Phosphorylation by Protein Kinase A Inhibits Nuclear Import of 5-Lipoxygenase*

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The enzyme 5-lipoxygenase initiates the synthesis of leukotrienes from arachidonic acid. Protein kinase A phosphorylates 5-lipoxygenase on Ser523, and this reduces its activity. We report here that phosphorylation of Ser523 also shifts the subcellular distribution of 5-lipoxygenase from the nucleus to the cytoplasm. Phosphorylation and redistribution of 5-lipoxygenase could be produced by overexpression of the protein kinase A catalytic subunit α, by pharmacological activators of protein kinase A, and by prostaglandin E2. Mimicking phosphorylation by replacing Ser523 with glutamic acid caused cytoplasmic localization; replacement of Ser523 with alanine prevented phosphorylation and redistribution in response to protein kinase A activation. Because Ser523 is positioned within the nuclear localization sequence-518 of 5-lipoxygenase, the ability of protein kinase A to phosphorylate and alter the localization of green fluorescent protein fused to the nuclear localization sequence-518 peptide was also tested. Site-directed replacement of Ser523 with glutamic acid within the peptide impaired nuclear accumulation; overexpression of the protein kinase A catalytic subunit α and pharmacological activation of protein kinase caused phosphorylation of the fusion protein at Ser523, and the phosphorylated protein was found chiefly in the cytoplasm. Taken together, these results indicate that phosphorylation of Ser523 inhibits the nuclear import function of a nuclear localization sequence, resulting in the accumulation of 5-lipoxygenase enzyme in the cytoplasm. As cytoplasmic localization can be associated with reduced leukotriene synthetic capacity, phosphorylation of Ser523 serves to inhibit leukotriene production by both impairing catalytic activity and by placing the enzyme in a site that is unfavorable for action.

Leukotrienes (LTs) are intercellular mediators that have important roles in pathologic as well as homeostatic inflammation (reviewed in Ref. 1). For instance, the overproduction of LTs contributes to a variety of diseases, including asthma (2), atherosclerosis (3), and fibrosis (4, 5). By contrast, the underproduction of LTs, as occurs in human immunodeficiency virus infection (6, 7) or malnutrition (8), results in compromised antimicrobial defense. The enzyme 5-lipoxygenase (5-LO) catalyzes the first two steps of LT synthesis from arachidonic acid. Therefore, the regulation of 5-LO action has been a focus of interest.

Previous studies have shown that the subcellular localization of soluble 5-LO before cell stimulation can affect the amount of LT secreted following cell stimulation. For example, import of 5-LO into the nucleus in neutrophils upon adherence increases LTB4 secretion upon subsequent stimulation (9). On the other hand, nuclear import of 5-LO following adherence rapidly inhibits LTC4 synthetic capacity in eosinophils (10). Whereas adherence can change 5-LO localization and LT production rapidly, cytokines alter these parameters more slowly. For example, interleukin-3 has been shown to increase the nuclear localization of 5-LO and increase LTC4 synthetic capacity, in eosinophils treated for 6 h (11). Also, differentiation of human cord blood-derived mast cells with interleukin-3 or interleukin-5 for 5 days increased both nuclear localization of 5-LO and LTC4 production upon cell stimulation (12). These results demonstrate that different factors can alter 5-LO localization and that 5-LO redistribution affects LT generation upon subsequent cell activation. However, little is known about the intracellular signaling pathways that alter 5-LO localization and consequent LT production.

We recently demonstrated that 5-LO can be phosphorylated by protein kinase A (PKA) on Ser523 (13). Numerous studies have demonstrated that factors that elevate cellular cAMP levels rapidly inhibit LT synthesis (e.g. Refs. 14 and 15). We found that phosphorylation of 5-LO, in cells or in vitro, as well as mimicking phosphorylation by substituting Ser523 with Glu, reduced the enzymatic activity of 5-LO. Thus, the direct phosphorylation of 5-LO by PKA may contribute to the reduction in LT synthesis that occurs following elevation of cellular cAMP.

Interestingly, Ser523 is embedded in one of the three nuclear localization sequences (NLS) of 5-LO, NLS518 (16). Because the function of a classical NLS is to bind with a karyopherin (importin) protein (17) to initiate import, we asked whether phosphorylation at Ser523 would inhibit the nuclear import of 5-LO. We report here, for the first time to our knowledge, that phosphorylation of 5-LO on Ser523 results in an accumulation of 5-LO in the cytoplasm.

EXPERIMENTAL PROCEDURES

Plasmids, Mutagenesis, and DNA Construction—Plasmids containing the wild type (WT) 5-LO or the S23A mutant of 5-LO, alone in pcDNA or fused to green fluorescent protein (GFP) in pEGFP, have been described previously (13, 18). Plasmid containing the NLS518 peptide of 5-LO (encoding Val134-Leu535) in fusion with GFP in pEGFP has also been described previously (16); substitution of Ser523 with Glu in 5-LO or the NLS518 peptide was performed using the QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s directions. The double mutant, mutNLS112+S271A, was produced by site-directed mutagenesis of Ser271 in the previously described and characterized mutNLS112 of GFP/5-LO (19), where mutNLS112 is R115Q/K117Q/R120Q. Mutations and protein frame reading were verified by DNA sequence analysis (DNA Sequencing Core, University of Michigan). All oligonucleotides were synthesized by Integrated DNA Tech-

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nologies Inc. (Coralville, IA). Plasmid and oligonucleotide sequences are available upon request. The plasmid encoding the mouse PKA catalytic subunit α (Ca) was a gift of Dr. Michael D. Uhler (Department of Biological Chemistry, University of Michigan).

Cell Culture, Transfection, and Immunoblotting—NIH 3T3 cells obtained from ATCC (Manassas, VA) were grown under 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% calf serum and 100 units/ml each of penicillin and streptomycin (complete medium). Cells were plated at 70% confluency and transfected with plasmids using Polyfect transfection reagent (Qiagen) following the manufacturer’s instructions. 16–20 h posttransfection, cells were treated with complete medium containing various compounds including 100 μM forskolin (Calbiochem) and with and without 200 μM 3-isobutyl-1-methylxanthine (Calbiochem), 1 mM each of N⁶-benzoyladenosine-(6-bnz-) cAMP, 8-β-hexylaminoadenosine-cAMP, 8-bromo (8-br-) cAMP, dibutyryl-cAMP (all from Biolog Life Science Institute, Germany), with and without 1–30 μM H89 or 1–30 μM SB203580 (Calbiochem) for 3 h or various time points from 0 to 8 h. Following treatment, cells were washed with PBS, harvested by a rubber policeman, and lysed with 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid (Sigma) with complete protease inhibitor and phosphatase inhibitor cocktails (Sigma). In some cases, cell pellets were directly lysed in 1X SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) and heated twice in boiling water for 3 min. Protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with the previously described rabbit polyclonal antibody raised against phosphorylated 5-LO Ser⁵²³ (titer: 1:3000) and antibodies against purified human leukocyte 5-LO (a generous gift of Dr. J. Evans, Merck Research Laboratories; titer: 1:3000) or GFP (Santa Cruz Biotechnology, Inc.; titer: 1:500) followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Amersham Biosciences). Densitometry was performed using NIH Image software.

Indirect Immunofluorescence and Confocal Microscopy—NIH 3T3 cells grown on two-well chamber slides (BD Falcon™) were transfected with 0.5 μg of plasmid DNA overnight, then washed, and treated as described. For immunostaining, cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 25 min at room temperature, and permeabilized with 0.3% Triton X-100 in PBS containing 0.1% bovine serum albumin. The cells were then blocked with 0.1% bovine serum albumin in PBS, 0.3% Triton X-100 and incubated with rabbit phospho-5-LO antibody (p5-LO, titer: 1: 200) for 1 h at room temperature. Cells were washed in permeabilization buffer and incubated with rhodamine-conjugated goat anti-rabbit secondary antibody (titer: 1:250) in blocking buffer for 1 h. Mounting was done in mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA). Cells were visualized and imaged using a Nikon E600 microscope equipped for epifluorescence and digital image capture using a SPOT RT camera. Confocal microscopy was performed with a Bio-Rad MRC-600 laser confocal microscope.

Quantitation of Subcellular Distribution following PKA Activation—As described previously (16), 3T3 cells at 16 h posttransfection were treated with 1 μM 8-br-cAMP for various time points from 0 to 6 h. After fixation with 4% paraformaldehyde, 100 positive cells were scored as to whether nuclear fluorescence was greater than, equal to, or less than cytosolic fluorescence. Care was taken to avoid damaged, dead, or autofluorescent cells. Results from at least three independent transfections were used for statistical analysis. As a second approach, 100 individual cells from each time point after 8-br-cAMP treatment were scored for cytosolic and nuclear fluorescence intensity. Using Adobe Photoshop 6.0, grayscale digital images were adjusted to include the full black-to-white range, and representative gray values, from 0 (white) to 100 (black), were obtained for the cytoplasm and nucleoplasm. Cytosolic and nuclear values for each cell were summed to give total cellular fluorescence, and the percent fluorescence values for the nuclear compartment were calculated.

Measurement of Intracellular cAMP Production—After (20), 3T3 cells were plated until confluent in 6-well tissue culture dishes in complete medium. The medium was then replaced with serum-free medium, and the cells were exposed to prostaglandin (PG) E₂, the EP₂-selective agonist butaprost, the EP₄-selective agonist ONO-AE1-329 (final concentration of each 1 μM), or vehicle for the times indicated. Culture supernatants were aspirated, and the cells were lysed by incubation for 20 min with 0.1 M HCl (22 °C), followed by disruption using a cell scrapper. Intracellular cAMP levels were determined by enzyme-linked immunosorbent assay kit according to the manufacturer (Cayman Chemical, Ann Arbor, MI). PGE₂ and butaprost (supplied as the free acid) were from Cayman Chemical; ONO-AE1-329 was a generous gift from ONO Pharmaceutical. Compounds were dissolved in Me₂SO₄, and stock solutions were stored at ~80 °C until used in assays. Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

Statistical Analysis—Statistical significance was evaluated by one-way analysis of variance, using p < 0.05 as indicative of statistical significance. Pairs of group means were analyzed using the Tukey-Kramer posttest.

RESULTS

Persistent Phosphorylation on Ser⁵²³ of 5-LO—As phosphorylation of proteins can be transient, the effects of phosphorylation may also be transitory. To clearly evaluate the effects of phosphorylation of Ser⁵²³ on the localization of 5-LO, we co-transfected NIH 3T3 cells with plasmids encoding GFP/5-LO with or without Ca. In cells expressing only GFP/5-LO, the majority of the fluorescence was in the nucleus, co-localizing with DAPI-stained DNA (Fig. 1, A–C). As expected, these cells were negative for p5-LO. In cells expressing GFP/5-LO with Ca, the fluorescence was outside of the nucleus, in the cytoplasmic compartment (Fig. 1D). Staining for p5-LO matched the fluorescence pattern of GFP/5-LO almost exactly (Fig. 1E). These results demonstrated that the overexpression of the active catalytic subunit of PKA with GFP/5-LO resulted in the accumulation of 5-LO in the cytoplasm, rather than in the nucleus.

The phosphorylation of specific residues on proteins can be mimicked by substitution of the residue with an acidic amino acid, such as glutamic acid. The replacement of Ser⁵²³ with Glu on GFP/5-LO resulted in a striking redistribution from the nucleus (WT, Fig. 2, A and B) to the cytoplasm (S523E, Fig. 2, C and D). Thus, a single amino acid change, like co-transfection with Ca, produced cytoplasmic localization of GFP/5-LO.

Activation of PKA Leads to Phosphorylation and Redistribution of 5-LO—To study the effects of PKA activation on 5-LO localization, we first tested different agonists for their ability to phosphorylate 5-LO. The adenylyl cyclase activator forskolin, with or without the phosphodiesterase IV inhibitor 3-isobutyl-1-methylxanthine, as well as PKA-specific agonist 6-bnz-cAMP and the cAMP analogues 8-br-cAMP and dibutyryl-cAMP, were all able to elicit phosphorylation of 5-LO on Ser⁵²³, as indicated by positive immunostaining using the p5-LO antibody (Fig. 3A). However, the B site activator of PKA, 8-β-hexylaminoadenosine-cAMP, appeared to be a poor agonist. The treatment of cells overexpressing 5-LO with Ser⁵²³ replaced with Ala did not show phos-
phorylation in response to the PKA-specific agonist 6-bnz-cAMP (Fig. 3B). Based on these results, additional experiments focused on the effects of 6-bnz-cAMP and 8-br-cAMP on 5-LO phosphorylation and localization.

The kinetics of phosphorylation of 5-LO by PKA was evaluated using 6-bnz-cAMP, a potent, selective activator of PKA (21). Although significant phosphorylation of 5-LO was evident within 1 h after the addition of 6-bnz-cAMP, phosphorylation continued to increase over 6 h (Fig. 4). Modest phosphorylation of Ser523, as indicated by positive staining with the p5-LO antibody, was evident in untreated cultures. Enhanced phosphorylation could be seen as early as 15 min after treatment (data not shown). Also, treatment with the phosphatase inhibitors okadaic acid or calyculin A significantly increased phosphorylation at earlier time points (data not shown). Taken together, these results indicate that PKA activation results in a relatively slow but continuous phosphorylation of 5-LO, countered in part by dephosphorylation by endogenous phosphatase activity, with phosphorylated 5-LO accumulating over time.

Because persistent phosphorylation altered 5-LO localization, it was of interest to determine whether a time-dependent redistribution of 5-LO paralleled the time-dependent phosphorylation. Live cell imaging of individual cells indicated that treatment with either 6-bnz-cAMP or 8-br-cAMP resulted in a significant change in the subcellular localization of 5-LO within 1 h in some cells, whereas other cells did not respond at all (data not shown). To quantitate the change in localization within a population of cells, cultures were treated with 1 mM 8-br-cAMP for various times, then fixed, photographed, and scored for subcellular distribution of GFP/5-LO. In untreated cells overexpressing GFP/5-LO, essentially all cells had fluorescent nuclei (Fig. 5A). Following PKA activation, the difference in the fluorescence between the nucleus and cytoplasm diminished, with some cells having nuclei that were darker than the cytoplasm (Fig. 5, B and C). Quantitative analysis of nuclear fluorescence of untreated cells indicated that most cells exhibited moderate (Fig. 5D, N1) or strong (Fig. 5D, N2) nuclear accumulation of WT 5-LO. PKA activation resulted in a decrease in the
percentage of cells with strong nuclear fluorescence and an increase in cells with predominantly cytoplasmic fluorescence. Cells overexpressing GFP/5-LO with the S523A mutation showed moderate to strong nuclear fluorescence that was not affected by PKA activation (data not shown). A statistically significant decrease in the percentage of cells with predominantly nuclear fluorescence, paralleled by a significant increase in cytoplasmic predominant cells, was documented at 6 h after 8-br-cAMP treatment (Fig. 6A). Immunofluorescent staining of these cells with the p5-LO antibody revealed strong positive staining within the cytoplasm (Fig. 6B). Thus, PKA activation produced a time-dependent redistribution of 5-LO with accumulation of phosphorylated 5-LO in the cytoplasm.

From these results, it was not clear whether PKA phosphorylation of 5-LO occurred in the cytoplasm, in the nucleus, or both. Further examination of cells treated with 8-br-cAMP for 6 h and stained for p5-LO revealed that several cells with more nuclear than cytoplasmic GFP/5-LO also stained positive for p5-LO. Confocal analysis of these cells indicated that the majority of the p5-LO was in the cytoplasm (Fig. 7, top and bottom), although some was evident within the nucleus, particularly at the periphery (Fig. 7, middle). To further test whether cytoplasmic 5-LO is phosphorylated more than nuclear 5-LO, we compared the ability of PKA to target WT GFP/5-LO versus a mutant developed to alter 5-LO localization but not activity. This mutant, with substitutions to inactivate NLS112 and block phosphorylation of Ser271, has significantly stronger cytoplasmic localization than the WT protein (Fig. 8A). Activation of PKA with 8-br-cAMP produced greater phosphorylation of the mutant than WT GFP/5-LO, as determined by immunoblot analysis (Fig. 8B). These results indicate that although PKA may be able to phosphorylate 5-LO within the nucleus there appears to be greater targeting of 5-LO within the cytoplasm.

Effect of p38 Mitogen-activated Protein Kinase (MAPK) Inhibition on PKA Modulation of 5-LO—5-LO can be phosphorylated on Ser271 in response to stresses that activate p38 MAPK (22). We asked whether phosphorylation of Ser271 was independent of p38 MAPK activity in

FIGURE 5. Visual and quantitative evaluation of time-dependent change in 5-LO localization following PKA activation. Cells overexpressing GFP/5-LO were treated with 1 mM 8-br-cAMP for 0 (A), 2 (B), or 4 (C) h and visualized by fluorescent microscopy. D, the subcellular distribution of GFP/5-LO in individual cells, with and without 8-br-cAMP treatment, was measured as described under “Experimental Procedures.” Values less than 45% nuclear fluorescence indicated nuclear fluorescence less than cytoplasmic (N<C), 45–55% indicated balanced distribution (N=C), 55–70% nuclear greater than cytoplasmic (N>C), and >70% nuclear much greater than cytoplasmic (N>>C); numbers near cells shown in A–C are representative. C, population of cells with cytoplasmic 5-LO; N1, N2, two populations of cells with differing amounts of nuclear accumulation of 5-LO. Results are from one experiment, representative of four independent experiments.

FIGURE 6. Quantitative and visual characterization of GFP/5-LO subcellular localization after treatment with 8-br-cAMP for 6 h. A, after treating GFP/5-LO-overexpressing cells with 8-br-cAMP (1 mM), subcellular distribution of fluorescence was scored as nuclear (N>C), balanced between nucleus and cytoplasm (N=C), or cytoplasmic (N<C) in 100 cells. *, p < 0.05 versus untreated. B, fluorescent visualization of total GFP/5-LO (GFP), phosphorylated GFP/5-LO (p5-LO), or DNA (DAPI). Results are from one experiment and are representative of three independent experiments.

FIGURE 7. Confocal localization of phosphorylated GFP/5-LO following PKA activation. Cells overexpressing GFP/5-LO were stimulated with 8-br-cAMP for 6 h, fixed, and stained for phospho523 by indirect immunofluorescence. Images are optical sections of individual cells showing fluorescence for total 5-LO (GFP) and phospho523 5-LO (p5-LO).
vivo by treating cells with the selective p38 MAPK inhibitor SB203580. The PKA/PKG inhibitor H-89 demonstrated a dose-dependent inhibition of phosphorylation of 5-LO by 8-br-cAMP, whereas SB203580 was without effect up to 30 μM (Fig. 9A). It has been reported that the IC50 of SB203580 on p38 MAPK is 0.5 μM (23). In addition, 3T3 cells overexpressing GFP/5-LO retained nuclear localization of 5-LO and did not stain with the p5-LO antibody when treated with 8-br-cAMP (1 mM) plus H-89 (30 μM) (Fig. 9B). The inhibition of p38 MAPK with SB203580 (30 μM) did not block cytoplasmic localization and phosphorylation of Ser523 on 5-LO in response to 8-br-cAMP (Fig. 9B).

**FIGURE 9.** 8-Br-cAMP induced 5-LO phosphorylation and inhibition of nuclear import are abolished by the PKA inhibitor, H89, but not by the p38 MAPK inhibitor, SB203580. A, 3T3 cells were transiently transfected with GFP/5-LO for 16 h, and then treated with Me2SO, 1mM 8-br-cAMP, or 1mM 8-br-cAMP plus different concentrations of H89 or SB203580 for 3 h. Cells were washed with PBS, directly lysed in 1× SDS-PAGE loading buffer, boiled, and separated by 10% SDS-PAGE. Proteins were assessed by immunoblot for phosphorylation on Ser523 (p5-LO) and total 5-LO via anti-GFP antibodies. B, GFP/5-LO transfected 3T3 cells cultured on chamber slides were treated with 1 mM 8-br-cAMP plus 30 μM of H89 or SB203580 for 3 h, then were fixed and stained for phospho 5-LO (p5-LO) and DNA (DAPI). The localization of GFP/5-LO was monitored by the green fluorescence of the GFP/5-LO fusion protein. Results are from one experiment, representative of three independent experiments.

**FIGURE 8.** Greater phosphorylation of a mutant of 5-LO with more cytoplasmic protein. A, comparison of the subcellular distribution of WT GFP/5-LO versus GFP/5-LO with inactivation of the NLS112 import sequence plus mutation of the Ser271 site (mutNLS112+S271A). Results are from five independent experiments. B, comparison of the phosphorylation of 5-LO at various time points after activation of PKA using 8-br-cAMP. The ratio of band densities (×100) from samples probed for phosphorylated 5-LO and total 5-LO is presented graphically. Results are from one experiment, representative of two independent experiments.

**PKA Can Phosphorylate and Alter the Localization of the NLS518 Peptide**—We previously reported that the NLS518 peptide was sufficient to drive nuclear accumulation of GFP (16). We asked whether phosphorylation of GFP-tagged NLS518 on Ser523 would alter nuclear import. As expected, GFP alone was distributed diffusely throughout the cell (Fig. 10A), whereas GFP/NLS518 was strongly accumulated within the nucleus (Fig. 10C). Substitution of Ser273 with Glu, to produce a phosphorylation mimic within the GFP/NLS518 construct, resulted in a relatively diffuse distribution, consistent with little or no active nuclear import (Fig. 10E). Co-transfection of 3T3 cells with GFP/NLS518 and Ca resulted in more cytoplasmic than nuclear GFP fluorescence, with strong positive staining with the p5-LO antibody, particularly in the cytoplasm (Fig. 11, A–C). Finally, activation of PKA with 8-br-cAMP also resulted in strong positive staining with p5-LO, again in the cytoplasm (Fig. 11, D–F). In this treatment, the effect of phosphorylation on localization becomes most apparent, as abundant fluorescent protein is able to be accumulated in the nucleus when NLS518 is not phosphorylated and the majority of phosphorylated NLS518 is found in the cytoplasm. Taken together, these results suggest that the NLS518 peptide itself can be targeted by PKA and that when phosphorylated its ability to drive nuclear import is impaired.

**PGE2 Increases cAMP, Causes Phosphorylation of 5-LO, and Increases Cytoplasmic GFP/5-LO Localization**—PGE2 can inhibit leukotriene synthesis via a cAMP-dependent process (24). PGE2 acts through four distinct G protein-coupled receptor subtypes (EP1–4), with distinct signaling pathways. Because the Gs-coupled EP2 and EP4 receptors activate adenylate cyclase activity (25), we measured changes in intracellular cAMP in response to PGE2 (Fig. 12A). Treatment of 3T3 cells with PGE2 (1 μM) provoked an immediate elevation of cAMP, resulting in a 50% increase in cAMP 5 min after PGE2 exposure. Levels remained...
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FIGURE 10. Effect of substitution of Ser^{523} with Glu on the subcellular distribution of the GFP/NLS^{518} fusion protein. Cells were transfected with plasmids encoding GFP (A and B), GFP/NLS^{518} (C and D) or GFP/NLS^{518} S523E (E and F). After 20 h, cells were fixed and stained with DAPI, then imaged for expression protein (A, C, and E) or nuclear (B, D, and F) localization. Results are from one experiment, representative of three independent experiments.

DISCUSSION

As 5-LO serves the key function of initiating LT synthesis from arachidonic acid, it represents a primary point for regulating the generation of these potent proinflammatory mediators. Several studies have demonstrated that changes in the subcellular localization of 5-LO in resting leukocytes significantly affects the amount of LT produced when those leukocytes are activated. For example, we have recently used molecular modification of the import sequences of 5-LO to show that LTB4 production decreased, as less 5-LO could be imported into the nucleus (26). In that study and others, the positioning of 5-LO in the cytoplasm of resting leukocytes typically correlated with reduced production of LTs upon cell stimulation. In this study, we have examined the effects of phosphorylation of Ser^{523} on the localization of 5-LO. This residue is nested in NLS^{518}, identified as RGRKSSGFPSVK^{530} (16). Previous work demonstrated that phosphorylation of Ser^{523} significantly reduced the intrinsic enzymatic activity of 5-LO (13). We report here, for the first time to our knowledge, that phosphorylation of Ser^{523} also blocks nuclear import, resulting in the accumulation of 5-LO in the cytoplasm. Thus, phosphorylation of 5-LO achieves two effects that both serve to reduce cellular LT generation: a direct molecular effect on the intrinsic catalytic activity of 5-LO and a cellular effect of placing 5-LO in a subcellular compartment that is less favorable for arachidonic acid metabolism.

A very surprising result is the apparently slow rate of phosphorylation of 5-LO following PKA activation. One contributing factor is the activity of endogenous phosphatases. Elevated CAMP levels increase activity of phosphatases (27, 28), suggesting that our results might underestimate the true rate of phosphorylation. Consistent with this interpretation, we found that pretreatment with the phosphatase inhibitors okadaic acid or calyculin A resulted in stronger phosphorylation of 5-LO at earlier time points (data not shown). Thus, phosphorylation of 5-LO by PKA may occur in minutes, as observed in response to PGE_{2} treatment.

FIGURE 11. Phosphorylation of the NLS^{518} peptide by the catalytic subunit of PKA and by PKA activation. A–C, 3T3 cells were co-transfected with plasmids encoding both GFP/NLS^{518} and Cα and, after 16 h, were fixed and stained for p5-LO. D–F, cells overexpressing GFP/NLS^{518} were stimulated with 8-br-cAMP for 6 h then were fixed and stained for p5-LO. Images show GFP/NLS^{518} (GFP), p5-LO and DNA (DAPI). Results are from one experiment, representative of four independent experiments.
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FIGURE 12. Effect of PGE₂ on cAMP production and the phosphorylation and localization of 5-LO. A, time course of cAMP generation in 3T3 cells exposed to prostaglandin E₂. 3T3 cells were treated with PGE₂ (1 μM) or vehicle control for the times indicated followed by determination of intracellular cAMP as described under “Experimental Procedures.” Experiments were performed in duplicate on two separate occasions, and a representative result is shown. Data are expressed as relative to the vehicle-treated control (basal cAMP level = 4.29 pmol/ml). B, cells overexpressing 5-LO were stimulated with vehicle (DMSO), PGE₂ (1 μM), or 6-bnz-cAMP (1 mM) for the indicated times, then analyzed by immunoblot for p5-LO or total 5-LO. C, localization of GFP/5-LO in 3T3 cells after treatment with PGE₂ (1 μM) for 6 h. Arrows, cells with cytoplasmic fluorescence. In a parallel experiment, cells overexpressing GFP/5-LO were stimulated with PGE₂ for 6 h and then were fixed, stained, and imaged for GFP/5-LO (D) or p5-LO (E).

Dephosphorylation by phosphatases, then, would serve to limit the time of phosphorylation and its impact on LT synthesis.

The positioning of Ser523 within NLS518 suggests that this NLS is functional in promoting import when Ser523 is not phosphorylated, with phosphorylation inhibiting import perhaps by interfering with docking of karyopherins with the NLS. Activation of PKA, as following PGE₂ treatment with PGE₂ (1 μM) or total 5-LO. C, localization of GFP/5-LO in 3T3 cells after treatment with PGE₂ (1 μM) for 6 h. Arrows, cells with cytoplasmic fluorescence. In a parallel experiment, cells overexpressing GFP/5-LO were stimulated with PGE₂ for 6 h and then were fixed, stained, and imaged for GFP/5-LO (D) or p5-LO (E).

In summary, we report here that elevation of intracellular cAMP also inhibits the release of arachidonic acid (37–39). This effect is rapid, whereas the effects of PKA modulators were heterogeneous, with phosphorylation as well as redistribution of 5-LO occurring faster in some cells, slower in others, and not at all in some cells (data not shown). This may be because of the fact that a non-synchronized cell culture is heterogeneous. The lag between phosphorylation and redistribution of 5-LO suggests that phosphorylation may be the initial factor affecting 5-LO activity, with redistribution serving to increase or prolong the effects of phosphorylation.

It is important to note that elevation of intracellular cAMP also inhibits the release of arachidonic acid (37–39). This effect is rapid, whereas the effects of PKA modulators were heterogeneous, with phosphorylation as well as redistribution of 5-LO occurring faster in some cells, slower in others, and not at all in some cells (data not shown). This may be because of the fact that a non-synchronized cell culture is heterogeneous. The lag between phosphorylation and redistribution of 5-LO suggests that phosphorylation may be the initial factor affecting 5-LO activity, with redistribution serving to increase or prolong the effects of phosphorylation.

Previously, we reported that PKA modulators did not alter the subcellular distribution of 5-LO in the first 30 min after treatment (13). As shown in Fig. 5, the changes in 5-LO localization are slow, with only modest changes observable at 2-h posttreatment. We also found that the effects of PKA modulators were heterogeneous, with phosphorylation as well as redistribution of 5-LO occurring faster in some cells, slower in others, and not at all in some cells (data not shown). This may be because of the fact that a non-synchronized cell culture is heterogeneous. The lag between phosphorylation and redistribution of 5-LO suggests that phosphorylation may be the initial factor affecting 5-LO activity, with redistribution serving to increase or prolong the effects of phosphorylation.
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serve to reduce LT synthesis, these events may be important in the resolution of inflammation.

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