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Arachidonic Acid Promotes Phosphorylation of 5-Lipoxygenase at Ser-271 by MAPK-activated Protein Kinase 2 (MK2)*

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We demonstrated previously that 5-lipoxygenase (5-LO), a key enzyme in leukotriene biosynthesis, can be phosphorylated by p38 MAPK-regulated MAPKAP kinases (MKs). Here we show that mutation of Ser-271 to Ala in 5-LO abolished MK2 catalyzed phosphorylation and clearly reduced phosphorylation by kinases prepared from stimulated polymorphonuclear leukocytes and Mono Mac 6 cells. Compared with heat shock protein 27 (Hsp-27), 5-LO was a weak substrate for MK2. However, the addition of unsaturated fatty acids (*i.e.* arachidonate 1–50 μM) up-regulated phosphorylation of 5-LO, but not of Hsp-27, by active MK2 *in vitro*, resulting in a similar phosphorylation as for Hsp-27. 5-LO was phosphorylated also by other serine/threonine kinases recognizing the motif Arg-Xaa-Xaa-Ser (protein kinase A, Ca^{2+}/calmodulin-dependent kinase II), but these activities were not increased by fatty acids. HeLa cells expressing wild type 5-LO or S271A-5-LO, showed prominent 5-LO activity when incubated with Ca^{2+}-ionophore plus arachidonate. However, when stimulated with only exogenous arachidonic acid, activity for the S271A mutant was significantly lower as compared with wild type 5-LO. It appears that phosphorylation at Ser-271 is more important for 5-LO activity induced by a stimulus that does not prominently increase intracellular Ca^{2+} and that arachidonic acid stimulates leukotriene biosynthesis also by promoting this MK2-catalyzed phosphorylation.

5-Lipoxygenase (5-LO)[1] catalyzes initial steps in formation of leukotrienes (LTs) and lipoxins, mediators and modulators of inflammatory and allergic reactions (1). In addition to phagocytes and B-lymphocytes, 5-LO was recently found also in dendritic cells, implying functions for LTs also in the adaptive part of the immune response (2, 3). Depending on the cell type, 5-LO is present in the cytosol but also in a nuclear soluble pool of resting cells. Upon cell stimulation, soluble 5-LO translocates to the nuclear membrane where it colocalizes with 5-lipoxygenase-activating protein (FLAP) and cytosolic phospholipase A_2, and initializes the formation of LTs (for review see Ref. 4). It was recently described that an N-terminal β-barrel domain of 5-LO is important for Ca^{2+}-stimulated membrane association (5–7). It appears that phosphorylation is another determinant of cellular LT biosynthesis (8, 9); cell stimulation leading to 5-LO activity activated p38 MAPK and its downstream targets (MAPKAP kinases (MKs)), which can phosphorylate 5-LO *in vitro*, (10–13). Interestingly, the p38 MAPK inhibitor SB 203580 inhibited antigen-induced LTC_4 production in sensitized mouse bone marrow-derived mast cells (14).

The mitogen-activated protein kinase (MAPK) superfamily in mammalian cells includes p38 MAPK, which is activated when cells are exposed to cytokines or various forms of cellular stress (for review see Ref. 14). For PMNL and other cell types, exogenous arachidonic acid (AA) resulted in phosphorylation and activation of p38 MAPK (15–17). In PMNL, AA also leads to activation of another MAPK, ERK1/2 (18). Activated p38 MAPK subsequently phosphorylates and activates downstream kinases such as MKs, as well as certain transcription factors (14). MK2 phosphorylates substrates at serine residues in the consensus motif hyd-Xaa-Arg-Xaa-Xaa-Ser, where hyd is a bulky hydrophobic amino acid such as Phe or Leu (19, 20). Some identified MK2 substrates are heat shock protein 27 (Hsp-27) (21, 22), lymphocyte-specific protein (23), serum response factor, CREB (cAMP-response element-binding protein), tyrosine hydroxylase, glycogen synthases (23, 24), and vimentin (25). We found that also 5-LO is a substrate for p38 MAPK-regulated MKs (10, 12). Here we demonstrate that Ser-271 is a phosphorylation site in 5-LO and that unsaturated fatty acids such as AA stimulate phosphorylation of 5-LO at Ser-271 by MK2. This phosphorylation site was more important for 5-LO activity in transfected cells stimulated with only exogenous AA as compared with cells stimulated with AA plus Ca^{2+}-ionophore.

EXPERIMENTAL PROCEDURES

Materials—Human transforming growth factor β was purified from outdated platelets as described (26). 1,25-Dihydroxyvitamin D_3 was from Biomol (Plymouth Meeting, PA); RPMI 1640 from Invitrogen; fetal calf serum, bovine insulin, protein kinase A (PKA) catalytic subunit, human recombinant Hsp-27, arachidic acid, linoleic acid, linolenic acid, palmitic acid, oleic acid, Ca^{2+}-ionophore A23187, and fMLP from Sigma; arachidonic acid from Nu-Chek (Elysian, MN); SB203580 from Calbiochem; [γ-^{32}P]ATP (110 TBq/mmol) from Amersham Biosciences; HPLC solvents from Rathburn Chemicals (Walkerburn, Scotland); activated GST-MK2 and Ca^{2+}/calmodulin-dependent kinase (CaMK) II

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[1] The abbreviations used are: 5-LO, 5-lipoxygenase; AA, arachidonic acid; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; ERK, extracellular signal-regulated kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; HEK-293 cells, human embryonic kidney 293 cells; Hsp-27, heat shock protein 27; IP, immunoprecipitate; LT, leukotriene; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein; MK, MAPKAP kinase; MM6 cells, Mono Mac 6 cells; PBS, phosphate-buffered saline, pH 7.4; PKA and PKC, protein kinase A and C, respectively; PMNL, polymorphonuclear leukocytes; SDS-b, 2× SDS-PAGE sample loading buffer; wt, wild type; 5-HETE, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5-HPETE, 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 13-HPODE, 13(*S*)-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid.

from Upstate Biotechnology (Lake Placid, NY); and oligonucleotides were from Cyber Gene (Huddinge, Sweden).

Cell Culture and Transient Transfections—Mono Mac 6 (MM6) cells were cultured and differentiated with transforming growth factor β and 1,25-dihydroxyvitamin D_3 as described (10). Cells were harvested by centrifugation (200 × *g*, 10 min at room temperature) and washed once in phosphate-buffered saline, pH 7.4 (PBS). Human polymorphonuclear leukocytes (PMNL) were isolated from leukocyte concentrates obtained from healthy donors at Karolinska Hospital. For incubations, MM6 cells and PMNL were finally resuspended in PGC buffer (PBS with 1 mM Ca^{2+} and 1 mg/ml glucose).

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 μg/ml streptomycin, 100 units/ml penicillin at 37 °C in a 5% CO_2 incubator. Plasmid DNA (pcDNA3.1-5LO, 10 μg) was transiently transfected into HeLa and HEK-293 cells using the Ca^{2+}-phosphate method (27), cultured for 48 h, and assayed for 5-LO activity.

Site-directed Mutagenesis, Expression, and Purification of 5-LO Proteins—The codon for Ser-271 in the plasmid pT3-5LO was mutated using the QuikChange™ kit from Stratagene as described (5). The mutated DNA was confirmed using the Applied Biosystem PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Life Sciences), followed by analysis on a Applied Biosystem PRISM 377 sequencer (carried out by KISeq, Core Facilities at Karolinska Institutet). *Escherichia coli* MV1190 was transformed with mutated and wild type (wt) DNA, and recombinant 5-LO proteins were expressed at 27 °C and purified as described (5). The mutated plasmid pcDNA3.1-5LO-S271A was prepared from pcDNA3.1-5LO (28) by replacement of the *Eco*RV to *Not*I fragment with the corresponding DNA fragment from pT3-5LO-S271A.

In-gel Kinase Assay—PMNL and differentiated MM6 cells (5×10^7 and 2.5×10^7, respectively, in 1 ml of PGC buffer) were stimulated with the indicated additives for 3 min at 37 °C. Incubations were stopped by addition of the same volume of 2× SDS-PAGE sample loading buffer (SDS-b) and heated for 6 min at 95 °C. Total cell lysates corresponding to 0.25×10^6 MM6 cells or 0.5×10^6 PMNL were loaded on 10% SDS-PAGE. A Mini Protean system (Bio-Rad) was used, and the separation gels contained 0.2 mg/ml purified recombinant human wt-5-LO or S271A-5-LO. After electrophoresis, 5-LO phosphorylation by activated kinases was analyzed by in-gel kinase assay as described previously (10).

Immunoprecipitation and in Vitro Kinase Assay—For preparation of immunoprecipitates (IPs), MM6 cell incubations were stopped by the addition of 2 volumes of ice-cold stop buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, and 2 mM Na_3VO_4) and cooled on ice. After about 2 min on ice, cells were pelleted (500 × *g*, 3 min, 4 °C) and lysed by the addition of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM NaF, 2 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM 4-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μM $ZnCl_2$, 10 μg/ml leupeptin, and 60 μg/ml soybean trypsin inhibitor). During 10 min in this buffer, the suspension was vortexed repeatedly (5 s bursts) to assure complete lysis. Supernatants were obtained by centrifugation of the lysates (16,000 × *g*, 10 min, 4 °C) and kept on ice. To immunoprecipitate MK2, supernatants corresponding to 2×10^7 MM6 cells were incubated with 5 μl of MK2-antibody (Santa Cruz Biotechnology) for 2 h at 4 °C. The immune complexes were precipitated (2 h at 4 °C) with 20 μl of protein A/G Plus-agarose (Santa Cruz Biotechnology) and washed twice with lysis buffer and twice with kinase buffer. For *in vitro* phosphorylation studies, purified recombinant wt-5-LO, S271A-5-LO, and recombinant Hsp-27 (40 pmol each) were preincubated in the absence or presence of fatty acids in kinase buffer (25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4) for 5 min at room temperature. Then, kinase (MK2-IPs, MK2, CaMKII, and catalytic subunit of PKA) was added and the reaction was started by the addition of ATP (100 μM) and [γ-^{32}P]ATP (100 μCi/ml). The final volume was 20 μl, and incubation time was 30 min at 30 °C. The reaction was terminated by the addition of the same volume of SDS-b and heating at 95 °C for 6 min. Samples (20 μl) were separated by SDS-PAGE, and proteins were first visualized by Coomassie staining to assure correct loading of protein. Phosphorylated proteins were then visualized by autoradiography and quantitated by densitometry using a Gel Doc 1000 instrument and the Molecular Analyst software (Bio-Rad), or alternatively they were analyzed with a Phosphoimager (Fuji FLA-3000). Here we define 1 milliunit of kinase activity (MK2, CaMKII, and the catalytic subunit of PKA) as incorporation of 1 pmol of phosphate into a standard substrate peptide. Since the standard substrates used by the suppliers are different for the different kinases, the unit amounts are not strictly comparable.

In-gel Digestion of 5-LO and Two-dimensional Phosphopeptide Mapping—Purified recombinant 5-LO (3 μg or 40 pmol) was phosphorylated *in vitro* by activated MK2 (10 milliunits) in the presence of ATP (100 μM) and [γ-^{32}P]ATP (2 μCi/ml) with or without 50 μM AA as described above. After separation of proteins by SDS-PAGE, 5-LO was excised from the gel, and in-gel digestion of 5-LO by trypsin was performed as described (29). The gel was extracted by acidic, basic, and lipophilic extraction methods to recover all peptides, and successful digestion was confirmed by MALDI analysis (matrix-assisted laser desorption/ionization). The tryptic digests were resuspended in glacial acetic acid and subjected to thin layer electrophoresis and subsequent ascending chromatography as described (30). After air drying the phosphopeptides were visualized using a Phosphoimager Fuji FLA-3000.

Subcellular Fractionation—Isolated human PMNL (3×10^7 in 1 ml PGC buffer) were incubated for 5 min at 37 °C with the indicated additives. Samples were chilled on ice, and nuclear and non-nuclear fractions were obtained after cell lysis by 0.1% Nonidet P-40 as described previously (11). Aliquots of nuclear and non-nuclear fractions were immediately mixed with the same volume of SDS-b, heated for 6 min at 95 °C, and analyzed for 5-LO protein by SDS-PAGE and immunoblotting.

Western Blot—Subcellular fractions or total cell lysates were separated by SDS-PAGE using a Mini Protean system (Bio-Rad) on a 4–15% linear gradient gel. After electroblot to nitrocellulose membrane (Hybond C, Amersham Biosciences), blocking with 5% nonfat dry milk in 50 mM TBS (Tris-HCl, pH 7.4, and 100 mM NaCl), membranes were washed and then incubated with primary antibody for overnight at 4 °C. A 5-LO column was used to produce an affinity-purified anti-5-LO antiserum (1551, AK7). Anti-p38 MAPK antibody was from Santa Cruz Biotechnology, and phospho-specific antibodies recognizing p38 MAPK (Thr-180/Tyr-182) were obtained from New England Biolabs and used as 1:2,000 dilution. Immunoreactive proteins were visualized using alkaline phosphatase-conjugated IgGs as described (5).

Determination of 5-Lipoxygenase Product Formation—Cells (5×10^6 PMNL, HeLa 2×10^6) in 1 ml of PGC buffer were stimulated by the addition of exogenous AA at the indicated concentrations with or without ionophore. After 5 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 ml of 1 N HCl, and then 200 ng of prostaglandin B_1 and 500 μl of PBS were added. 5-LO metabolites were extracted and analyzed by HPLC as described (31). 5-LO activity is expressed as pmol of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans*-isomers, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid, 5-HPETE and 5-HETE.

RESULTS

Ser-271 Is Required for 5-LO Phosphorylation by Kinases from MM6 Cells and PMNL—We showed that 5-LO is a substrate for p38 MAPK-regulated MKs *in vitro*, and we identified a MK2 phosphorylation motif (hyd-Xaa-Arg-Xaa-Xaa-Ser) within the primary sequence of 5-LO with Ser-271 as the putative phosphorylation site (10). To determine the sites of 5-LO phosphorylation by MKs, Ser-271 was mutated to alanine. Phosphorylation of wt-5-LO and S271A-5-LO was analyzed by in-gel kinase assays using lysates from activated MM6 cells and PMNL as sources for active kinases. Using wt-5-LO as substrate, lysates of ionophore-stimulated MM6 cells and ionophore (or fMLP)-stimulated PMNL contained 5-LO kinase activities migrating at ~40, 47, and 55 kDa (Fig. 1). These bands agree which the positions of MK2 and MK3 (compare Ref. 10). With samples from ionophore-stimulated MM6 cells, particularly the kinase activity migrating at 47 kDa (presumably MK2) was reduced when mutated S271A-5-LO was used as substrate. However, kinase activities at 55 and 40 kDa were still observed. When mutated S271A-5-LO was used as substrate for kinases prepared from PMNL, activities migrating at 47 and 40 kDa were very weak, but a 55-kDa kinase activity remained, which is best seen with the sample from cells stimulated with fMLP. It appears that kinase activities migrating close to 40 kDa were different in MM6 cells and PMNL. This activity from MM6 cells remained with S271A-5-LO as substrate, but for PMNL samples (apparently two bands) it was

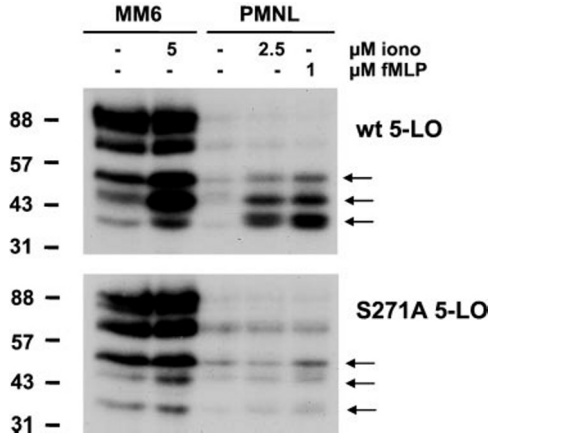

FIG. 1. **Phosphorylation of wild-type and mutated 5-LO proteins by cellular kinases.** MM6 cells (2.5×10^7) and PMNL (5×10^7) in 1 ml of PGC buffer were stimulated with ionophore or fMLP at the indicated concentrations for 3 min at 37 °C. Incubations were terminated by addition of the same volume of SDS-b, vortexed, and heated at 95 °C for 6 min. Aliquots of total cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel that had been polymerized in the presence of 0.2 mg/ml either wt- or S271A-5-LO. 5-LO phosphorylation was analyzed by an in-gel kinase assay as described under "Experimental Procedures." The *arrows* indicate kinase activities migrating close to 55, 47, and 40 kDa.

practically absent with S271A-5-LO as substrate. Since MK3 recognizes the same motif as MK2 (32–34), this further confirms that the PMNL 40 kDa band is MK3. In summary, Ser-271 is important for phosphorylation of 5-LO, but kinases recognizing other motifs were present, particularly in extracts from stimulated MM6 cells. The MM6 cell kinase activities migrating above 57 kDa appeared also without 5-LO in the gel, which is believed to reflect autophosphorylation of various kinases (10).

Arachidonic Acid Enhances 5-LO Phosphorylation by MK2 in Vitro—To estimate the efficiency of 5-LO phosphorylation by MK2, we compared 5-LO and Hsp-27 as substrates for MK2, by *in vitro* kinase assays. Hsp-27 is phosphorylated by MK2 at three serine residues, most efficiently on Ser-82 followed by Ser-78 and Ser-15 (33). Purified 5-LO (40 pmol) or human Hsp-27 (40 pmol) was incubated with the same amounts of active recombinant MK2 in *in vitro* kinase assays, and phosphorylated proteins were visualized and quantitated after SDS-PAGE. As shown in Fig. 2*A*, 5-LO is a rather weak substrate for activated MK2 (10 milliunits) compared with Hsp-27. Densitometric analysis of phosphorylated bands obtained after audioradiography revealed that MK2 is ~20–30-fold more active toward Hsp-27 than toward 5-LO. Similar differences in phosphorylation were obtained when the amount of substrates or amount of MK2 was varied. Co-incubation of 5-LO together with Hsp-27 did not reduce phosphorylation of Hsp-27, indicating that 5-LO had no inhibitory effect on kinase activity of MK2 or any phosphatase activity (data not shown).

AA dose-dependently enhanced 5-LO phosphorylation by MK2, about 3-fold at 1 μM AA and up to about 30-fold at 50 μM AA as shown in Fig. 2*B* (*left panel*). Thus, in the presence of AA, MK2 efficiently phosphorylates 5-LO, comparable with Hsp-27. Ca^{2+} (0.01–1 mM) and phosphatidylcholine (20 μg/ml), as well as cellular soluble or particulate fractions (which can increase catalytic activity of 5-LO *in vitro*) gave no such up-regulation but rather reduced MK2 activity toward 5-LO in the absence or presence of AA (data not shown). In contrast to 5-LO, no increase in phosphorylation of Hsp-27 was obtained after adding AA to the reaction mixture (Fig. 2*B*, *right panel*). Higher amounts of MK2 augmented Hsp-27 phosphorylation dose-dependently, indicating that substrate supply was not a limiting factor.

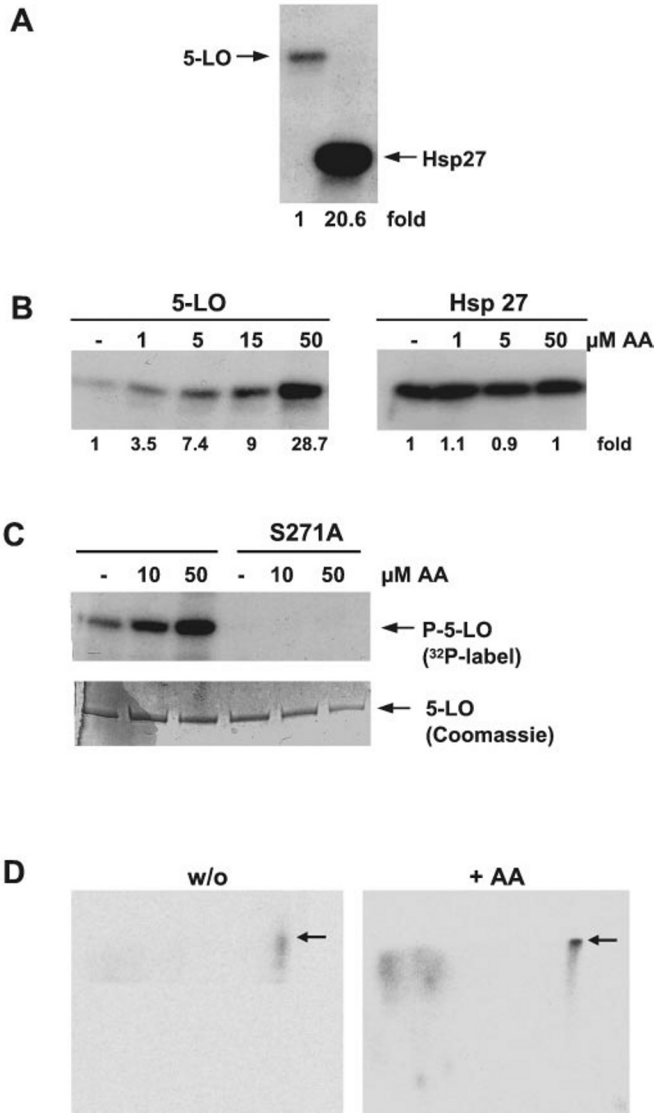

FIG. 2. **Arachidonate stimulates 5-LO phosphorylation by MK2 at Ser-271.** Phosphorylation of 5-LO proteins and Hsp-27 by MK2 was determined by *in vitro* kinase assays as described under "Experimental Procedures." The same amounts (40 pmol) of 5-LO proteins and Hsp-27 was incubated with 10 milliunits of active MK2 and the indicated amounts of AA. After incubation at 30 °C for 30 min, the samples were heated and aliquots were subjected to SDS-PAGE. Phosphorylated proteins were visualized by autoradiography and quantitated by densitometry. *A*, comparison of phosphorylation of 5-LO and Hsp-27. The relative phosphorylation of the proteins (shown *under* the lanes) was calculated as the ratio of the radioactivity intensities of phosphorylated Hsp-27 and phosphorylated 5-LO. Results are representative of three separate experiments. *B*, effects of increasing amounts of AA on MK2 activity toward 5-LO and Hsp-27. The relative phosphorylation of 5-LO and Hsp-27, respectively, was calculated as the ratio of the radioactivity intensities within each series. Results are representative of three separate experiments. *C*, phosphorylation of wt- and S271A-5-LO proteins by MK2 and effects of AA. *D*, two-dimensional tryptic phosphopeptide maps of 5-LO, phosphorylated in the absence (*w/o*, *left panel*) or presence of AA (*right panel*). 5-LO (40 pmol) was incubated with 10 milliunits of active MK2 with or without 50 μM AA for 30 min at 30 °C, and proteins were separated by SDS-PAGE. After in-gel digestion of 5-LO by trypsin, the phosphopeptides were separated in two dimensions on thin layer chromatography plates as described under "Experimental Procedures." The *arrows* indicate the major phosphopeptide.

This effect of AA could be related to phosphorylation of other sites in 5-LO, in addition to Ser-271. Therefore, we investigated whether AA induced phosphorylation of the S271A mutant by MK2. The same amounts of wt- and S271A-5-LO proteins were used in *in vitro* kinase assays with active MK2 as kinase (Fig.

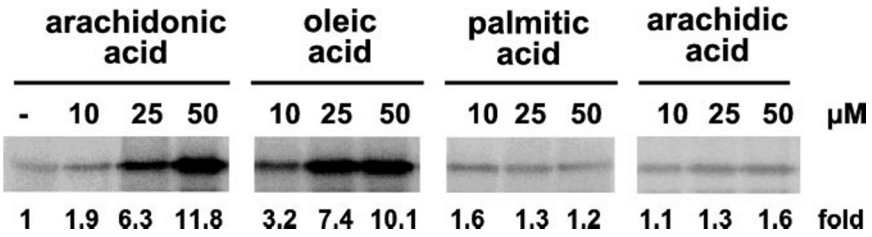

FIG. 3. **Only unsaturated fatty acids stimulate 5-LO phosphorylation by MK2.** The effects of fatty acids on 5-LO phosphorylation were determined by *in vitro* kinase assays as described under "Experimental Procedures." 5-LO (40 pmol) was incubated with 10 milliunits of MK2 in the absence or presence of the indicated fatty acids at 10, 25, or 50 μM. Proteins were separated by SDS-PAGE, and phosphorylated 5-LO was visualized and quantitated by Phosphoimager. The relative phosphorylation of the samples was calculated by the ratio of the radioactivity intensities, and the control without fatty acid was set as 1. Results are representative of three separate experiments.

2*C*, *upper panel*). Prior to drying and exposure of the gels, Coomassie staining was performed to ensure correct loading of 5-LO proteins (*lower panel*). As shown in Fig. 2*C*, S271A-5-LO was not a substrate for MK2 *in vitro* neither in the absence or presence of AA.

Enhanced 5-LO phosphorylation in the presence of AA might be due to phosphate incorporation at additional sites of 5-LO by MK2, which require initial phosphorylation at Ser-271. Therefore, two-dimensional phosphopeptide analyses of 5-LO incubated with MK2 in the presence or absence of AA (50 μM) were performed. As seen in Fig. 2*D*, only one major phosphopeptide was detectable in both samples. For the sample derived from 5-LO that was phosphorylated in the presence of 50 μM AA, the signal of this phosphopeptide was much more intense as compared with the control. Thus, AA apparently increases 5-LO phosphorylation at one major site (Ser-271) but does not lead to phosphorylation at additional sites. The faint spots on the left of the plates (seen for both samples) might result from incomplete trypsin digestion.

Unsaturated but Not Saturated Fatty Acids Stimulate Phosphorylation of 5-LO—We also determined the capability of other long chain fatty acids to stimulate phosphorylation of 5-LO by MK2. The results in Fig. 3 illustrate that oleic acid (C18:1) was effective. Compared with AA, the concentration of oleic acid required for the same effect appeared to be slightly lower. Similar dose-dependent up-regulation of 5-LO phosphorylation by MK2 was observed with linoleic acid (C18:2) and linolenic acid (C18:3) at 10–50 μM (data not shown). In contrast, arachidic acid (C20:0) (the saturated derivative of AA) or palmitic acid (C16:0) up to 50 μM caused only marginal enhancement of 5-LO phosphorylation (Fig. 3). Also oxygenated metabolites of polyunsaturated fatty acids (13-HPODE, 5-HPETE, LTB_4) gave only slight (about 2–4-fold) increase in 5-LO phosphorylation (not shown). No enhancement of phosphorylation was obtained with esterified fatty acids (oleyl-acetyl-glycerol, mixed phosphatidylcholines; data not shown). These findings indicate that efficient phosphorylation of 5-LO by MK2 *in vitro* requires the presence of free unsaturated fatty acids.

Arachidonic Acid Specifically Stimulates 5-LO Phosphorylation by MK2 but Not by CaMKII or PKA—The MK2 phosphorylation motif, hyd-Xaa-Arg-Xaa-Xaa-Ser, is also recognized by other basic amino acid-directed Ser/Thr kinases (for example PKC, PKA, and CaMK II and IV). Thus, we tested whether kinases other than MKs could phosphorylate 5-LO in *in vitro* kinase assays (Fig. 4). The catalytic subunit of PKA as well as CaMKII phosphorylated 5-LO in a dose-dependent manner. Interestingly, PKA and CaMKII also phosphorylated Hsp-27 (not shown). The amounts of kinases used are given in units as provided by the suppliers. Please note that the unit definitions for these kinases are based on different synthetic substrates and may not be directly comparable. As found for MK2, PKA was unable to phosphorylate S271A-5-LO, indicating that PKA also acts on the Ser-271 residue (not shown). As demonstrated in Fig. 5, the presence of AA (or oleic acid, not shown) during the kinase reaction did not enhance the phosphorylation rates of CaMKII and PKA toward 5-LO. In contrast, fatty acids rather inhibited the kinase activity of CaMKII toward 5-LO as well as its autophosphorylation (Fig. 5) as observed by other investigators (35).

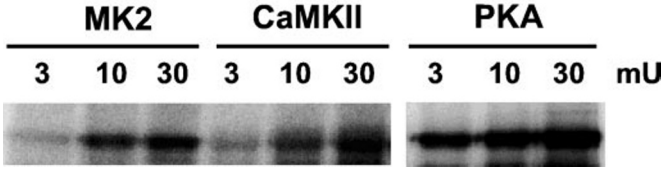

FIG. 4. **5-LO is phosphorylated by CaMKII and PKA.** 5-LO phosphorylation was determined by *in vitro* kinase assay as described under "Experimental Procedures" except that 1 mM $CaCl_2$ and 50 μg/ml calmodulin were added to incubations with CaMKII. In all incubations 40 pmol of 5-LO was used. One milliunit of each kinase (MK2, CaMKII, and the catalytic subunit of PKA) incorporates 1 pmol of phosphate into a standard substrate peptide. Since the standard substrates are different for the different kinases, the unit amounts are not strictly comparable. Proteins were separated by SDS-PAGE, and phosphorylated 5-LO was visualized by Phosphoimager. Results are representative of three separate experiments.

Effect of Phosphorylation on Ca^{2+}-stimulated 5-LO Catalytic Activity in Vitro—To determine the effects of 5-LO phosphorylation by MK2, PKA, and CaMKII on the activity of the enzyme, purified recombinant 5-LO (0.2 μg) was preincubated with 10 milliunits of kinase in kinase buffer with 100 μM ATP (20 μl final volume). After 30 or 60 min at 30 °C, 5-LO (0.1 μg in 10 μl) was added to a 5-LO activity assay substrate mix (990 μl, containing Ca^{2+}, phosphatidylcholine, ATP, 13-HPODE, and AA) to start the 5-LO reaction (compare Ref. 5). After 10 min the incubation was terminated, and 5-HPETE plus 5-HETE formation was determined by HPLC. In another set of incubations, kinases were added simultaneously (no preincubation) with the substrate mix. There was no appreciable effect on 5-LO activity, and at most about a 1.2-fold up-regulation was observed. Also, the activity of purified mutated S271A-5-LO protein in *in vitro* assay was comparable with the activity of wt-5-LO.

Arachidonic Acid Activates p38 MAPK-regulated 5-LO Kinases in Leukocytes—It was shown previously that p38 MAPK is activated by AA in a cell type-specific manner, for example in PMNL, HL60, and HeLa cells but not in a T cell line (Jurkat) (15). To determine the activation of p38 MAPK and 5-LO kinases by AA in MM6 cells and PMNL, cells were stimulated with increasing concentrations of AA for 3 min and lysed by the addition of SDS-b, and total cell lysates were prepared. Activation of p38 MAPK-regulated 5-LO kinases was analyzed by in-gel kinase assays using 5-LO as substrate. As shown in Fig. 6*A*, AA (10–100 μM) led to activation of 5-LO kinases in MM6 cells, particularly at 47 kDa (presumably MK2), which is sim-

ilar to the positive control (cells stimulated with ionophore) and to results with ionophore-stimulated MM6 cells in Fig. 1. In other experiments, activation of the 47-kDa kinase in MM6 cells was apparent already at 3 μM AA (not shown). Activation of MK2 in MM6 cells was determined also after immunoprecipitation of the kinase and subsequent *in vitro* kinase assay using 5-LO as substrate. 5-LO kinase activity was increased in MK2-IPs from cells stimulated with AA in a dose-response fashion (see Fig. 6*B*). At the highest concentration of AA (100 μM) 5-LO kinase activity was almost as high as that obtained with MK2-IPs from cells stimulated with 5 μM ionophore A23187. Activation of p38 MAPK was determined by Western blotting using an antibody that detects only the dually phosphorylated (activated) form of the kinase. Stimulation of MM6 cells with AA led to activation of p38 MAPK (Fig. 6*C*), which seemed to correlate with the activation of 5-LO kinases (compare Fig. 6, *panels A* and *B*). Similarly, 5-LO kinase activity was obtained in AA-stimulated PMNL when cell lysates were analyzed by in-gel kinase assay (Fig. 6*D*). In contrast to cells stimulated with ionophore, lysates of AA-challenged PMNL gave only weak bands for the 40-kDa kinase (presumably MK3).

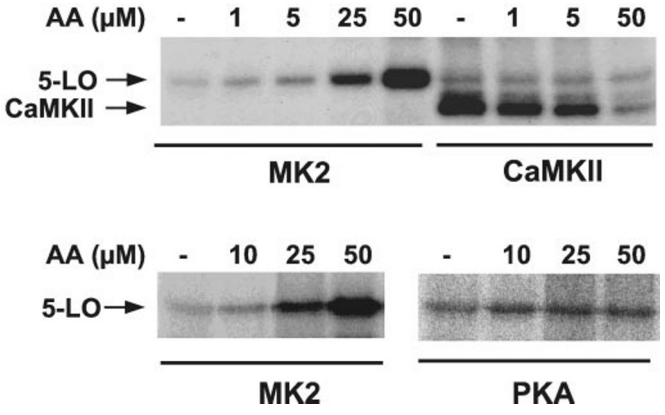

FIG. 5. **Arachidonic acid stimulates 5-LO phosphorylation by MK2 but not by CaMKII and PKA.** Effects of AA on phosphorylation of 5-LO by different kinases was determined by *in vitro* kinase assays as described under "Experimental Procedures" except that 1 mM $CaCl_2$ and 50 μg/ml calmodulin were added to incubations with CaMKII. 40 pmol of 5-LO and 10 milliunits of MK2, CaMKII, and the catalytic subunit of PKA were used. For unit definitions, see the legend for Fig. 4. Proteins were separated by SDS-PAGE, and phosphorylated 5-LO was visualized by autoradiography. Results are representative of three separate experiments.

AA Stimulates 5-LO Activity and Translocation to the Nucleus in PMNL—In a recent report it was shown that exogenous AA induced 5-LO enzyme activity and translocation to the nucleus in adenosine-depleted PMNL (36), and we have confirmed that AA also contributes to nuclear translocation of 5-LO in MM6 cells (11). Here we found that stimulation of PMNL with AA (5–50 μM) resulted in a modest but dose-dependent 5-LO activation (Fig. 7*A*). For comparison, in different experiments the 5-LO activity induced by 40 μM AA was 10–20% of the activity obtained after stimulation with ionophore only (2.5 μM) without the addition of exogenous AA (data not shown). Already in resting cells, a small amount of 5-LO was associated with the nucleus, but a high concentration of AA

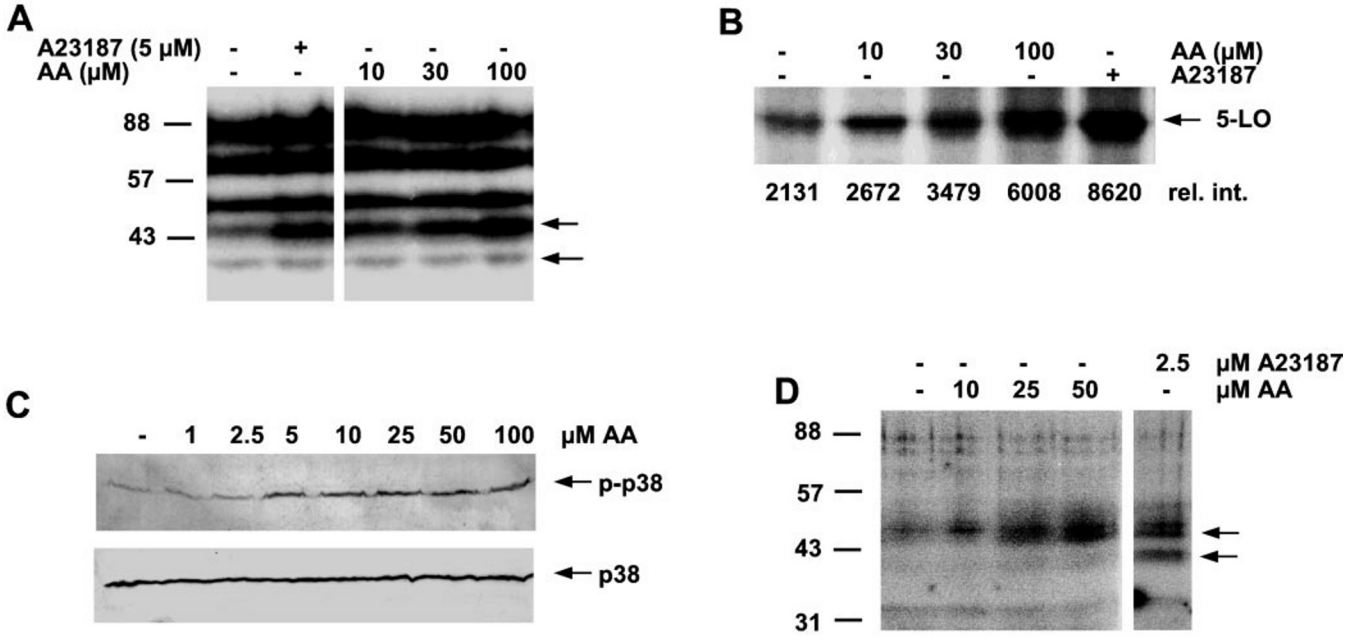

FIG. 6. **Stimulation of p38 MAPK and p38 MAPK-regulated 5-LO kinases by AA.** *A*, MM6 cells (2.5×10^6) in 100 μl of PGC buffer were stimulated with ionophore or AA at the indicated concentrations for 3 min at 37 °C. Incubations were terminated by addition of the same volume of SDS-b, vortexed, and heated at 95 °C for 6 min. Aliquots (0.25×10^6) of total MM6 cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel that had been polymerized in the presence of 0.2 mg/ml 5-LO. 5-LO phosphorylation was analyzed by in-gel kinase assay as described under "Experimental Procedures." Results are representative of two separate experiments. The *arrows* indicate kinase activities migrating close to 47 and 40 kDa. *B*, MM6 cells (1×10^7 in 1 ml of PGC buffer) were incubated for 3 min at 37 °C as indicated, and MK2 was immunoprecipitated as described under "Experimental Procedures." MK2-IPs were incubated with purified recombinant 5-LO in the presence of 50 μM AA, ATP (100 μM), and [γ-^{32}P]ATP (2 μCi/ml). The final volume was 20 μl, and incubation time was 30 min at 30 °C. Proteins were separated by SDS-PAGE, and phosphorylated proteins were analyzed with a Fuji FLA-3000 Phosphoimager. *C*, MM6 cells (2.5×10^6) in 100 μl of PGC buffer were stimulated with AA at the indicated concentrations for 3 min at 37 °C. Incubations were terminated by addition of the same volume of SDS-b, vortexed, and heated at 95 °C for 6 min. Aliquots (0.25×10^6) of total MM6 cell lysates were electrophoresed, and immunoblotting was performed using a specific antibody that detects the dually phosphorylated form of p38 MAPK (*upper panel*). Equal sample loading was demonstrated with anti-p38 MAPK antibodies (*lower panel*). *D*, PMNL (5×10^6) in 100 μl of PGC buffer were stimulated with ionophore or AA at the indicated concentrations for 3 min at 37 °C. Incubations were terminated by addition of the same volume of SDS-b, vortexed, and heated at 95 °C for 6 min. Aliquots (0.5×10^6) of total PMNL cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel that had been polymerized in the presence of 0.2 mg/ml 5-LO. 5-LO phosphorylation was analyzed by in-gel kinase assay. The *arrows* indicate kinase activities migrating close to 47 and 40 kDa.

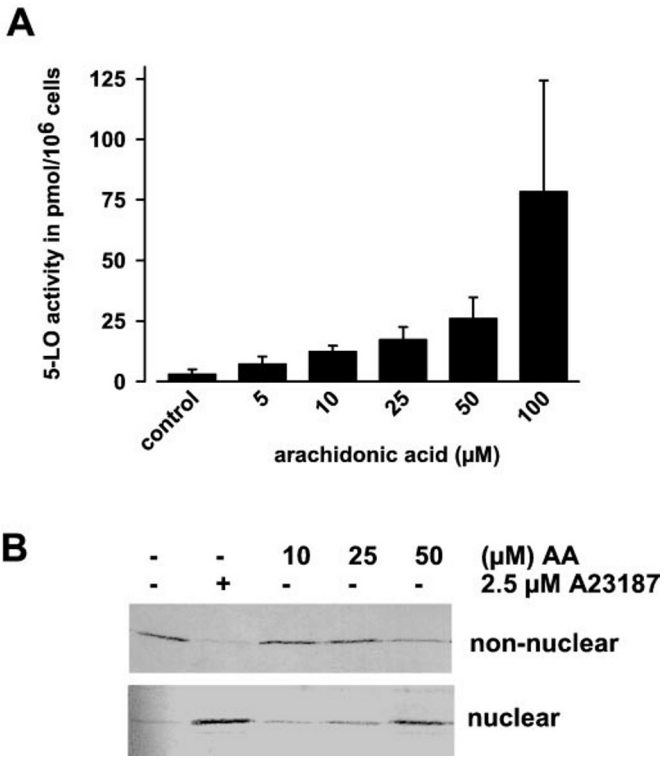

FIG. 7. **Effects of arachidonic acid on 5-LO product formation and 5-LO distribution in PMNL.** *A*, for determination of 5-LO product formation in intact cells, human PMNL (7.5×10^6 in 1 ml of PGC buffer) were stimulated with the indicated concentrations of AA for 5 min at 37 °C. 5-LO product formation was determined by HPLC as described under "Experimental Procedures." Results are given as mean ± S.E., $n = 3$. *B*, for determination of 5-LO distribution, PMNL (3×10^7 in 1 ml of PGC buffer) were stimulated with the indicated additives for 5 min at 37 °C. Cell fractionation and immunoblotting was performed as described under "Experimental Procedures." Pairwise samples (*non-nuclear* and *nuclear*) correspond to the identical cell numbers. Similar results were obtained in two additional independent experiments.

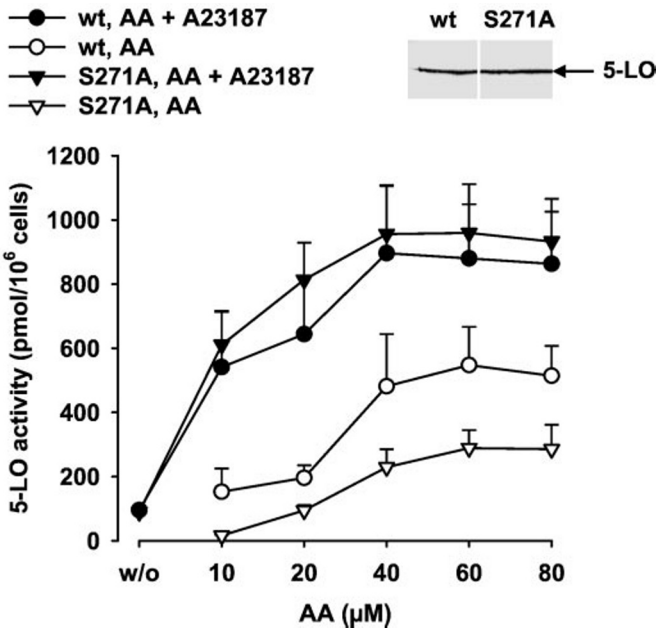

FIG. 8. **5-LO product formation in HeLa cells transformed with wt-5-LO and S271A-5-LO.** HeLa cells were transiently transformed with plasmids pcDNA3.1-5LO or pcDNA3.1-5LO-S271A (10 μg). Cells (2×10^6) were resuspended in 1 ml of PGC buffer and stimulated with the indicated concentrations of AA in the absence of or together with 10 μM ionophore for 10 min at 37 °C. 5-LO product formation was determined by HPLC. Results are given as mean ± S.E., $n = 4$. For one set of HeLa samples (total cell lysates corresponding to 0.1×10^6 cells) the expression of 5-LO proteins was analyzed by Western blot (*inset*).

was required (50 μM) for a clearly increased translocation of 5-LO to nuclear structures, comparable with that obtained with ionophore (Fig. 7*B*). Thus, we could confirm that exogenous AA leads to 5-LO translocation in PMNL, but stimulation of activity was seen at lower concentrations of AA than were needed to induce translocation. We performed these experiments in the absence of adenosine deaminase, which possibly explains the high concentration of AA required to induce translocation of 5-LO to the nucleus (50 μM as compared with 3 μM in Ref. 36).

Role of Ser-271 for 5-LO Product Formation in Transformed Cells—HeLa cells were transiently transformed with plasmids encoding wt-5-LO or S271A-5-LO. The expression levels of wt-5-LO and S271A-5-LO were similar, as determined by Western blot (*inset* in Fig. 8). Transformed cells were stimulated with ionophore plus exogenous AA or only with exogenous AA, and 5-LO products were determined by HPLC. As shown in Fig. 8, HeLa cells expressing wild type or mutated 5-LO, stimulated with both ionophore (10 μM) and AA (0–80 μM), gave similar prominent product formations. However, when transformed cells were stimulated with AA only (10–80 μM), 5-LO activity for the S271A mutant was significantly lower as compared with wt-5-LO. Particularly at 10 μM AA, 5-LO activities were strikingly different. 5-LO activity for the S271A mutant was 15 ± 2 pmol/10^6 cells compared with 155 ± 70 pmol/10^6 cells for the wt-5-LO. At 40–60 μM AA, for S271A-5-LO activities after incubation with AA were 24–30% of activities after incubation with AA plus ionophore; the corresponding numbers for wild-type 5-LO were 54–63%. Similar results were obtained also after transfections of HEK-293 cells. This indicates that phosphorylation at Ser-271 is more important for activation of 5-LO in cells stimulated only with AA as compared with cells receiving both ionophore and AA.

DISCUSSION

In a previous paper (10) we reported that MKs prepared from stimulated leukocytes could phosphorylate 5-LO *in vitro*, which could be one factor determining cellular 5-LO activity. Here we show that Ser-271 is a phosphorylation site in 5-LO. Thus, mutation of Ser-271 to Ala resulted in a protein that was no longer a substrate for active recombinant MK2 *in vitro*. Also, when S271A-5-LO was used as substrate in in-gel kinase assays, phosphorylation by kinases prepared from PMNL (probably MK2 and MK3) was severely hampered. Phosphorylation by kinases prepared from MM6 cells was also reduced (particularly the 47-kDa band containing MK2), but other kinase activities remained, indicating that MM6 cell kinases may recognize also other phosphorylation sites in 5-LO.

In comparison with Hsp-27, 5-LO was a rather poor substrate for MK2 *in vitro*. However, the addition of AA or other unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid) to the reaction mixture strongly stimulated phosphorylation of 5-LO by MK2. In the presence of 50 μM AA, phosphorylation of 5-LO and Hsp-27 were about equal. It was required that the fatty acid be non-esterified; oxygenated fatty acids (*e.g.* 13-HpODE, 5-HPETE, LTB_4) were less effective, and saturated fatty acids (arachidic acid and palmitic acid) had no effect. The motif hyd-Xaa-Arg-Xaa-Xaa-Ser (where hyd is a bulky hydrophobic residue) is recognized by MK2/3 and also by other kinases. Indeed, the catalytic subunits of PKA and CaMKII were also found to phosphorylate 5-LO *in vitro*. However, neither MK2-mediated phosphorylation of Hsp-27 nor 5-LO phosphorylation by other 5-LO kinases (PKA or CaMKII) were increased by AA. Rather, the activity of CaMKII was reduced,

and it was shown previously that fatty acids inhibit the activity of CaMKII (35) as well as of PKA type II holoenzyme but not of the catalytic subunit of PKA (37). The nature of the specific AA effect is unknown. Binding of AA may lead to a conformation change of 5-LO that favors the accession of Ser-271 by MK2. In this context, it is of interest that 5-LO (and other lipoxygenases) may have two fatty acid binding sites, one catalytic and one regulatory (38–40). Another possibility was that binding of AA to 5-LO could lead to exposure of another MK2 phosphorylation site in 5-LO. However, also in the presence of AA, there was no phosphorylation of S271A-5-LO by MK2 *in vitro*, and two-dimensional phosphopeptide mapping of trypsinized 5-LO revealed one major phosphopeptide after *in vitro* phosphorylation in the absence as well as presence of AA (Fig. 2*D*). Because phosphate incorporation into this peptide was much higher for the sample derived from 5-LO that was phosphorylated in the presence of AA, it seems that AA promotes 5-LO phosphorylation at Ser-271.

AA is released from phospholipids in many cell types (41), and it is well established that transcellular mechanisms for the release and uptake of free AA between cells occur (42, 43). In addition to its function as substrate for eicosanoid biosynthesis, AA modulates several signaling pathways at multiple levels. Free AA can modify the activity of phospholipases, protein kinases, G-proteins, adenylate, and guanylate cyclases, as well as ion channels (for review see Ref. 44). PKC is directly activated by cis-unsaturated fatty acids such as AA or oleate *in vitro*, and attention has been directed to the role of AA in activating PKC under physiological conditions (44, 45). AA can also lead to activation of p38 MAPK pathways in PMNL (15, 17) and in mammary carcinoma cells, where the activation of MK2 also was determined (16). The activation of p38 MAPK in human and rat PMNL seems to be independent of conversion to eicosanoids, but apparently it is partially PKC-mediated (15, 17). Also an involvement of Rac1 has been described in AA-induced p38 MAPK activation (46, 47); and quite recently it was presented that in Rat2-RacN17 cells (expressing a dominant negative Rac1 mutant), ionomycin-induced translocation of a GFP-5-LO fusion protein construct was impaired (48). We confirmed the activation of p38 MAPK and MK2 by AA for MM6 cells and PMNL (Fig. 6). AA is the most abundant free fatty acid in intact cells, and it has biological activity at concentrations that can exist in stimulated cells (1–20 μM) (15). In isolated islets of Langerhans, glucose was found to increase cell-associated free AA up to 75 μM (49). In our study, 1–50 μM AA led to an up to 29-fold increase of 5-LO phosphorylation by MK2 *in vitro*. Similar promotion of 5-LO phosphorylation by MK2 was observed with oleate, which was somewhat more effective than AA. Oleate is the most abundant (100–150 μM) extracellular free fatty acid in plasma (50), and it was demonstrated that oleate is released during stimulation of macrophages by lipopolysaccharide (51). It is intriguing that AA leads to activation of p38 MAPK in neutrophils, which in turn phosphorylates and activates MK2/3, and that AA also specifically stimulates the phosphorylation of 5-LO by MK2. Furthermore, AA is the most common 5-LO substrate, being converted to LTA_4; AA has been implicated in a novel pathway for non-capacitative Ca^{2+} entry (52). Thus, it appears that AA dose-dependent stimulation of LT biosynthesis (Fig. 6*A*) involves several mechanisms.

In optimized enzyme assays providing cofactors required for full activity of 5-LO *in vitro*, phosphorylation of 5-LO by MK2 (or PKA or CaMKII) caused no change in the (prominent) catalytic activity of the isolated enzyme. Also, in this assay mutated S271A-5-LO protein had approximately the same activity as 5-LO. These results indicate that phosphorylation may not directly affect catalysis of 5-LO *in vitro*. However, phosphorylation of 5-LO apparently alters product formation in intact cells. Thus, when eukaryotic cell lines lacking expression of endogenous 5-LO (HeLa, HEK-293) were transiently transfected with wt-5-LO and with the mutant S271A-5-LO, formation of 5-LO products was significantly lower for the mutant (than for wt-5-LO) when the cells were stimulated with AA only as compared with cells stimulated with AA and ionophore (Fig. 8). In PMNL stimulated with AA, the intracellular Ca^{2+} concentration increases but not to the same extent as caused by Ca^{2+} ionophores (13, 53, 54). In analogy with cytosolic phospholipase A_2 (55, 56), it seems possible that phosphorylation of 5-LO at Ser-271 is more important for 5-LO activity when cells are subjected to a stimulus that does not lead to a profound increase in intracellular Ca^{2+}. This is in accordance with our previous observation that sodium arsenite (which stimulates p38 MAPK, which in turn activates MK2) led to 4-fold increase in 5-LO activity in PMNL also receiving exogenous AA and platelet-activating factor, whereas sodium arsenite had no effect on cells also receiving ionophore (10). We also found that different forms of cell stress together with exogenous AA (no ionophore) was sufficient to stimulate 5-LO activity in BL41-E95-A lymphocytes and in PMNL even after chelation of Ca^{2+} (12, 13).

In a recent study utilizing adenosine-depleted PMNL, it was shown that AA-induced Ca^{2+} mobilization depends on conversion to LTB_4, and a model for AA-induced LT biosynthesis by autocrine stimulation was presented (36). This model implied that already membrane-bound 5-LO catalyzed an initial burst of LTB_4 biosynthesis in the absence of measurable Ca^{2+} mobilization. One could visualize that AA-induced phosphorylation of 5-LO as described in this study could contribute to such activation of 5-LO. An N-terminal C2-like β-barrel domain in 5-LO binds Ca^{2+} and mediates association of 5-LO with phospholipids (5–7). A comparison with other enzymes containing Ca^{2+} binding C2 domains suggests how phosphorylation could increase activity of 5-LO. For protein kinase C βII, it was proposed that phosphorylation at Ser-660 (outside of the C2 domains) stimulated activity by increasing the Ca^{2+} affinity and thus the affinity for phosphatidyl serine (57). For cytosolic phospholipase A_2, it was concluded that the C2 domain together with another region of the protein (subject to phosphorylation) both contributed to membrane binding and thus activity (55, 56). It appears possible that phosphorylation of 5-LO at Ser-271 could stimulate 5-LO activity by similar mechanisms.

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