Protein Kinase A Inhibits Leukotriene Synthesis by Phosphorylation of 5-Lipoxygenase on Serine 523


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Abbreviations: AA, arachidonic acid; Calpha, protein kinase A catalytic subunit α; CREB, cAMP response element-binding protein; FLAP, 5-lipoxygenase activating protein; GFP, green fluorescent protein; LO, lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.
Summary

Leukotrienes (LTs) are lipid messengers generated by leukocytes that drive inflammation and modulate neighboring cell function. The synthesis of LTs from arachidonic acid is initiated by the enzyme 5-lipoxygenase (5-LO). We report for the first time that LT synthesis is inhibited by the direct action of protein kinase A (PKA) on 5-LO. The catalytic subunit of PKA directly phosphorylated 5-LO in vivo and in vitro and inhibited activity in intact cells and in vitro. Mutation of Ser523 on human 5-LO prevented phosphorylation by PKA and restored LT synthesis. Treatment with PKA activators inhibited LTB₄ synthesis in 3T3 cells expressing wild type 5-LO but not in cells expressing the S523A mutant of 5-LO. The mechanism of inhibition of LTB₄ synthesis did not involve either reduced membrane association of activated 5-LO or redistribution of 5-LO from the nucleus to the cytoplasm. Instead, PKA phosphorylation of recombinant 5-LO inhibited in vitro activity, as did co-transfection of cells with 5-LO plus the catalytic subunit of PKA. Also, substitution of Ser523 with glutamic acid, mimicking phosphorylation, resulted in total loss of 5-LO activity. These results indicate that PKA phosphorylates 5-LO on Ser523, which inhibits the catalytic activity of 5-LO and reduces cellular LT generation. Thus, PKA activation, as can occur in response to adenosine, prostaglandin E₂, β-adrenergic agonists, and other mediators, is a means of directly reducing 5-LO activity and LT synthesis that may be important in limiting inflammation and maintaining homeostasis.
Introduction

Leukotrienes (LTs) are intercellular messengers that are secreted by leukocytes and modulate the actions of neighboring cells (reviewed in (1)). For example, LTB4 potently attracts and activates leukocytes and in this way plays an important role in the normal inflammatory response. Indeed, the underproduction of leukotrienes results in impaired host defense, manifested as susceptibility to infection (2). However, the overproduction of LTB4 and cysteiny LTs contributes to several diseases, most notably chronic inflammatory diseases, including asthma (3), fibrosis (4) and atherosclerosis (5). Thus, processes that affect the capacity of leukocytes to synthesize and secrete leukotrienes are of clinical importance.

The enzyme 5-lipoxygenase (5-LO), in concert with the 5-LO activating protein (FLAP), initiates LT synthesis from arachidonic acid (AA), catalyzing its hydroperoxidation and dehydration to produce LTA4. This intermediate can then be converted by LTA4 hydrolase to give LTB4 or by LTC4 synthase to produce LTC4. Also important in LT generation is the source of the substrate, AA. This fatty acid may be derived endogenously, released from membrane phospholipids by phospholipases (PL), including the group IV cytosolic PLA2 (cPLA2) (7). Alternatively, AA may be exogenously derived, released from membrane phospholipids of neighboring cells by various PLA2s.

It was first demonstrated in 1970 that cyclic AMP (cAMP) can inhibit the release of cysteiny LTs, known then as slow-reacting substance of anaphylaxis (8). cAMP is formed from ATP by the action of adenylyl cyclase, an integral membrane protein which is activated by Gsα-protein coupled receptors following ligand binding. Elevated cAMP activates protein kinase A (PKA) by binding to its two inhibitory subunits, causing the release and activation of the catalytic PKA subunits. Specific phosphodiesterases hydrolyze cAMP to AMP to limit signaling
through this pathway. Since the seminal finding in 1970, subsequent studies have shown that LT synthesis is inhibited by ligands that activate Gsα-protein coupled receptors (9-11), by direct activation of adenylyl cyclase (12,13), by direct elevation of cAMP (14), and by inhibition of phosphodiesterases (15,16). Inhibition of LT synthesis by PKA activation has been demonstrated in all cells that produce LTs, including neutrophils (9,10,17), mast cells and basophils (12,18), eosinophils (15,19), and macrophages (20). These results suggest that the inhibition of LT synthesis by PKA activation is a broad and important phenomenon.

Although it is clear that PKA activation inhibits LT synthesis, our understanding of the mechanism remains incomplete. Activation of PKA can lead to phosphorylation of cPLA2 (19,21), and can decrease the release of AA (22,23). Alternatively, activation of PKA can be without effect (24) or can increase (25) the release of AA in other circumstances, perhaps because AA may be released by PLA2s other than cPLA2. There are, to our knowledge, no reports of PKA inhibition of either the 12-LO or 15-LO pathways, suggesting that these pathways are able to obtain AA during PKA activation and that these LOs are not inhibited by PKA. The production of LTB4 (10,17,20) as well as cysteinyl leukotrienes (12,15,18,19) is inhibited by activation of the PKA pathway, indicating that the actions of PKA are not directed at LTA4 hydrolase or LTC4 synthase. Taken together, these findings suggest that PKA may have strong effects at an early step in the synthesis of LTs from AA. In this study, we asked whether PKA could directly phosphorylate 5-LO and whether phosphorylation affected the enzymatic activity of 5-LO.
Experimental Procedures

Sequence and Structural Analysis – Amino acid sequences were obtained from Swiss-Prot from the ExPaSy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics. Primary accession numbers for proteins are: 5-LOs, human P09917, mouse P48999, rat P12527, hamster P51399; 15-LOs, rabbit P12630 and human P16050; human platelet-type 12-LO P18054. Alignment of protein sequences was performed using ClustalW (26). Structural analysis utilized the resolved structure of rabbit 15-LO (PDB: 11ox) and a published theoretical model of the 5-LO catalytic domain (27). Prosite analysis of 5-LO was performed at ExPaSy (http://us.expasy.org/cgi-bin/scanprosite).

Plasmids, Mutagenesis and DNA Construction – Plasmids containing the wild type (WT) 5-LO or the S271A mutant of 5-LO, alone in pcDNA or fused to GFP in pEGFP, have been described previously (28-30). Substitution of Ser522 or Ser523 with Ala or Ser523 with Glu on these plasmids was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following manufacturer’s directions. To generate GST fusion constructs, WT and mutant 5-LO cDNA sequences were amplified from the respective GFP/5LO plasmids by high fidelity PCR using Pfu DNA polymerase (Stratagene). The amplified cDNA fragments were purified and ligated into the PGEX-4T3 expression vector (Pharmacia) in frame with the carboxyl terminus of the GST coding sequence. Mutations and protein frame reading were verified by DNA sequence analysis (DNA Sequencing Core, University of Michigan). All oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Plasmid and oligonucleotide sequences are available upon request. The plasmid encoding the mouse PKA catalytic subunit α (plasmid: pCMVCα; protein: Calpha) was a gift of Dr. Michael D. Uhler (Department of Biological Chemistry, University of Michigan).
Protein Purification and In Vitro PKA Phosphorylation Assay – GST fusion proteins were expressed in *E. coli* strain BL21 and purified on glutathione-Sepharose beads (Pharmacia) following manufacturer’s instructions. Purified proteins were separated on a 10% SDS-polyacrylamide gel and stained by Coomassie blue to verify the integrity and relative concentrations of expressed proteins. To test 5-LO phosphorylation in vitro by PKA, respective GST-5LO fusion proteins (3 µg) were suspended in buffer containing 50 mM Tris-Cl (pH7.4), 10 mM MgCl₂, 1 mM DTT, 10 mM β-glycerophosphate, 50 µM ATP, 20 µCi [γ-³²P] ATP (Amersham), and 10 units of the catalytic subunit of PKA (P2645, Sigma) with the final volume of 30 µl per reaction. As a positive control, similar amount of cAMP response element-binding protein (CREB) produced in an *E.coli* expression system as a maltose binding protein fusion protein (MW=81 kDa, purchased from Biomol, Plymouth Meeting, PA) was used to compare the relative efficiency of in vitro PKA phosphorylation. To test the specificity of PKA phosphorylation, 5 µM of PKA inhibitor peptide (P0300, Sigma) was added in the reaction mixture to test the specificity of PKA phosphorylation. Phosphorylation reactions were conducted at 30 °C for 40 min and terminated by the addition of 6 µl of 6X SDS-PAGE loading buffer. ³²P-labelled fusion proteins were resolved on a 10% SDS-polyacrylamide gel, dried and detected by autoradiography.

Cell Culture, Transfection, Cellular LTB₄ Analysis and Immunoblotting – NIH 3T3 cells obtained from ATCC (Manassas, VA) were grown under 5% CO₂ in DMEM (Invitrogen Life Technologies) with 10% calf serum and 100 units/ml each penicillin and streptomycin. To evaluate the effect of PKA phosphorylation of 5-LO on cellular LTB₄ synthesis, 1.6x10⁶ cells were co-transfected with 2 µg of p5-LO or p5-LOS523A and increasing amounts of pCMVα using Polyfect transfection reagent (Qiagen) following manufacturer’s directions. To measure
the effect of PKA inhibitor or activators on cellular LTB₄ synthesis, 3T3 cells were transfected
with pGFP/5-LO or pGFP5-LOS523A and, 16 h after transfection, cells were treated with 10 µM
H-89 (Calbiochem) for 2 h, or 50 µM forskolin (Calbiochem) plus 45 µM 3-isobutyl-1-
methylxanthine (IBMX, Calbiochem) for 15 min, or 1 mM dibutyryl cAMP (Sigma) for 15 min.
To stimulate LTB₄ production, cells were incubated with serum-free DMEM containing 1.6 mM
CaCl₂, 10 µM calcium ionophore A23187 and 10 µM AA at 37 °C for 15 min. Immunoreactive
LTB₄ in conditioned media was quantitated by ELISA (Cayman Chemical, Ann Arbor, MI)
following supplier’s instructions. The detection limit for LTB₄ was 4 pg/ml; cross-reactivity for
AA, 5-HETE, LTC₄, LTD₄ and LTE₄ was <0.01%. Following stimulation, cells were lysed in
SDS-PAGE sample buffer, boiled and 20 µl total cell protein was separated by SDS-PAGE and
transferred to nitrocellulose. Membranes were probed with rabbit polyclonal antibodies raised
against purified human leukocyte 5-LO (a generous gift of Dr. J. Evans, Merck Research
Laboratories, Rahway NJ; titer 1:3000) (31) or GFP (Santa Cruz Biotechnology, Inc.; titer 1:500)
followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence
detection (Amersham). Calpha was detected using a monoclonal antibody against human α
catalytic subunit of PKA (BD Transduction Laboratories, 1:1000). Phosphorylated 5-LO was
detected using a polyclonal antibody against RKSS* developed by PhosphoSolutions
(Aurora, CO). Densitometry was performed using NIH Image software. LTB₄ synthesis by
transfected cells was normalized for 5-LO protein expression.

Kinase Assay for PKA – 3T3 cells transfected with p5-LO or p5-LO/S523A and different
amounts of pCalpha plasmid were sonicated in extraction buffer containing 25mM Tris-
HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol and protease
inhibitor cocktail (Roche Diagnostics). PKA activity was determined by measuring the
transfer of $[^{32}P]$-labeled phosphates to a phosphocellulose filter-bound peptide substrate using the SignaTECT PKA assay kit (Promega, Madison, WI) following the manufacture’s instructions. Briefly, the kinase reaction was initiated by adding 6.5 µg of protein lysate with 100 µM biotinylated Kemptide (LRRASLG) to 25 µl of reaction mixture. After incubation at 30 °C for 5 min, the reaction was terminated by adding 12.5 µl of 7.5 M guanidine hydrochloride. A 10 µl aliquot of the reaction mixture was spotted to a phosphocellulose filter, washed and PKA activity was measured using an LS 6000TA liquid scintillation counter.

*Analysis of Eicosanoid Profile of 5-LO Metabolism* – To evaluate the effect of PKA activity on the formation of different 5-LO products, 3T3 cells (1.6x10^6 cells) were co-transfected with 2 µg of p5-LO or p5-LOS523A, 2 µg of plasmid encoding FLAP (a generous gift of Dr. Timothy D. Bigby, Veterans Affairs San Diego Healthcare System, San Diego, CA) and 2 µg of pCMVCα or its parent vector pCMVneo. Cells were harvested 16 h post-transfection, washed with PBS and incubated with 1 ml serum-free DMEM containing 1.6 mM CaCl₂, 10 µM calcium ionophore A23187 and 10 µM AA (Cayman Chemicals) plus ~30,000 dpm $[^3H]$ AA (Dupont-New England Nuclear, Boston, MA) at 37 °C for 15 min. Conditioned media were extracted using $C_{18}$ Sep-Pak Cartridges (Waters Corp., Milford, MA) and radiolabeled eicosanoids were separated by reverse-phase HPLC as previously described (32). The 5-LO products LTB₄ and 5-HETE were identified by co-elution with authentic standards (Biomol), and quantitated using an on-line radioactivity detector. Radiolabeled products were expressed as a percentage of total recovered radioactivity. LTB₄ and 5-HETE synthesis in the presence of Calpha (versus CMV-neo) were expressed as percentage of control.
Ca\textsuperscript{2+}-Dependent Membrane Translocation – To assess the effect of PKA phosphorylation on 5-LO membrane translocation in cell-free conditions, 2 µg of GFP/5-LO or S523A constructs were co-transfected with 6 µg of pCMVCα or pCMVneo into 3T3 cells and, 20 hr post-transfection, cells were homogenized and analyzed as described (33), with minor modification. Briefly, cells were sonicated on ice 3 times, 20 seconds each in KPB-1 buffer (100 mM NaCl, 2 mM EGTA, 1 mM DTT, 50 mM potassium phosphate buffer (pH 7.1), and protease inhibitor cocktail. Cell lysates were centrifuged at 10,000 x g for 15 min at 4 °C. Supernatants were then divided equally into two parts, one to which CaCl\textsubscript{2} was added (4 mM final concentration), and the other to which vehicle (water) was added. After 5 min at room temperature, samples were further centrifuged at 100,000 x g for 70 min at 4 °C. The resulting supernatant was designated soluble fraction (S), and the pellet, washed and re-suspended in KPB-1 buffer, was designated pelletable microsomal fraction (P). To assess 5-LO membrane translocation in intact cells, transfected cells were stimulated with vehicle (DMSO) or 10 µM A23187 in media for 20 min at 37 °C. After stimulation, cells were washed twice and sonicated in KPB-1 buffer, and soluble and pelletable microsomal fractions were prepared as described above. Protein concentrations of fractions were determined by a modified Coomassie dye binding assay (Pierce), and 10 µg protein per sample were separated by SDS-PAGE and evaluated by immunoblot as described above, with detection by GFP antibody.

Cell Free 5-LO Enzymatic Activity Assay – Recombinant human 5-LO (2 µg; gift from Merck Frosst, Montreal, Canada) was treated with PKA catalytic subunit with or without PKA inhibitor peptide, as described above, for in vitro phosphorylation. Alternatively, 3T3 cells (1.6x10\textsuperscript{6} cells/100 mm dish) were co-transfected with 2 µg of p5-LO or p5-LOS523A and 2 µg of pCMVCα or pCMVneo. Cells were harvested 16 h post-transfection, washed with PBS, and
sonicated for 90 sec in 10 sec bursts on ice in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1mM DTT, and protease inhibitor cocktail. Lysates were centrifuged (6000 x g, 10 min, 4 °C) to remove cell debris. The 5-LO activity of recombinant 5-LO or cell lysates (200 µg total protein) was determined in 0.25 ml reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 0.6 mM CaCl₂, 0.1 mM EDTA, 0.1 mM ATP, 12 µg/ml phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), 20 µM AA (Cayman Chemicals), including ~100,000 dpm [³H] AA (Dupont-New England Nuclear, Boston, MA) and 10 µM 13(S)-hydroperoxy-9-cis-11-trans-octadecadienoic acid (Cayman Chemicals). After 30 min incubation at room temperature, reactions were stopped by adding 1 ml of ether/methanol/1 M citric acid (30:4:1, v/v/v) and mixed thoroughly. The mixture was centrifuged at 5000 rpm, 5 min, and the upper phase was collected, evaporated under nitrogen and stored at −70 °C. Lipid residues were dissolved in 250 µl of 50% acetonitrile/trifluoroacetic acid (1000:1, v/v) and 50% water/trifluoroacetic acid (1000:1, v/v), and analyzed by reverse-phase HPLC as described previously (34). There were no LTB₄/LTB₄ isomers detected in the cell lysates; 5-HPETE and 5-HETE co-eluted as a single peak, clearly separated from un-metabolized AA. 5-LO specific activity was first calculated as percent conversion of radiolabeled AA to 5-HPETE/5-HETE and standardized for 5-LO expression. Final results were expressed as percentage of control.

To study 5-LO enzyme kinetics with or without phosphorylation, 4 µg of p5-LO and 4 µg of pCMVCα or pCMVneo were co-transfected into 3.2x10⁶ 3T3 cells. Cells were harvested 16 h post-transfection, washed with PBS, and sonicated for 90 sec in 10 sec bursts on ice in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1mM DTT, and protease inhibitor cocktail. Lysates were centrifuged (6000 x g, 10 min, 4 °C) to remove cell debris. The 5-LO activity of cell lysate in different cold AA concentrations (1, 3, 10, 30, 60, 100, 300, 1000
μM) was determined as described above and was first calculated as percent conversion of radiolabeled AA to 5-HPETE/5-HETE and standardized for 5-LO protein expression. Total products formed were expressed as pmol/min/5-LO densitometric unit. \( V_{\text{max}} \), \( K_m \) and \( V/ K_m \) were determined using GraphPad Prism software (Version 3.02, Graph-Pad Software Inc., San Diego, CA).

To compare the intrinsic enzymatic activity of 5-LO and its mutant proteins S523A and S523E in vitro, 4 μg of plasmids expressing 5-LO, 5-LOS523A and S523E were transfected into cells and, 16 h post-transfection, the cell free activity in each lysate was determined as described above.

**Microscopic Imaging of Living Cells** – 3T3 cells on chamber slides were transfected with GFP/5-LO and mounted on a temperature-regulated (37 °C) stage on a Leica DMI RB fluorescent inverted microscope. After pretreatment with forskolin or vehicle (DMSO) for 20 min, cells were stimulated with 10 μM A23187 in calcium-containing medium. Fluorescent images were taken before and after 15 min of stimulation.

**Statistical Analysis** – Statistical significance was evaluated by one-way ANOVA, using p<0.05 as indicative of statistical significance. Pairs of group means were analyzed using the Tukey-Kramer post test.
Results

*Phosphorylation of 5-LO on Ser523 by PKA* – The human 5-LO protein sequence was scanned for consensus patterns using Prosite (35), which revealed two sites on 5-LO that matched the pattern of [RK](2)-x-[ST] for cAMP- and cGMP-dependent protein kinase phosphorylation sites. One site is RRcT, located at amino acids 246 to 249 (human 5-LO residues and numbering). Alignment of primary sequences of different LOs indicated that the critical 249 Ser/Thr site was not conserved across 5-LOs from different species, being replaced with Arg in hamster (Fig. 1A). Also, the pattern RRST was found on human 12-LO, indicating that this site is not unique to 5-LO. A second phosphorylation site was located at amino acids 520 to 523. Sequence alignment showed that the [RK](2)-x-[ST] pattern was common to all 5-LO species and unique to 5-LO (Fig. 1B). No sites matching the [RK](2)-x-[ST] pattern were found on FLAP. For these reasons, experiments were focused on the 520-523 residues of 5-LO.

To evaluate if 5-LO is a direct target for PKA phosphorylation in vitro, we performed an in vitro PKA kinase assay using purified GST/5-LO fusion protein as target. Autoradiography of reaction products showed that the 104 kD fusion protein was phosphorylated by PKA and phosphorylation was inhibited by a PKA inhibitor peptide (PKI, Fig.2A). Since GST is not phosphorylated by PKA (e.g., (36)), this suggested that 5-LO was the target of PKA. Phosphorylation of 5-LO by PKA was comparable to that of CREB, a known PKA substrate; Coomassie staining of gels demonstrated comparable loading of purified proteins (Fig. 2B). Substitution of Ser523 with Ala on GST/5-LO strongly reduced phosphorylation as compared to WT GST/5-LO (Fig. 2B). Slight residual phosphorylation suggested that an additional residue could be targeted by PKA under these optimal conditions. Mutation of the MAP kinase-activated protein kinase site Ser271 did not reduce phosphorylation by PKA (Fig. 2B). These
results indicated that 5-LO is phosphorylated, like CREB, by PKA in vitro, and Ser523 is the major site for phosphorylation by PKA.

*Inhibition of LTB₄ Synthesis by Calpha: Role of Ser523* — Previous studies indicated that 3T3 cells lacked endogenous 5-LO and did not secrete detectable LTB₄ when stimulated with the calcium ionophore A23187 (data not shown). When cells were transiently transfected with 5-LO and stimulated with 10 µM ionophore without added AA, they synthesized 161.0 ± 13.5 pg/ml LTB₄. When a matched set of transfected cells, expressing a comparable amount of 5-LO, were stimulated with ionophore plus 10 µM AA, they produced 5,779 ± 320 pg/ml LTB₄. Thus, endogenous PLA₂-derived AA contributed less than 3% of the substrate utilized by 5-LO when transfected cells were stimulated in the presence of added AA. Co-transfection of cells with 5-LO and the plasmid encoding Calpha resulted in significant inhibition of LTB₄ synthesis when cells were stimulated with ionophore plus AA (Fig. 3A). Increasing the amount of the Calpha plasmid, pCMV-Cα, increased the amount of expressed Calpha and decreased LTB₄ synthesis in co-transfected cells. Expression of 5-LO protein was similar for all conditions, and the dose-dependent inhibition of LTB₄ production associated with increasing Calpha was apparent when LTB₄ amount was normalized for 5-LO expression (Fig. 3A). These results indicated that LTB₄ synthesis can be dose-dependently inhibited by increasing Calpha expression, even when cells are provided with abundant exogenous AA.

In parallel experiments, cells were co-transfected with the S523A mutant of 5-LO and the Calpha plasmid and stimulated with calcium ionophore plus AA. Lysates of cells over-expressing the S523A mutant had substantial 5-LO activity, indicating that this mutation did not impair catalytic function. Co-transfection with S523A 5-LO and 0.5-2 µg of pCMV-Cα did not significantly decrease LTB₄ synthesis, compared to cells transfected with S523A 5-LO alone.
(Fig. 3B). These results indicated that Ser523 is important for the effects of Calpha on LTB₄ synthesis.

Additional experiments addressed the correlations between Calpha amount, PKA activity, and the phosphorylation of 5-LO in intact cells. Using a novel antibody against phosphorylated S523 in the RKSS context of 5-LO developed by PhosphoSolutions, weak phosphorylation of WT 5-LO was detected with no Calpha and strong phosphorylation occurred with all levels of Calpha co-transfection (Fig. 4A). No phosphorylated 5-LO was detected in the S523A 5-LO, with or without Calpha co-transfection. Quantitative analysis indicated maximal phosphorylation of 5-LO (Fig. 4B) associated with maximal PKA activity (Fig. 4C) when WT 5-LO was co-transfected with 1-4 μg pCalpha. Thus, co-transfection of 5-LO with Calpha increases cellular PKA activity and phosphorylation of 5-LO on S523.

To determine the effects of Calpha overexpression on all of the 5-LO products that are generated from exogenous AA by 3T3 cells, radiolabeled metabolites from transfected cells stimulated with ionophore plus AA were separated by HPLC. Cells transfected with WT 5-LO alone produced predominantly prostaglandin E₂, with LTB₄ and 5-HETE being minor peaks; co-expression of 5-LO and Calpha resulted in loss of both LTB₄ and 5-HETE peaks (data not shown). Cells co-transfected with 5-LO and FLAP produced increased and comparable amounts of LTB₄ and 5-HETE, as well as detectable PGE₂. When Calpha was co-expressed with 5-LO and FLAP, both LTB₄ and 5-HETE were significantly decreased (Fig. 5A). No peaks corresponding to LTA₄ or LTB₄ degradation products were evident. The reduction in 5-LO product formation could not be attributed to altered expression of 5-LO or FLAP in the cells, as verified by immunoblotting (Fig. 5B). In contrast, when 5-LO harboring the S523A mutation
was cotransfected with FLAP and Calpha, neither LTB$_4$ and 5-HETE were significantly decreased, compared to cells without Calpha (Fig. 5A); the protein expression of 5-LO and FLAP in the cells was also comparable, as verified by immunoblotting (Fig. 5B). These findings suggested that the inhibitory effect of Calpha on 5-LO metabolism directly resulted from its inhibition of 5-LO, and that Ser523 is pivotal for this inhibition.

**Role of Ser523 in the Effects of Native PKA** – Proliferating 3T3 cells express native Calpha (Fig. 3) and may be expected to manifest a low level of PKA activation. Consistent with this, when 3T3 cells over-expressing GFP/5-LO were pretreated with 10 µM H-89, a selective PKA inhibitor, for 2 h and then stimulated with 10 µM A23187 plus 10 µM AA, LTB$_4$ synthesis was significantly greater compared to cells pretreated with vehicle (Fig. 6A). In contrast, when cells were transfected with GFP/5-LO containing the S523A mutation, H-89 pretreatment had no significant effect on LTB$_4$ synthesis upon subsequent stimulation with A23187 plus AA (Fig. 6A).

The effect of different PKA activators on LTB$_4$ synthesis was also tested. When 3T3 cells over-expressing GFP/5-LO were first treated with the adenylyl cyclase activator forskolin (50 µM) plus the phosphodiesterase inhibitor IBMX (45 µM) for 20 min and then stimulated with 10 µM A23187 plus 10 µM AA, LTB$_4$ synthesis was significantly decreased relative to vehicle pretreated cells (Fig. 6B). Similarly, inhibition of LTB$_4$ synthesis was also found when transfected cells were pretreated with the stable cAMP analogue, dibutyryl cAMP (1 mM) for 20 min and then stimulated (Fig. 6B). In contrast, cells over-expressing GFP/5-LO with the S523A mutation did not show decreased LTB$_4$ generation with either pretreatment (Fig. 6B). Taken together, these experiments indicated that Ser523 in 5-LO is necessary and important for suppression of LTB$_4$ synthesis mediated by endogenous native PKA.
Mechanism of PKA-Mediated Inhibition of 5-LO: Membrane Translocation vs. Catalytic Activity

Activation of p38 MAP kinase has been shown to promote a necessary step in LTB₄ synthesis, namely membrane translocation of 5-LO (37). In neutrophils, PKA activation can block p38 MAP kinase activation and impair membrane association of 5-LO (14). The effects of PKA activity on membrane association of GFP/5-LO in 3T3 cells were tested by both cell-free and intact cell approaches. When 3T3 cells, co-transfected with pGFP/5-LO and pCMVneo, the parent vector of pCMV-Cα, were fractionated into soluble (S) and pelletable (P) microsomal membrane fractions in the absence of calcium, GFP/5-LO was detected in both fractions (Fig. 7A, left). When calcium was present during fractionation, significantly less GFP/5-LO was in the soluble fraction and more was in the membrane fraction, demonstrating calcium-dependent membrane association. Cells over-expressing both GFP/5-LO and Calpha, which led to impaired LTB₄ synthesis (Fig. 3), showed normal membrane association of 5-LO: GFP/5-LO was present in both fractions in the absence of calcium and membrane-associated in the presence of calcium (Fig. 7A). Since the presence of Calpha could not impair calcium-mediated in vitro membrane association of GFP/5-LO, mutation of Ser523 should not be expected to affect this process; indeed, the S523A mutant of GFP/5-LO showed normal membrane-association upon exposure to calcium, with or without over-expressed Calpha (Fig. 7A).

Parallel experiments were performed to evaluate translocation of 5-LO in intact cells (Fig. 7A, right). In these experiments, cells over-expressing GFP/5-LO (WT or the S523A mutant) with or without Calpha, were fractionated (without calcium) into soluble or microsomal membrane fractions, either before or after stimulation with calcium ionophore. As observed during the in vitro analysis, GFP/5-LO was evident in both soluble and microsomal fractions in all unstimulated combinations. Stimulation of cells with the calcium ionophore A23187 resulted...
in a sharp reduction in soluble and increase in pelletable GFP/5-LO in all combinations. This indicated that the presence of Calpha, which inhibited LTB$_4$ synthesis by WT 5-LO, did not significantly affect membrane association of 5-LO in 3T3 cells.

The ability of PKA activation to block translocation of GFP/5-LO to membranes was also assessed visually by live cell microscopy. In resting 3T3 cells over-expressing GFP/5-LO, 5-LO is strongly accumulated in the nucleus but also evident in the cytoplasm (Fig. 7B), as described previously (28). Distribution within the nucleus is heterogeneous and patchy, while the distribution in the cytoplasm is even and diffuse. Following activation of live cells with ionophore, 5-LO was observed to move over time, resulting in a rim of fluorescence at the nuclear envelope with additional fluorescence at cytoplasmic membranes (primarily endoplasmic reticulum)(Fig. 7B). Pretreatment of cells with 100 µM forskolin for 20 min did not block the redistribution of GFP/5-LO to membranes (Fig. 7B). Forskolin pretreatment also did not delay the rate of redistribution of GFP/5-LO (data not shown). Taken together, these experiments suggest that PKA activation does not inhibit LTB$_4$ synthesis by blocking translocation of 5-LO in ionophore-stimulated 3T3 cells.

The subcellular localization of 5-LO is an important determinant of LTB$_4$ synthesis, with placement of 5-LO within the nucleus allowing greater LTB$_4$ production than 5-LO in the cytoplasm (30). As shown in Fig. 7, pretreatment with 100 µM forskolin did not cause movement of 5-LO from the nucleus to the cytoplasm. Treatment with forskolin (50 µM) plus IBMX (45 µM), which inhibited LTB$_4$ synthesis (Fig. 6), also did not promote nuclear export of 5-LO (Fig. 8). Similarly, treatment with H-89 (10 µM), which enhanced LTB$_4$ synthesis (Fig. 6), did not promote nuclear import of 5-LO (Fig. 8). This indicates that phosphorylation of 5-LO by PKA does not cause redistribution of 5-LO in the time frame used in these experiments.
Since PKA activation could not be shown to alter 5-LO translocation or cause redistribution of 5-LO, we tested whether PKA could directly decrease 5-LO enzymatic activity. The 5-LO activity of recombinant human 5-LO treated with the catalytic subunit of PKA was significantly less than that of recombinant 5-LO treated identically but with PKA inhibitor peptide (Fig. 9A). Analysis of lysates from cells transfected with 5-LO with or without Calpha, adjusted for 5-LO expression, indicated that phosphorylation reduced Vmax from 100.8 to 42.1, increased the Km of 5-LO from 16.5 to 75.8 µM AA, and decreased V/Km from 6.1 to 0.56 (Fig. 9B). Whereas co-expression of Calpha with 5-LO significantly reduced the 5-LO activity of cell lysates, co-expression of Calpha with the S523A mutant of 5-LO did not reduce lysate 5-LO activity (Fig. 9C). Immunoblotting of lysates showed that 5-LO protein expression was similar across all samples while Calpha protein was elevated when co-transfected (Fig. 9C). Thus, PKA directly reduced 5-LO enzymatic activity, this was associated with a 10.9-fold decrease in the V/Km of 5-LO and inhibition of 5-LO by Calpha was eliminated when Ser523 was altered to Ala.

The prediction that insertion of a large negatively-charged side chain at site 523 inhibited catalytic activity was tested by replacing Ser523 with Glu (E). The lysates from cells overexpressing either WT 5-LO or the S523A 5-LO mutant showed comparable conversion of AA to the products 5-HPETE and 5-HETE, which co-elute as a single peak (Fig. 10A). In contrast, lysates from cells overexpressing S523E 5-LO produced no detectable product. Statistically, there was no significant difference between WT and S523A 5-LO, whereas the S523E mutant had less AA conversion to product (Fig. 10B). All three constructs had comparable protein expression levels (Fig. 10B, inset). These results support the model that phosphorylation at Ser523 inhibits 5-LO catalytic activity.
Discussion

LTs are potent lipid mediators that evoke a large number of responses in target cells and tissues, including inflammation. It seems reasonable that mechanisms must exist for preventing the initiation of this signaling pathway, as well as for blunting or extinguishing the pathway after it has been initiated. We report here, for the first time, that activation of the cAMP signaling pathway directly inhibits LT synthesis through the phosphorylation of 5-LO by PKA. We provide evidence that PKA phosphorylates 5-LO on Ser523 and that mutation of Ser523 ameliorates the changes in LTB₄ synthesis caused by cAMP elevation and PKA activation. The motif that is recognized by Calpha is conserved across species, so that human 5-LO can be targeted by mouse Calpha, as shown in this study. Phosphorylation of 5-LO by PKA directly inhibits the catalytic activity of 5-LO, presumably through allosteric changes resulting from interactions between phosphorylated Ser523 and residues near Thr444. The direct inhibition of 5-LO following PKA activation, then, serves as a way for endogenous mediators, such as adenosine and PGE₂, and therapeutic agents, such as β-adrenergic agonists, to rapidly reduce the capacity of leukocytes to generate LTs. This represents an additional mechanism by which PKA can regulate LT synthesis, beyond known actions to inhibit cPLA₂ activity and 5-LO translocation.

Examination of the crystal structure of rabbit reticulocyte 15-LO (1LOX.pdb; (38)) places the residue (Gln514), that corresponds to Ser523 on human 5-LO, at the periphery of the molecule (Fig. 11A). This site was clearly distinct from the residues that correspond to those identified as lining the catalytic pocket of 15-LO (39,40). According to this model, Ser523 projects toward residues 441-444 (Lys-Asp-Leu-Thr), with closest association to the positive Lys441 and negative Asp442 (Fig. 11B). Phosphorylation of Ser523 then would insert a large,
negatively charged polar phosphate group toward the 441-444 segment and potentially change conformation through residues linked to either 523 or 441-444, or both. Ser523 is close to an α-helix that includes both His550, which is essential for iron binding, and Gln557, which corresponds to the pocket-defining Gln547 on 15-LO (39). The 441-444 group is connected to three residues, Phe421, Ala424, and Asn425 which are critical for metabolism of AA by 15-LO (40,41). Thus, phosphorylation of Ser523 may cause allostERIC changes that produce a 10-fold reduction in the V/Km of 5-LO (Fig. 9).

As noted above, a wide variety of treatments that elevate cAMP will inhibit LT synthesis. This effect is wide-spread, being observed in all leukocytes. PKA activation inhibits cPLA₂ (22,23), a major source of AA for LT generation (7). PKA activation can also block activation of p38 MAP kinase in neutrophils (14,42), and this can reduce LT synthesis by impeding the translocation of 5-LO to nuclear membranes (14). Our results show that activated PKA inhibits LT synthesis in 3T3 cells, even when AA is added and translocation of 5-LO is not impaired. This inhibition of 5-LO activity occurs through phosphorylation on a PKA target that is conserved across different 5-LO species. That PKA inhibits three different enzymes that can contribute to LT synthesis (cPLA₂, p38 MAP kinase, 5-LO) suggests that this is a physiologically important mechanism. The direct inhibition of 5-LO by PKA is particularly significant since LT synthesis can proceed without cPLA₂ activity (43) or p38 MAP kinase signaling (44), but not without 5-LO action. The possibility that PKA may modulate other enzymes in the 5-LO pathway, including FLAP, LTA₄ hydrolase and LTC₄ synthase, remains to be clarified.

As a rule, LT synthesis is normally restricted to leukocytes, because only leukocytes express 5-LO. Biologically, the activation of the 5-LO pathway is an early event in the response
to infection and serves to promote the inflammatory response through a variety of mechanisms, including the recruitment of additional leukocytes. These inflammatory leukocytes may elaborate additional LTs, providing a positive feedback mechanism to amplify the inflammatory response. Infection also induces new transcription of several genes, including cyclooxygenase-2, which will eventually lead to the synthesis and secretion of prostaglandins at the inflammatory site. Certain prostaglandins, most notably PGE$_2$, PGD$_2$ and prostacyclin, bind specific $G_{\alpha}$-coupled receptors, leading to cAMP elevation and PKA activation. The resulting suppression of LT production will interrupt the positive feedback loop, stopping the progression of the inflammatory process. Failure to activate PKA may allow persistent production of LTs, leading to chronic inflammatory disease.

The importance of PKA-mediated suppression of LT synthesis may extend beyond host defense to LT-driven fibrosis. In an animal model of lung fibrosis, tissue damage induced by bleomycin results in the rapid and sustained production of LTs and fibrosis (45). Mice lacking the 5-LO gene cannot make LTs following bleomycin damage and are protected from fibrosis. In humans, the relentlessly progressive disease, idiopathic pulmonary fibrosis, is characterized by chronic LT overproduction (4,46). By contrast, macrophages (47) and fibroblasts (48) from lungs of patients with idiopathic pulmonary fibrosis have a severely impaired capacity to synthesize prostaglandins. It is attractive to think that this deficiency in prostaglandin production leads to a failure to activate PKA, allowing chronic LT generation and fibrosis.

Just as both the overproduction and the underproduction of LTs can contribute to disease pathogenesis, it seems likely that either under- or over-activity of PKA signaling may be clinically relevant. For example, LTs are overproduced during active systemic lupus erythematosus (49). Interestingly, profound deficiencies of both type I and type II PKA isozyme
activities are found in T lymphocytes from patients with systemic lupus erythematosus (50). There may be additional examples where aberrant signaling between PKA and 5-LO contributes to disease. This, of course, suggests the potential for indirect therapeutic modulation of the 5-LO pathway by targeting the PKA pathway.

In summary, this study demonstrates a previously unrecognized and novel means for the regulation of LT biosynthesis by PKA, namely, the direct phosphorylation of 5-LO on Ser523 by PKA. This action, in combination with other known effects of PKA on cPLA₂ and p38 MAP kinase signaling, indicates that activation of cAMP signaling is a powerful mechanism for inhibiting LT synthesis. Under normal conditions, this pathway may be important in maintaining homeostasis and limiting the progression of inflammation. Dysfunctional signaling through cAMP and PKA, then, may result in the production of abnormal levels of LTs and contribute to a variety of diseases, including those characterized by chronic inflammation or fibrosis.

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References


Figure Legends

**Fig. 1.** Potential targets for PKA phosphorylation. Sequences in human 5-LO that conform to the pattern recognized by cAMP- and cGMP-dependent protein kinases were identified using Prosite; sequence alignments for selected LOs were performed using ClustalW, with asterisks and colons indicating identical and similar residues, respectively. **A,** Sequence at Thr249. **B,** Sequence at Ser523.

**Fig. 2.** Phosphorylation of human 5-LO on Ser523 by PKA in vitro. **A,** Bacterially expressed GST/5-LO fusion protein eluted from glutathione-sepharose beads was used as substrate in *in vitro* kinase reactions using purified catalytic subunit of PKA in the absence and presence of 5 µM of PKA inhibitor, PKI. Kinase reactions were conducted at 30 °C for 40 min in reaction mixtures containing [γ-32P] ATP. Proteins were separated by SDS-PAGE and 32P-labelled GST/5-LO proteins detected by autoradiography. Arrow indicates 104 kD phosphorylated GST/5-LO. **B,** *In vitro* PKA kinase assay of purified CREB (+PKI), GST/5-LO, and S523A and S271A mutants of GST/5-LO. 32P-Labeled proteins were detected by autoradiography (Phos); protein loading was evaluated by Coomassie blue staining.

**Fig. 3.** Calpha expression dose-dependently inhibited LTB4 biosynthesis by WT 5-LO but not the S523A mutant of 5-LO. 2 µg of plasmids encoding WT 5-LO (**A**) or S523A 5-LO (**B**) were co-transfected with increasing amounts of the plasmid pCMV-Cα, with the empty pCMVneo plasmid used to balance DNA per transfection. 16 h post-transfection, cells were stimulated with 10 µM A23187 plus 10 µM AA and secreted LTB4 was measured by ELISA. 5-LO and Calpha proteins in cell pellets were evaluated by immunoblot using antibodies against 5-LO and the α catalytic subunit of PKA, respectively. LTB4 synthesis for each cotransfection was normalized.
for 5-LO expression and is expressed as percentage of control. Data are means ± SE of n = 3 independent transfections; *, p< 0.05 versus control.

**Fig. 4.** Detection of the direct phosphorylation of 5-LO by PKA in intact 3T3 cells. 3T3 cells were transfected with 2 µg of p5-LO or p5-LO/S523 with different doses of plasmid pCalpha, total DNA in each transfection was complemented to 6 µg using pCMV-neo, the parent vector of PKA Calpha. 16 h post transfection, cells were harvested and 15 µg of total protein lysate from each transfection was separated by SDS-PAGE and probed using antibodies against phospho 5-LO and 5-LO. Also, lysates were tested for PKA activity assay as described in Experimental Procedures. A, Western blot to detect the direct phosphorylation of 5-LO or 5-LO/S523A by PKA. B, Densitometric analysis of phospho 5-LO normalized for total 5-LO. C, PKA kinase activity in cell lysates after transfected with different doses of pCalpha.

**Fig. 5.** Co-expression of Calpha inhibited both LTB4 and 5-HETE production from 3T3 cells expressing WT but not S523A 5-LO. WT or S523A 5-LO plasmid (2 µg) were co-transfected with FLAP (2 µg) and pCMV-Cα (2 µg) plasmid. In negative controls, empty pCMVneo plasmid was used in lieu of pCMV-Cα. 16 h post-transfection, cells were stimulated with 10 µM A23187 and 10 µM AA with trace [3H] AA. Secreted radiolabeled eicosanoids were separated by HPLC as indicated in *Experimental Procedures*. The 5-LO products LTB4 and 5-HETE are expressed as percent of control. A, LTB4 and 5-HETE synthesis from cells expressing WT or S523A 5-LO in the presence or absence of Calpha. B, Western blots of 5-LO, FLAP and Calpha protein. Data are means ± SE of n = 3 independent transfections; *, p< 0.05 versus control.

**Fig. 6.** Effect of pharmacological modulators of PKA on LTB4 generation by WT or S523A 5-LO. A, 3T3 cells were transfected with WT or S523A GFP/5-LO overnight, pretreated with the
PKA inhibitor H-89 (10 µM) or vehicle (DMSO) for 2 h, then stimulated with 10 µM A23187 and 10 µM AA. LTB₄ in conditioned media was measured by ELISA and normalized for 5-LO protein in the corresponding cell pellet. LTB₄ synthesis is expressed as percent of control. B, Cells were transfected as above, pretreated with forskolin (50 µM) plus IBMX (45 µM), or db-cAMP (1mM) or vehicle (DMSO) for 20 min and then stimulated as above. LTB₄ was measured by ELISA and normalized for 5-LO protein; LTB₄ synthesis is expressed as percent of control. Data are means ± S.E of n = 3 independent transfections; *, p< 0.05 versus control.

**Fig. 7.** Effect of elevated PKA activity on calcium-dependent translocation of 5-LO. A, Ca²⁺-dependent translocation in vitro and in intact cells during increased expression of Calpha. WT or S523A GFP/5-LO was co-transfected with either pCMV-Cα or pCMV-neo into 3T3 cells. After 16 h, cells were fractionated (as described in Methods) with or without Ca²⁺, or stimulated with or without 10 µM A23187 (20 min, 37 °C) before fractionation. Cells were fractionated into soluble (S) and pelletable microsomal (P) fractions and were examined by immunoblotting using antibodies against GFP based on equal loading (10 µg/well) of total protein. B, Ca²⁺-dependent membrane translocation following forskolin treatment. Cells expressing WT GFP/5-LO were pretreated with or without forskolin (100 µM, 20 min) and then fluorescently imaged before (rest.) or 15 min after (act.) activation with 10 µM A23187 (37 °C).

**Fig. 8.** Effect of PKA modulators on the subcellular localization of 5-LO. 3T3 cells were transfected with GFP/5-LO and, after 20 hr, imaged live before and at 10 min intervals after the addition of forskolin (50 µM) plus IBMX (45 µM) or H-89 (10 µM). Images of representative cells at the start (Initial) and after 30 min are presented.

**Fig. 9.** Effect of PKA on 5-LO activity. A, Effect of in vitro phosphorylation of recombinant human 5-LO by PKA on 5-LO activity. B, Kinetic analysis of the effect of phosphorylation of
expressed 5-LO by Calpha on 5-LO activity. Cells were co-transfected with WT 5-LO and pCMV-Cα (or CMVneo). After 16 h, 5-LO activity was evaluated using varying substrate concentrations; product formation was adjusted for enzyme expression. C, Effect of Calpha on the 5-LO activity of lysates from cells expressing WT and S523A 5-LO. Plasmids (2 µg) encoding WT or S523A 5-LO were co-transfected with pCMV-Cα (2 µg) or pCMVneo into 3T3 cells. Recombinant 5-LO and cell lysates were tested for their capacity to convert AA to 5-HPETE/5-HETE as described in Experimental Procedures. Results were normalized for 5-LO protein from immunoblots and are presented as percent of control. Normal and elevated expression of Calpha was detected by anti-PKA Cα antibody. Data are means ± SE of n = 3 independent transfections; *, p< 0.05 versus co-transfection with pCMV-neo.

Fig. 10. Comparison of the intrinsic enzymatic activity of WT, S523A and S523E 5-LO. Plasmids (4 µg) expressing WT and mutant 5-LO were transfected into cells and the 5-LO activity of lysates determined. A, HPLC separation of radio-labeled 5-HPETE/5-HETE (products) from un-metabolized AA in WT, S523A and S523E cell lysates. B, Statistical evaluation of percent conversion of AA to 5-HPETE/5-HETE by WT, S523A and S523E 5-LO. Inset, immunoblots of representative samples using 5-LO antibodies. Data are means ± SE of n = 3 independent transfections; *, p< 0.05 versus transfection with WT or with S523A, n.s., not statistically significant.

Fig. 11. Secondary structure of the catalytic domain of 5-LO and residues associated with Ser523. A, Localization of Ser523 to the periphery of the catalytic domain, away from the proposed pocket for AA binding (in black). B, Detailed structure showing Ser523 in close proximity to and oriented toward Lys441, Asp442, and Thr444. Ser523 is physically linked to His550, which binds the iron atom, and Gln557. The 441-444 strand is physically linked to
Ala424, Asn425 and Phe421 which, with Gln557, correspond positionally to residues identified in 15-LO that are important for enzymatic activity.
A
hum 5LO IRRCTELP
rat 5LO IKRCTELP
ham 5LO IKRCRELP
mou 5LO IKRCTALP
rab 15LO LRRSVQLP
hum 15LO LRRSAHLP
hum 12LO LRRSTSLP
:::*:***

B
hum 5LO GRKSSGFP
rat 5LO GRKASGFP
ham 5LO GRKASGFP
mou 5LO GKKASGFP
rab 15LO GAQKQGFP
hum 15LO GAQDRGFP
hum 12LO QAQDRGFP
:::***

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
**Fig. 9**

**A**

AA Conversion (\%)

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**B**

5-HETE/5-HPETE (pmol/min/5-LO unit)

Log AA (\(\mu M\))

- 5-LO+CMVneo
- 5-LO+Calpha

**C**

AA Conversion (% of control)

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5-LO

Calpha

Fig. 9
Fig. 10
Fig. 11
Protein kinase A inhibits leukotriene synthesis by phosphorylation of 5-lipoxygenase on serine 523
Ming Luo, Sandra M. Jones, Susan M. Phare, Michael J. Coffey, Marc Peters-Golden and Thomas G. Brock

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