Phosphorylation of 5-Lipoxygenase at Ser^523 by Protein Kinase A Determines Whether Pioglitazone and Atorvastatin Induce Proinflammatory Leukotriene B_4 or Anti-Inflammatory 15-Epi-Lipoxin A_4 Production

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Phosphorylation of 5-Lipoxygenase at Ser\(^{523}\) by Protein Kinase A Determines Whether Pioglitazone and Atorvastatin Induce Proinflammatory Leukotriene B\(_4\) or Anti-Inflammatory 15-Epi-Lipoxin A\(_4\) Production

Yumei Ye,*† Yu Lin,* Jose R. Perez-Polo,† Barry F. Uretsky,* Zaiming Ye,† Brian C. Tieu,‡ and Yochai Birnbaum1*†

The 5-lipoxygenase (5LO) produces leukotriene B\(_4\) and 15-epi-lipoxin-A\(_4\) (15-epi-LXA\(_4\)). Phosphorylation at Ser\(^{523}\) by protein kinase A (PKA) prevents 5LO shift to the perinuclear membrane. Atorvastatin and pioglitazone up-regulate 15-epi-LXA\(_4\) production in the heart. We assessed whether phosphorylation of 5LO by PKA determines whether 5LO interacts with the membranous cytosolic phospholipase A\(_2\) (cPLA\(_2\)) to produce leukotriene B\(_4\) or with cyclooxygenase-2 (COX2) to produce 15-epi-LXA\(_4\). Rats received either pioglitazone, atorvastatin, pioglitazone plus atorvastatin, vehicle, or LPS. Rat myocardial cells were incubated with pioglitazone plus atorvastatin, pioglitazone plus atorvastatin plus H-89 (PKA inhibitor), H-89, or vehicle for 8 h. Pioglitazone and atorvastatin did not affect total 5LO expression. However, both increased 5LO levels in the cytosolic fraction. H-89 caused a shift of 5LO to the membranous fraction in atorvastatin- and pioglitazone-treated rats. Pioglitazone and atorvastatin increased phospho-5LO levels. H-89 attenuated this increase. Both pioglitazone and atorvastatin increased COX2 levels in the cytosolic fraction and the membranous fraction. H-89 prevented this increase. Pioglitazone and atorvastatin increased cPLA\(_2\) expression in the membranous fraction. This effect was not attenuated by H-89. Pioglitazone plus atorvastatin increased 15-epi-LXA\(_4\) levels. H-89 attenuated the effect of pioglitazone plus atorvastatin. Pioglitazone plus atorvastatin plus H-89 increased leukotriene B\(_4\) levels. Coimmunoprecipitation showed that without H-89, atorvastatin and pioglitazone induced an interaction between 5LO and COX2 in the cytosolic fraction, whereas when H-89 was added, 5LO interacted with cPLA\(_2\) on the membranous fraction. The 5LO phosphorylation determines whether 15-epi-LXA\(_4\) (anti-inflammatory) or leukotriene B\(_4\) (inflammatory mediator) is produced. The Journal of Immunology, 2008, 181: 3515–3523.

Both the peroxisome proliferator-activated receptor \(\gamma\) agonist pioglitazone ([\(\text{4-[(2-(5-ethylpyridin-2-yl)ethoxy)]-[methyl][thiazolidine-2,4-dione}}\]) (PIO)\(^2\) (1, 2) and the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, atorvastatin ([\(\text{7-[(2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxy-heptanoic}}\]) acid) (ATV) (3, 4) have anti-inflammatory properties, reducing serum markers of inflammation, including C-reactive protein, IL-6, and TNF-\(\alpha\). However, the underlying mechanisms of their anti-inflammatory properties are unknown. We have shown that both PIO and ATV increase the production of 15-epi-lipoxin A\(_4\) ((5S,6R,15R)-5,6,15-trihydroxy-7,9,13-trans-1-cis-eicosatetraenoic acid) (15-epi-LXA\(_4\)), a lipid mediator with strong anti-inflammatory and inflammation-resolving properties (5). The 15-epi-LXA\(_4\) has been shown to inhibit the production of IL-6, TNF-\(\alpha\) (6–9), and IL-8 (10); inhibit neutrophil activation (chemotaxis, adhesion, and transmigration across the vascular endothelium) (11); and function as a local anti-inflammatory mediator involved in protein and diverse human diseases, including airway inflammation and asthma; arthritis; graft vs host disease; and multiple cardiovascular, gastrointestinal, periodontal disease and kidney disease (12–15). It has become apparent that in addition to their anti-inflammatory properties, 15-epi-LXA\(_4\) and lipoxin A\(_4\) have distinct properties, inducing resolution of inflammation (15). A recent study suggested that 15-epi-LXA\(_4\) binds to the same receptor as serum amyloid A and overrides the antiapoptotic effects of serum amyloid A on neutrophils (16). Moreover, 15-epi-LXA\(_4\) facilitates phagocytosis of apoptotic neutrophils by macrophages (17).

Claria and Serhan (18) found that 15-epi-LXA\(_4\) is an eicosanoid produced by an interaction between neutrophils and HUVECs after incubation with aspirin. Aspirin causes acetylation of cyclooxygenase-2 (COX2), leading to the production of (15R)-5-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid (15R-HETE) that is converted by 5-lipoxygenase (5LO) to 15-epi-LXA\(_4\) (18). It has been found that nonsteroidal anti-inflammatory agents other than aspirin, and selective COX2 inhibitors do not share this property and are unable to generate 15-epi-LXA\(_4\) (18). Moreover, selective COX2 inhibitors prevent 15-epi-LXA\(_4\) generation by aspirin (19).

Shortly thereafter, it was found that cytochrome P-450 in the liver can metabolize arachidonic acid (AA) to 15R-HETE, and...
thus, the liver is a rich source of 15-epi-LXA4 (20, 21). However, in contrast to COX2, cytochrome P-450 induced by aspirin generates both 15R-HETE (40%, which is converted to 15-epi-LXA4) and 5(S)-hydroperoxy-6-trans-8,11,14-9-eicosatetraenoic acid (60%, which is converted to lipoxin A4) (20). In a human lung adenocarcinoma cell line, aspirin increases the generation of both lipoxin A4 and 15-epi-LXA4 (22). In contrast, we have previously shown that in the rat heart, both ATV and PIO caused a much larger increase in 15-epi-LXA4 than in lipoxin A4, suggesting that the COX2 and not P-450 is the major source of 15R-HETE in the rat heart (5). Moreover, valdecoxib, a selective COX2 inhibitor, completely abrogated the increase in 15-epi-LXA4 levels by both ATV and PIO (5). Although it is still unclear how PIO activates COX2, it seems that S-nitrosylation of COX2 by inducible NO synthase triggers the production of 15-epi-LXA4 by ATV (23).

However, 5LO also catalyzes the oxygenation of AA to 5(S)-hydroperoxy-6-trans-8,11,14-9-eicosatetraenoic acid, and the further dehydration to the allylic epoxide leukotriene (LT) A4, the membrane (24 –26). This shift involves Ca2+-induced binding of the C2-like domain to phospholipids) migrate to the perinuclear membrane and, therefore, cannot use AA to produce LT. Instead, it interacts with COX2 in the cytoplasm, producing 15-epi-LXA4, which is probably modified by S-nitrosylation at Cys526 by inducible NO synthase (5, 23, 34). We compared the effect of ATV and PIO, singly and in combination, and LPS on myocardial expression of total and Ser523 P-5LO. We then determined the localization of 5LO in rat cardiomyocytes pretreated with PIO plus ATV or LPS. Subsequently, we used rat cardiomyocytes in culture to assess whether: 1) PIO plus ATV increased 5LO phosphorylation and prevented shift of 5LO to the membranes, leading to enhanced 15-epi-LXA4 production; 2) inhibiting PKA with H-89 prevented 5LO phosphorylation by PIO plus ATV and caused shift of 5LO to the membranes, resulting in enhanced production of LTs instead of 15-epi-LXA4.

Finally, we used coimmunoprecipitation to show that the production of 15-epi-LXA4 occurs by interaction between COX2 and 5LO in the cytosolic fraction, whereas the production of LT occurs by an interaction of cPLA2 and 5LO on the membranous fraction of the cell.

Materials and Methods

Animal care

Male Sprague-Dawley rats received humane care in compliance with The Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996).

In vivo experiment

Experiment 1. Rats received the following: 1) PIO (10 mg/kg/day); 2) ATV (10 mg/kg/day); 3) PIO (10 mg/kg/day) plus H-89 (20 mg/kg); 4) PIO and LPS (5 mg/kg). Rats received the following: 1) PIO (10 mg/kg/day); 2) ATV (10 mg/kg/day); 3) PIO (10 mg/kg/day) plus H-89 (20 mg/kg); 4) PIO and LPS (5 mg/kg).
ATV (10 mg/kg/day) plus H-89 (20 mg/kg); 5) H-89 (20 mg/kg); or 6) water alone (control). PIO and ATV were suspended in water and administered by gastric gavage once daily for 3 days; H-89 was dissolved in DMSO (final concentration 5% v/v) and injected i.p. on the third day. Rats in groups 5 and 6 received water by gastric gavage once daily for 3 days. Rats in groups 1, 2, and 6 received i.p. injection of 5% DMSO. Sixteen hours after injection, rats were euthanized and the hearts were explanted for further analyses.

**Experiment 2.** Rats received 3-day pretreatment with the following: 1) water (control); 2) PIO (10 mg/kg/day); 3) ATV (10 mg/kg/day); 4) PIO (10 mg/kg/day) plus ATV (10 mg/kg/day); or 5) LPS (10 mg/kg).

PIO and ATV were administered by oral gavage once daily as above; LPS was administered i.v. In addition, rats in groups 1–4 received i.v. saline on the fourth day, whereas rats in group 1 and 5 received water by gastric gavage once daily for 3 days and the LPS injection on the third day. Sixteen hours after the injection, rats were euthanized and the hearts were explanted for further analyses.

**In vitro study**

Cardiac myocytes were isolated from adult Sprague-Dawley rats (250–300 g, male). Animals were heparinized (1000–2000 U i.p.) 5 min before being anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) given i.p., and the hearts were removed and placed in ice-cold heart medium solution (in mmol/L: 112 NaCl, 5.4 KCl, 1 MgCl2, 9 NaH2PO4, and 11.1 D-glucose; supplemented with 10 HEPES, 30 taurine, 2DL-carnitine, and 2 creatine (pH 7.4)). The hearts were perfused retrogradely in a Langendorff apparatus with Ca2+-free heart medium for 5 min at 5 ml/min at 37°C, followed by perfusion with Ca2+-free heart medium containing 210 U/mg collagenase II (Worthington Biochemical) for 20 min. After perfusion, both ventricles were removed from the heart and minced in collagenase II-containing heart medium for 10–15 min. The cell solution was then washed several times to remove collagenase II and re-exposed to 1.2 mM Ca2+ over 25 min to produce Ca2+-tolerant cardiac myocytes. Myocytes were then plated in 4% FBS on laminin (2 μg/cm2)-coated plates for 1 h

**FIGURE 3.** a. Representative immunoblots (a, c) and densitometric analyses (b, d) of 5LO levels in the cytosolic and membranous fraction of the rat heart tissue. There were no detectable levels of 5LO in the nuclear fraction (data not shown). Both ATV and PIO increased 5LO levels in the cytosolic fraction without a detectable effect on the membranous levels. H-89 alone had no effect on 5LO levels; however, in combination with either ATV or PIO, there was a shift of 5LO from the cytosolic fraction to the membranous fraction. Values represent mean ± SEM of four animals in each group. *, p < 0.001 vs controls; #, p < 0.001 with vs without H-89.

**FIGURE 4.** Immunofluorescence of 5LO (red), myosin (green), and DAPI (blue) of myocardium of rat treated with PIO plus ATV, LPS, or sham. In the PIO plus ATV-treated rat, there is increased expression of 5LO in the cytosol of cells stained positive for myosin (cardiomyocytes). In the LPS-treated rat, the 5LO is expressed around the nuclei of the cardiomyocytes (magnification ×120).
and incubated at 37°C in 5% CO₂ for 12–24 h before experiments (35). Cells were incubated with the following: 1) vehicle (0.07% ethanol); 2) PIO (10 μM) plus ATV (10 μM); 3) H-89 (0.1 μM); or 4) PIO plus ATV plus H-89 for 12 h. The supernatants were collected directly for 15-epi-LXA₄ and LTB₄ analyses by ELISA, and the cells were harvested for immunoblotting. In addition, cells were plated in eight-chamber slides, received the same treatment as above, and were used for immunohistochemical staining.

ELISA. Samples (50 μl) of the supernatant were used following the manufacturers’ instructions for the 15-epi-LXA₄ and LTB₄ immunoassay kits (36). Data are expressed as ng/μg protein (15-epi-LXA₄) or pg/μg protein (LTB₄).

Whole-cell fractions. The hearts were rapidly explanted, rinsed in cold PBS (pH 7.4) containing 0.16 mg/ml heparin to remove RBC and clots, frozen in liquid nitrogen, and stored at −80°C. Myocardial samples from the anterior left ventricular wall were homogenized in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and the total protein concentration was determined using the protein assay kit (Bio-Rad).

Separation of the cytosolic, membranous, and nuclear fraction

Myocardial samples (0.25 g) were homogenized in buffer (50 mM Tris (pH 7.5), 1 mM EDTA, 2 mM EGTA, 10 mM benzamidine, 0.3% 2-ME, 50 μg/ml PMSF, 25 mM NaN₃, 1 mM Na₂VO₃, and 1% proteinase inhibitor) with a homogenizer (PowelGen Model 125; Fisher Scientific). The lysates were centrifuged at 1000 × g for 15 min. The resultant supernatant contained the cytosolic and membrane fraction, and the pellets contained the nuclear fraction. The latter was solubilized in a buffer containing 0.3% Triton X-100 (1 h, 4°C) and then centrifuged at 105,000 × g for 1 h. The resultant supernatants of this second step were then collected as the nuclear fractions. The 1000 × g supernatants containing cytosolic and membrane fractions were centrifuged at 105,000 × g for 1 h, and the supernatant was collected as the cytosolic fractions. Pellets from this 105,000 × g spin to remove insoluble debris. The supernatants were then collected as the membranous fraction. Protein concentrations were determined by using the protein assay kit (Bio-Rad).

Immunoblotting. The protein samples (60 μg) in loading buffer were run in 4–20% Tri-HCl Ready Gel at 100 V for 2 h until the desired m.w. bands were separated. After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 193 mM glycine, 0.1% SDS, and 10% methanol), and the proteins were transferred to nitrocellulose membranes. The expression of the proteins was assessed by standard SDS-PAGE Western immunoblotting. The protein signals were quantified by an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin stain signal. Data are expressed as a ratio between the band corresponding to the protein of interest and the corresponding β-actin signal density.

Immunohistochemical study. Immunofluorescent labeling was performed on paraffin sections (5 μm) of 4% formaldehyde-fixed rat cardiac tissue, as described previously (37). The primary Abs were mouse anti-myosin IgG, rabbit anti-COX2 IgG, and rabbit anti-S5LO IgG, which were diluted in

**FIGURE 6.** Confocal microscopy (magnification ×120) of a cardiac myocyte treated with PIO plus ATV plus H-89. There is localization of the 5LO (red) around the nuclear membrane, but not inside the nucleus (blue).
Materials.
H-89, anti-\(\beta\)-actin mAbs, and anti-myosin mAbs were purchased from Sigma-Aldrich; anti-5LO and anti-Ser523 P-5LO Abs, polyclonal anti-COX2 Abs, and LTB\(_4\) enzyme immunoassay kit from Cayman Chemical; and anti-cPLA\(_2\), Abs from Cell Signaling Technology. DAPI was purchased from Vector Laboratories; goat anti-mouse Alexa 488 Abs from Molecular Probes; and universal negative controls for mouse and rabbit IgG. All the slides were viewed under an Olympus BX51 microscope (images recorded by a DP70 Digital camera (Olympus Optical)) or confocal microscope (Bio-Rad 2100).

Commmunoprecipitation. For coimmunoprecipitation, whole-cell lysates, cytosolic fractions, and membranous fractions (500 μg) were incubated with anti-SLO Abs for 4 h, followed by overnight incubation at 4°C with protein A-agarose. The agarose beads were collected by centrifugation, and SDS-PAGE Western immunoblotting was performed with the supernatant fraction. The anti-SLO precipitates were subjected to immunoblotting with anti-COX2 or anti-cPLA\(_2\) Abs.

Real-time PCR
Equal amounts of total cellular RNA were reverse transcribed with oligo(dT) primer by use of AMV reverse transcriptase (Applied Biosystems). Transcribed cDNAs (40 ng) were used for real-time PCR with specific primers, as follows: rat ALOX5 (ALOX5F, AGCCAACAAGATTGTTCC CATCGC; ALOX5R, TGGCAATACCGAACCCTGAC) and rat GAPDH (5'-ACCCCAAATGTACCCGTTG-3', 5'-TACTCTTGGAG GCCATGTA-3'). The threshold cycle (C\(_\text{t}\)) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Expression of the ALOX5 relative to the GAPDH was calculated as the difference among groups. *p < 0.001 vs PIO plus ATV; †, p < 0.005 vs sham; ‡, p = 0.009 vs PIO plus ATV plus H-89.

Results
In vivo studies
At first, we used immunoblotting to assess the effect of PIO and ATV, alone or with H-89, on myocardial levels of total SLO and P-SLO in the whole cell homogenate (n = 4 in each group) (Fig. 1). PIO and ATV did not affect total SLO concentration, but they increased myocardial levels of Ser\(^{523}\) P-SLO, whereas H-89 alone did not affect total SLO or P-SLO levels; however, it completely blocked the increase in P-SLO by both PIO and ATV. We compared the effects of PIO and ATV with that of LPS on SLO phosphorylation (n = 4 in each group) (Fig. 2). PIO and ATV alone or in combination caused a significantly greater increase in SLO phosphorylation than LPS.

For further characterization of the effects of PIO and ATV on SLO expression and translocation, we repeated the immunoblotting separately in the cytosolic, membranous, and nuclear fractions of the myocardial cells (n = 4 in each group) (Fig. 3). PIO and ATV caused a small, yet significant increase in SLO levels in the cytosolic fraction. In contrast, they had no detectable effect on the SLO levels in both the cytosolic and membranous fractions. On the other hand, when H-89 was combined with either PIO or ATV, there were significant decreases in SLO levels in the cytosolic fraction and significant increases in the membranous fraction, suggesting translocation of SLO from the cytosolic fraction to the membranous fraction. There was no expression of SLO in the nuclear fraction in all groups studied (data not shown).

Subsequently, we used immunofluorescence to localize SLO in vivo in myocardial tissue of rats pretreated with LPS, PIO plus ATV, or vehicle alone (n = 2 in each group). PIO plus ATV caused enhanced staining of SLO in the cytoplasm of myocytes, whereas LPS caused migration of SLO to the perinuclear membrane without an apparent increase in overall intensity (Fig. 4).

In vitro studies
We further characterized the SLO localization in adult rat cardiomyocyte cultures after incubation with PIO plus ATV in the presence and absence of H-89, a specific PKA inhibitor. The SLO was expressed in the cytoplasm of myosin-positive cells (experiments were repeated twice) (Fig. 5). PIO plus ATV enhanced SLO staining in cytoplasm. H-89 alone had no effect on SLO expression or distribution; however, when combined with PIO plus ATV, there was a shift of SLO toward the perinuclear region. Confocal microscopy (×120 magnification) shows that in the PIO plus ATV plus H-89-treated cells, SLO is localized around, but not inside the nucleus (Fig. 6).

PIO plus ATV had no effect on total SLO levels (data not shown), but did increase P-SLO levels in the cell cultures (n = 4 in each group) (Fig. 7). The SLO phosphorylation was almost completely blocked by H-89. PIO plus ATV increased the levels of 15-epi-LXA\(_4\), H-89 alone had no effect; however, it attenuated the PIO plus ATV augmentation of 15-epi-LXA\(_4\) levels (n = 4 in each group) (Fig. 8, a and c). Finally, whereas PIO plus ATV alone and H-89 alone had no significant effect on LTB\(_4\), H-89 given together with PIO plus ATV significantly increased myocardial LTB\(_4\) levels (Fig. 8, b and d).

Commmunoprecipitation
Using whole-cell lysate, we found that in the control animals there was no coimmunoprecipitation of either COX2 or cPLA\(_2\) with SLO (n = 3 in each group). However, in rats treated with either...
PIO or ATV, COX2, but not cPLA2, precipitated with 5LO. In contrast, in rats treated with PIO or ATV in combination with H-89, 5LO precipitated with cPLA2, but not with COX2 (Fig. 9). To further characterize the location of these interactions, we repeated the coimmunoprecipitation in the cytosolic and membranous fractions of the same hearts (n = 3 in each group) (Fig. 10). Coimmunoprecipitation of 5LO with COX2 in the ATV- or PIO-treated rats occurred only in the cytosolic fraction. In contrast, there was an increased interaction between cPLA2 and 5LO in the membranous fraction in rats treated with H-89 combined with PIO or ATV.

**RT-PCR**

To confirm that adult rat myocardial cells express 5LO, we used RT-PCR. White blood cells isolated from rat blood at basal condition or 16 h after stimulation with 5LO served as positive controls (n = 4 in each group) (38). Both adult rat cardiomyocytes and white blood cells express mRNA for 5LO (Fig. 11). PIO, ATV, and H-89 alone or in combination did not affect 5LO mRNA levels in the cardiomyocytes. LPS increased 5LO expression in the white blood cells.

**Discussion**

In the present study, we are showing that both PIO and ATV augmented 5LO phosphorylation at Ser$^{523}$ in the rat myocardium, and that this effect was blocked by H-89, a PKA inhibitor (Fig. 1). In contrast, the proinflammatory stimulation with LPS caused significantly less Ser$^{523}$ phosphorylation of 5LO (Fig. 2). Both PIO and ATV caused a small increase in 5LO levels in the cytosolic fraction (Fig. 3) without a detectable change in total 5LO cell levels (Fig. 1), suggesting translocation of 5LO into the cytosolic fraction. In contrast, inhibiting PKA activity with H-89 prevented the Ser$^{523}$ phosphorylation of 5LO by PIO and ATV (Fig. 1) and caused a shift of 5LO to the membranous fraction (Fig. 3). Under these conditions, 5LO communoprecipitated with cPLA2 (Figs. 9 and 10) and metabolized the AA, generated by cPLA2, into LTB$_4$, a strong inflammatory mediator. In contrast, when 5LO...
was prevented from shifting by Ser523 phosphorylation, it interacted with COX2 in the cytosolic fraction (Fig. 10) to generate 15-epi-LXA4 (Fig. 8a), a potent anti-inflammatory mediator.

H-89 alone had no effect on 5LO translocation, and 15-epi-LXA4 and LTB4 production. However, H-89 caused translocation of the PIO- and ATV-activated 5LO and induced the production of LTB4, suggesting that both ATV and PIO activate 5LO in a yet undefined pathway. It might be that phosphorylation at other sites, Ca2+, ATP, or lipid hydroperoxide, activates 5LO (24, 39). It is probable that LPS activates 5LO, and because most of the activated 5LO is not phosphorylated at Ser523, it is translocated to the perinuclear membrane.

Thus, it seems that Ser523 phosphorylation of 5LO by PKA not only prevents LT production, but also facilitates 15-epi-LXA4 production, and, therefore, is a key factor in determining whether the end products will be pro- or anti-inflammatory mediators. Until recently, it was thought that 5LO is expressed mainly in inflammatory cells (polymorphonuclear leukocytes, monocytes/macrophages, mast cells, B lymphocytes, dendritic cells, and foam cells in human atherosclerotic tissue) (24, 40). However, there is growing evidence that cardiomyocytes participate in innate immunity (41, 42). It has been shown that cardiomyocytes respond to various injuries by producing some of the mediators that are classically associated with cells of the innate immune system (43). We have previously shown that 5LO is expressed in cardiac myocytes (5, 23). In the present study, we are showing that rat cardiomyocytes express 5LO mRNA (Fig. 11) and protein (Figs. 1–7).

In resting human leukocytes, 5LO is found mainly in the endoplasmic reticulum; however, in stimulated leukocytes, 5LO is found in the perinuclear envelope (44). This may explain the discrepancy between the increased cytoplasmic staining of 5LO seen with immunofluorescence after incubation with ATV plus PIO (Fig. 5) and the fact that PIO, ATV, and LPS did not change total 5LO levels in whole-cell lysates (Figs. 1 and 2). Indeed, we found that both PIO and ATV increased 5LO levels in the cytosolic fraction (Fig. 3). Thus, like other stress response signaling molecules, intracellular shifting may play a bigger role than cellular levels. It might be also that different compartmentalization of 5LO in lipid droplets also changes the intensity of 5LO staining with immune fluorescence (45, 46). cPLA2 is a membrane-bound enzyme, generating AA from the membrane phospholipids (24–26). In non-stimulated rat basophilic leukemia cells, cPLA2 is found in the cytoplasm, but after stimulation it migrates to the perinuclear membrane (47). In quiescence cells, COX2 is preferentially bound to the nuclear envelope; however, in some cells COX2 can be found inside the nucleus and/or in the endoplasmic reticulum (48). However, upon stimulation, cytoplasmic accumulation of COX2 has been described in endothelial cells (48, 49). Interestingly, we found that the interaction between COX2 and 5LO occurred only in the cytosolic fraction and not in the membranous fraction. In contrast, the interaction between cPLA2 and 5LO occurred as expected in the membranous fraction.

Because the in vivo studies have shown that both PIO and ATV up-regulate COX2 expression and activity and increase the production of 15-epi-LXA4, we combined the two drugs in the in vitro experiments. We have previously shown that PIO and ATV have additive effects on 15-epi-LXA4 levels. PIO is indicated for the treatment of diabetes, and ATV for the treatment of hypercholesterolemia. Because hypercholesterolemia is commonly detected in diabetic patients, many patients are receiving both drugs in the clinical setting.

It could be that defects in 5LO phosphorylation could explain the muscle symptoms and/or elevation of muscle and liver enzymes associated with statin therapy. Moreover, it is plausible that in patients with 5LO phosphorylation deficits, statins may not decrease inflammation and, thus, may have fewer effects on atherosclerosis.

The 5LO-activating protein (FLAP, also known as ALOX5AP) activates 5LO and facilitates the production of LTs (50, 51). Increased production of LTs due to gene mutations in 5LO (52) and FLAP in Caucasians (53–57) and Japanese (58), and LTA4 hydroxylase in African-American population (59) has been associated with an increased risk of stroke and/or myocardial infarction. It is unclear how statins and/or PIO affect AA metabolism in patients with these mutations. It might be that by Ser523 phosphorylation of 5LO, statins, and PIO prevent 5LO translocation and, therefore, attenuate the proinflammatory state. In contrast, it is

**FIGURE 9.** Coimmunoprecipitation of the whole-cell lysate. The 5LO precipitated with COX2 in the ATV and PIO group, whereas 5LO precipitated with cPLA2 in the ATV plus H-89 and PIO plus H-89 groups. There were three animals in each group.

**FIGURE 10.** Coimmunoprecipitation of 5LO with COX2 and cPLA2 in the cytosolic and membranous fractions. In both the ATV and PIO groups, 5LO precipitated with COX2 in the cytosolic, but not the membranous fraction. In contrast, the interaction between cPLA2 and 5LO in the ATV plus H-89 and PIO plus H-89 occurred mainly in the membranous fraction. There were three animals in each group.

**FIGURE 11.** RT-PCR of 5LO mRNA. There is expression of 5LO in both adult rat cardiomyocytes and white blood cells. PIO, ATV, and H-89 alone or in combination did not affect 5LO expression. In contrast, LPS significantly increased 5LO expression in the white blood cells. Values represent mean ± SEM of four samples in each group. *, *p < 0.001 vs control.
possible that these agents cannot prevent this intracellular shift and, by up-regulating cPLA₂ (31–33) and activating 5LO, they augment the production of LTs in patients with such mutations. cPLA₂ generates AA, which has been shown to promote activation and translocation of 5LO to the perinuclear membrane (66). We are currently exploring this issue in several experimental models. There are also implications in medical fields other than atherosclerosis, because the increased production of LTs by COX2 and 5LO has been implicated with increased risks for colon cancer (61), Alzheimer’s disease (62–64), and asthma (65). Because statins may reduce the risk of colon cancer (66) and the progression of Alzheimer’s disease (67), it is plausible that statins (and PIO) may have a role in preventing the membranous shift of 5LO and, hence, the production of LTs in various disease states.

Phosphorylation as a mechanism responsible for the translocation of apoptotic mediators to the perinuclear membrane in response to oxidative stress is not restricted to 5LO; Bcl-2 and Bcl-xl are also phosphorylated and inactivated as antiapoptotic proteins in response to trauma (68, 69).

In conclusion, not only inflammatory cells, but also myocardial and endothelial cells (our unpublished data) can produce AA metabolites such as LTB₄ and 15-epi-LXA₄ in response to various stimuli. The 5LO phosphorylation at Ser²⁵² by PKA prevents the membranous shift of 5LO and, thus, the production of LTB₄. Instead, the cytosolic-bound 5LO processes 15R-HETE, produced by cytosolic COX₂, resulting in the production of 15-epi-LXA₄, a potent anti-inflammatory mediator. Prevention of 5LO translocation toward the perinuclear membrane by PKA-mediated phosphorylation at Ser²⁵² may explain in part the anti-inflammatory and antiatherosclerosis effects of statins and PIO.

Limitations

We have used ELISA for measurements of LTB₄ and 15-epi-LXA₄. This method is highly sensitive, but less specific than mass spectrometry.

Disclosures

The authors have no financial conflict of interest.

References


