann et al., 2004). Such an activation of granulocytes opposes the general observation that extracts of *B. serrata* or isolated BAs exert anti-inflammatory properties in several cellular experimental settings (Krieglstein et al., 2001; Syrovets et al., 2005) or animal models (Sharma et al., 1989; Gupta et al., 1994) and finally also in studies on human subjects (Gupta et al., 1998; Gerhardt et al., 2001).

Recently, Syrovets et al. (2005) showed that in activated human monocytes, BAs down-regulate TNFα expression via a direct inhibition of IκB kinases, providing a molecular basis for the anti-inflammatory properties of BAs. The result from the present investigation focusing on central signaling pathways in monocytes provides additional evidence for an anti-inflammatory implication of AKBA at the cellular level. Thus, AKBA decreased the basal $[Ca^{2+}]_i$, prevented agonist-

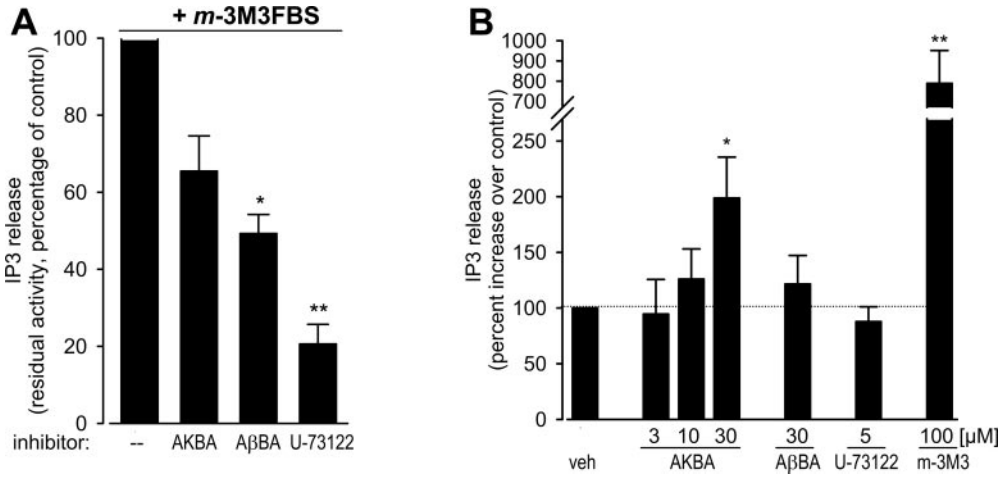

Fig. 6. Effects of BAs on IP_3 formation. A, prewarmed MM6 cells (1.2×10^7/ml PGC buffer) were treated with vehicle (DMSO), AKBA (30 μM), AβBA (30 μM), or U-73122 (5 μM) for 20 s prior to stimulation with *m*-3M3FBS (100 μM) for 15 s. Incubations were stopped by the addition of 0.2 volumes of ice-cold $HClO_4$ (20%, v/v), and extraction and evaluation of IP_3 released were determined according to the manufacturer's instructions (IP_3 [^{3}H] Biotrak Assay System; GE Healthcare). Data are expressed as percentage of vehicle-treated (*m*-3M3FBS-stimulated) control ± S.E., $n = 3$ to 4. Statistical analysis was performed prior to normalization, $p < 0.05$ (*) or <0.01 (**). B, prewarmed MM6 cells (1.2×10^7/ml PGC buffer) were incubated with vehicle (DMSO), AKBA (3, 10, and 30 μM), AβBA (30 μM), U-73122 (5 μM), or *m*-3M3FBS (100 μM) for 15 s. Incubations were stopped by the addition of 0.2 volumes of ice-cold $HClO_4$ (20%, v/v). The subsequent extraction procedure is identical to the description in A. Data are expressed as percentage of vehicle-treated (unstimulated) control ± S.E., $n = 3$ to 4. Statistical analysis was performed prior to normalization, $p < 0.05$ (*) or <0.01 (**).

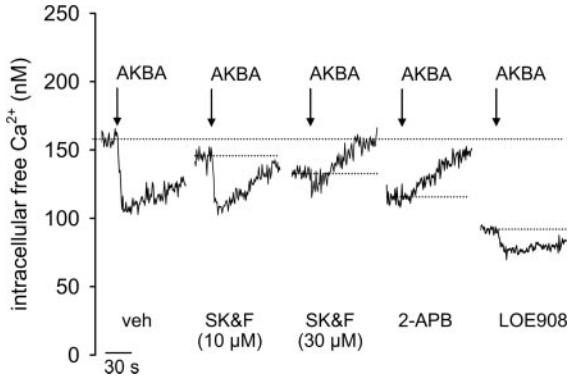

Fig. 7. Effects of plasma membrane Ca^{2+} channel inhibitors on Ca^{2+} homeostasis and on the actions of AKBA. Fura-2-loaded MM6 cells (3×10^6/ml PG buffer) were preincubated with vehicle (veh, DMSO), SK&F96365 (10 or 30 μM), 2-APB (50 μM), or LOE908 (10 μM) for 2 min at 37°C in the presence of 1 mM $CaCl_2$. Then, cells were stimulated with AKBA (30 μM), and $[Ca^{2+}]_i$ was determined. Traces are representative for three to four independent determinations.

induced Ca^{2+} mobilization, and blocked the activation of ERK1/2 and $p38^{MAPK}$, signaling events that are determinants for typical functional monocyte/macrophage responses (Gijon and Leslie, 1999; Chen et al., 2001). Interestingly, TNFα generation and NF-κB activation in monocytic cells may depend on Ca^{2+} (Pollaud-Cherion et al., 1998; See et al., 2004), providing a possible link between interference with Ca^{2+} and down-regulation of NF-κB and TNFα.

Apparently, in view of the opposite, agonistic effects on PMNLs and HL-60 cells, AKBA and KBA exert disparate effects on certain cellular processes, depending on the cell type. Thus, in monocytic cells, AKBA may be regarded as pharmacologically active compound that suppresses important signaling events, implying anti-inflammatory functionality. PMNLs that are terminally differentiated are involved in acute inflammatory responses, whereas monocytes act more in chronic inflammation and can undergo differentiation prior to function. Indeed, opposite effects on Ca^{2+} homeostasis in analogy to AKBA are obvious in leukocytes exposed to arachidonic acid that decreases $[Ca^{2+}]_i$ in peritoneal macrophages (Randriamampita and Trautmann, 1990) and in MM6 cells (D. Poeckel and O. Werz, unpublished data) but on the other hand evokes Ca^{2+} mobilization in PMNLs (Naccache et al., 1989).

Many effector enzymes like phospholipases, 5-LO, and protein kinases respond to and are regulated by an elevation of $[Ca^{2+}]_i$, leading to functional monocyte responses including lipid mediator and superoxide release, degranulation, and cytokine generation (Bernardo et al., 1988; Pollaud-Cherion et al., 1998). Among the four major β-configurated BAs present in ethanolic extracts of *B. serrata* gum, AKBA was most potent, whereas the 11-methylene derivatives were hardly active; also, the absence of the 3-*O*-acetyl group led to a loss of efficacy. Similarly, for interference with so far all defined molecular pharmacological targets, i.e., 5-LO (Safayhi et al., 1992), human leukocyte elastase (Safayhi et al., 1997), topoisomerases I and II (Syrovets et al., 2000), as well as IκB kinases (Syrovets et al., 2005), AKBA possesses the highest potency, being of considerable pharmacological interest (Ammon, 2002).

The effects of AKBA in MM6 cells showed similar characteristics as the PLC inhibitor U-73122 (Bleasdale et al., 1990) that was found to block acute and chronic inflammatory responses in vivo (Hou et al., 2004). Indeed, both U-73122 and AKBA rapidly decreased the basal $[Ca^{2+}]_i$ of resting cells but also caused an immediate drop of the elevated $[Ca^{2+}]_i$ after challenge with PAF, displaying comparable kinetics. Moreover, both agents reduced agonist-evoked Ca^{2+} mobilization, which in fact is a characteristic for monocyte activation by external stimuli (Kim et al., 1992; Bernardo et al., 1997; Li et al., 2002). Such Ca^{2+}-antagonizing activity of AKBA or U-73122 was evident for agonists (fMLP, PAF, or *m*-3M3FBS) that act via the PLC/IP_3 pathway. In contrast, initial Ca^{2+} fluxes induced by the ER/SR-Ca^{2+}-ATPase inhibitor TG or by the Ca^{2+}-ionophore ionomycin, which both

circumvent PLC/IP_3 for Ca^{2+} mobilization (Gouy et al., 1990), were unaffected by either U-73122 or AKBA. Experiments conducted to determine the duration of the Ca^{2+} suppressing effects, either in resting or in agonist-challenged cells, revealed rather transient efficacy of U-73122, whereas AKBA-mediated antagonism was sustained and long-lasting, implying that the compounds most likely operate through differing mechanisms.

Another common feature of U-73122 and AKBA was their ability to inhibit the PAF-induced release of Ca^{2+} from internal storage sites. Hence, based on the inhibitory profile and characteristics to affect Ca^{2+} homeostasis, it first appeared reasonable that the Ca^{2+}-modulating effects of AKBA could be due to interference with PLC, which is a defined molecular target of U-73122 (Bleasdale et al., 1990). On the other hand, interference of AKBA with the IP_3 receptor could be a plausible explanation. Surprisingly, however, AKBA significantly inhibited TG-induced Ca^{2+} mobilization from internal stores and also the sustained elevation of $[Ca^{2+}]_i$ of TG-treated cells in Ca^{2+}-containing buffer, suggesting that AKBA may influence Ca^{2+} homeostasis, at least in part, independent of PLC or IP_3. An important finding that favors a PLC-independent mechanism is the failure of AKBA to efficiently suppress the release of intracellular IP_3. Strikingly, in contrast to AKBA, AβBA clearly failed to counteract *m*-3M3FBS-induced Ca^{2+} mobilization, even though it was more efficient than AKBA in inhibiting *m*-3M3FBS-evoked IP_3 formation. Based on these discrepancies, inhibition of PLC is no satisfying explanation for the potent impairment of $[Ca^{2+}]_i$ induced by AKBA. This hypothesis is further supported by the fact that AKBA on one hand even slightly increased basal IP_3 levels about 2-fold, which should actually lead to Ca^{2+} release from internal storage sites. However, in contrast, there is a strong decrease in the basal $[Ca^{2+}]_i$ under these conditions.

The AKBA-induced loss of intracellular (cytoplasmic) Ca^{2+} may result from different processes such as extrusion of intracellular Ca^{2+} to the extracellular space, stimulation of Ca^{2+} storage (uptake) into intracellular sites (e.g., by activation of a Ca^{2+}/ATPase), or interference with ion channels allowing Ca^{2+} influx. Our studies using selective inhibitors of various plasma membrane Ca^{2+} influx channels imply that AKBA might act (at least in part) by inhibition of SOCC and/or NSCC. Thus, inhibitors of SOCC and/or NSCC mimicked the loss of $[Ca^{2+}]_i$ observed with AKBA and were able to inhibit the subsequent decrease of $[Ca^{2+}]_i$ induced by AKBA. Of interest, also for U-73122, inhibition of plasma membrane Ca^{2+} channels has been accounted for reduced $[Ca^{2+}]_i$ (see Feisst et al., 2005, and references therein). In contrast, voltage-gated N-and L-type Ca^{2+} channels, SR-Ca^{2+} release channels (neomycin), or the Na^+-Ca^{2+} exchanger do not seem to mediate the effects of AKBA. It is conceivable that a block of SOCC/NSCC may shift the balance between Ca^{2+} influx and Ca^{2+} extrusion toward predominant extrusion that, as a result, leads to impaired $[Ca^{2+}]_i$. However, more detailed experiments are required to elucidate the molecular targets and mechanisms underlying the complex regulation of Ca^{2+} homeostasis by AKBA in MM6 cells, which would go beyond the scope of this study.

Besides antagonizing Ca^{2+}, AKBA potently prevented fMLP-induced activation of $p38^{MAPK}$ and ERKs. These MAPK pathways play pivotal roles in the transduction of external mediators to many cellular processes and are strongly implicated in inflammatory disorders (Herlaar and Brown, 1999; Johnson and Druey, 2002). Among the pharmacological strategies for intervention with inflammation, inhibitors of ERKs may possess potential for the treatment of inflammatory and neuropathic pain (Ji, 2004). $p38^{MAPK}$ inhibitors have been developed to treat for example rheumatoid arthritis (Pargellis and Regan, 2003) and Crohn's disease (Hommes et al., 2002), inflammatory disorders that in fact have been successfully treated with *B. serrata* extracts (Gerhardt et al., 2001). Of interest, recently also U-73122 was shown to reduce lysophosphatidylcholine-induced $p38^{MAPK}$ activation in monocytic THP-1 cells (Jing et al., 2000).

In summary, our data show that AKBA is capable to suppress central signaling events in human monocytic cells, typically important for functional monocyte responses at inflammatory sites. These findings may be added to the list of pharmacological actions of BAs assumed to contribute to the effects of *B. serrata* extracts observed in animal models and in clinical studies of humans and may be another step forward to the elucidation of the cellular and molecular basis of the anti-inflammatory properties of BAs.

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9 Publication List

1 **Poeckel, D.**; Tausch, L.; Altmann, A.; Feisst, C.; Klinkhardt, U.; Graff, J.; Harder, S.; Werz, O., Induction of central signalling pathways and select functional effects in human platelets by beta-boswellic acid. *Br J Pharmacol* **2005**, 146, (4), 514-524.

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3 **Poeckel, D.**; Tausch, L.; Kather, N.; Jauch, J.; Werz, O., Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase. *Mol Pharmacol* **2006**, 70, (3), 1071-1078.

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11 Kather, N.; Tausch, L.; **Poeckel, D.**; Werz, O.; Herdtweck, E.; Jauch, J., Immobilisation of Boswellic acids at EAH Sepharose™ for "target fishing". *Tetrahedron* **2006**, submitted.

Präsentationen bei Kongressen

Vorträge (oral presentations)

Poeckel D., Altmann A., und Werz O.. Ein neuer Aktivator für humane Thrombocyten. β-Boswelliasäure induziert die Ca^{2+}-Mobilisierung, MAP-Kinase- und 12-Lipoxygenase-Aktivierung. DPhG-Doktorandentagung, Freudenstadt (2004).

Poeckel D.. Arachidonic acid metabolites as mediators of β-boswellic acid-induced platelet activation. EU Graduate School GRK 757, Summer Seminar, Arnsburg bei Lich (2005).

Poeckel D.. Short oral presentations at the EU Graduate School Seminars (see next page)

Posterpräsentationen (poster presentations)

Poeckel D., Altmann A., Steinhilber D., Werz O.. Induction of arachidonic acid release and 12-lipoxygenase product formation by the platelet agonist β-boswellic acid. 2nd International Conference on Phospholipases A_2, Berlin (2004).

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Weitere Kongressteilnahme

Signal transduction pathways as therapeutic targets, Luxemburg (2004).

Teilnahme an Seminaren des EU-Graduiertenkollegs GRK 757

Winter School, Karolinska Institute Stockholm, Januar 2003
Summer Seminar, Kloster Arnsburg, August 2003
Winter School, Karolinska Institute Stockholm, Mai 2004
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(deutsch – englisch – schwedisch – holländisch – französisch – finnisch – spanisch – portugiesisch – polnisch - klingonisch – tschechisch – lettisch – türkisch – ungarisch – estnisch – italienisch – isländisch)

11 Curriculum Vitae

PERSÖNLICHES

Name, Vorname	Pöckel, Daniel
Geburtsdatum und –ort	2. Oktober 1976 in Frankfurt am Main
Staatsangehörigkeit	deutsch
Familienstand	verheiratet

SCHUL- UND HOCHSCHULAUSBILDUNG

1983 – 1986	Freiligrathschule (Grundschule), Frankfurt am Main
1986 – 1996	Helmholtzschule (Gymnasium), Frankfurt am Main
Mai 1996	Abitur
1997 – 2002	Studium der Biochemie an der Universität Frankfurt am Main
August 1999 – Dezember 1999	Auslandssemester an der University of Surrey, Guildford, Großbritannien
Juli 2001 – August 2001	Diplomprüfungen in Biochemie, Biophysik, Zellbiologie und Pharmakologie
Oktober 2001 – April 2002	Diplomarbeit im Max-Planck-Institut für Biophysik, Frankfurt am Main
April 2002	Diplom (Biochemie)
August 2002 – September 2005	Durchführung der Promotionsarbeit im Institut für Pharmazeutische Chemie an der Universität Frankfurt am Main
August 2002 – August 2005	Stipendiat des EU-Graduiertenkollegs „Role of Eicosanoids in Biology and Medicine“
April 2004 – Juli 2004	Forschungsaufenthalt im Institut für Medizinische Chemie II, Karolinska Institut, Stockholm, Schweden
Oktober 2005 – Mai 2006	Fortsetzung der Promotionsarbeit im Institut für Pharmazeutische Chemie an der Universität Tübingen

PRAKTIKA, BERUFSERFAHRUNG, WEITERBILDUNG, LEHRTÄTIGKEIT

Juli 1997 – August 1997	Praktikum (6-wöchig) im Paul-Ehrlich-Institut, Langen, Abteilung „HIV/Proteinforschung“
April 1999 – September 1999	Kurs „Wissenschaftsjournalismus“ (Dr. E. Nolde), Fachbereich Medizin, Universität Frankfurt am Main
Januar 2000 – März 2000	Werkstudent (10-wöchig) bei der BC Biochemie GmbH, Industriepark Höchst, Frankfurt am Main
Oktober 2000 – März 2001	Studium der Informatik (1. Semester), Universität Frankfurt am Main
Juni 2002 – Juli 2002	Werkstudent (7-wöchig) bei der Aventis Deutschland GmbH, Abteilung „Metabolic Diseases“, Industriepark Höchst, Frankfurt am Main
August 2005 – September 2005	Fortbildung „Ökonomisches Grundlagenwissen“ (40-stdg.), Zentrum für Weiterbildung, Universität Frankfurt am Main
seit Oktober 2005	Leiter des Praktikums „Instrumentelle Analytik“ im Studiengang Pharmazie, Universität Tübingen

WEHRERSATZDIENST

1996 – 1997	Melanchthongemeinde Frankfurt am Main

AKADEMISCHE LEHRER

Meine akademischen Lehrer neben Prof. Dr. Oliver Werz und Prof. Dr. Dieter Steinhilber waren:

Prof. Dr. Dr. H. Fasold
Prof. Dr. E. Bamberg
Prof. Dr. C. Grewer
Prof. Dr. H. Rüterjans
Prof. Dr. B. Ludwig
Prof. Dr. G. Lambrecht
Prof. Dr. H. Zimmermann
Prof. Dr. J. Bereiter-Hahn
Prof. Dr. A. Starzinski-Powitz