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 stimuli 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, Ca2+/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 Ca2+/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 Ca2+ and that arachidonic acid stimulates leukotriene biosynthesis also by promoting this MK2-catalyzed phosphorylation.

5-Lipoxygenase (5-LO) 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 A2, 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 Ca2+ 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 LTC4 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 Ca2+/ionophore.

EXPERIMENTAL PROCEDURES

Materials—Human transforming growth factor β was purified from outdented platelets as described (26). 1,25-Dihydroxyvitamin D3 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, arachidonic acid, linoleic acid, linolenic acid, palmitic acid, oleic acid, Ca2+/ionophore A23187, and DMLP from Sigma; arachidonic acid from Nu-Chek (Elysian, MN); SB203580 from Calbiochem; [γ-32P]ATP (110 TBq/mmol) from Amersham Biosciences; HPLC solvents from Rathburn Chemicals (Walkerburn, Scotland); activated GST-MK2 and Ca2+/calmodulin-dependent kinase (CaMK) II...
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 D3 as described (10). Cells were harvested by centrifugation at 1,000 g for 5 min at room temperature and resuspended 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 Ca2+ and 1 mg/ml glucose).

Human recombinant 5-LO, S271A-5-LO, and recombinant Hsp-27 (40 pmol each) were transfected into cells with an electroporation protocol 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 inner desorption/ionization). The tryptic digests were resubmitted 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 x 107 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 Protein system (Bio-Rad) on a 4–15% gel. After electrophoresis, 5-LO phosphorylation by activated kinases was analyzed by in-gel kinase assays 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, 50 mM EDTA, and 0.5% NP-40, 2 mM Na3VO4, and 100 units/ml penicillin at 37°C for 6 min. Samples (20 μl) were separated by SDS-PAGE, and proteins were transferred 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 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-Lipoxgenase Product Formation—**Cells (5 x 106 PMNL, HeLa 2 x 105) 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 B3, and 500 μM 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 106 cells, which includes LTB4, and its all-trans-isomers, 5(5),12(15)-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) in 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 PMNL were shown to phosphorylate S271A-5-LO, indicating that this substrate has a serine residue that is phosphorylated by a MK2-like kinase. However, kinase activities at 55 and 40 kDa were observed with wt-5-LO when mutated S271A-5-LO was used as substrate. Therefore, 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
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-78 followed by Ser-271 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. 2A, 5-LO is a rather weak substrate for activated MK2 (10 milliunits) compared with Hsp-27. Densitometric analysis of phosphorylated bands obtained after autoradiography 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. 2B (left panel). Thus, the presence of AA, MK2 efficiently phosphorylates 5-LO, comparable with Hsp-27. Ca<sup>2+</sup> (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. 2B, right panel). Higher amounts of MK2 augmented Hsp-27 phosphorylation dose-dependently, indicating that substrate supply was not a limiting factor.
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 were ineffective. As seen in Fig. 2D, 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.

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. 2D, 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.

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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).

Effect of Phosphorylation on Ca2+-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 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 Ca2+, 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, HU80, AAH 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. 6A, 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. 6B). 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. 6C), 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. 6D). In contrast to cells stimulated with ionophore, lysates of AA-challenged PMNL gave only weak bands for the 40-kDa kinase (presumably MK3).

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 adenine-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. 7A). 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

![Figure 5](image)

**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 CaCl₂ 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.

![Figure 6](image)

**FIG. 6.** Stimulation of p38 MAPK and p38 MAPK-regulated 5-LO kinases by AA. A, MM6 cells (2.5 × 10⁶) 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 × 10⁶) 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 (Fig. 6D). In contrast to cells stimulated with ionophore, lysates of AA-challenged PMNL gave only weak bands for the 40-kDa kinase (presumably MK3) of 5-LO.

The effects of AA on 5-LO phosphorylation were also investigated in vitro. Aliquots (0.2 mg/ml 5-LO) were stimulated with ionophore or AA at the indicated concentrations for 3 min at 37 °C. Proteins were separated by SDS-PAGE, and phosphorylated 5-LO was visualized by autoradiography. Results are representative of three separate experiments.

**A**

![Image](image)

**B**

![Image](image)

**C**

![Image](image)

**D**

![Image](image)
Arachidonic Acid Promotes Phosphorylation of 5-Lipoxygenase

**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-HETE, LTB_4) were less effective, and saturated fatty acids (arachidonic 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 lipooxygenases) 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 trypticized 5-LO revealed one major phosphopeptide after in vitro phosphorylation in the absence as well as absence of AA (Fig. 2D). 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 intracellular 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 lipo polysaccharide (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 LTA4; AA has been implicated in a novel pathway for non-capacitative Ca2+ entry (52). Thus, it appears that AA dose-dependent stimulation of LT biosynthesis (Fig. 6A) 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 Ca2+ concentration increases but not to the same extent as caused by Ca2+ ionophores (13, 53, 54). In analogy with cytosolic phospholipase A2, 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 Ca2+. 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 Ca2+ (12, 13).

In a recent study utilizing adenosine-depleted PMNL, it was shown that AA-induced Ca2+ mobilization depends on conversion to LTB4, 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 LTB4 biosynthesis in the absence of measurable Ca2+ 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 Ca2+ and mediates association of 5-LO with phospholipids (5–7). A comparison with other enzymes containing Ca2+ 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 Ca2+ affinity and thus the affinity for phosphatidyl serine (57). For cytosolic phospholipase A2, 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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