Augmentation of Myocardial Production of 15-Epi-Lipoxin-A₄ by Pioglitazone and Atorvastatin in the Rat

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Background—Both statins and thiazolidinediones have antiinflammatory properties. However, the exact mechanisms underlying these effects are unknown. We investigated whether atorvastatin (ATV) and pioglitazone (PIO) increase the myocardial content of lipoxin-A₄ and 15(R)-epi-lipoxin-A₄ (15-epi-LXA₄), both arachidonic acid products with strong antiinflammatory properties.

Methods and Results—In experiment 1, rats received 3-day pretreatment with water; PIO 2, 5, or 10 mg · kg⁻¹ · d⁻¹; ATV 2, 5, or 10 mg · kg⁻¹ · d⁻¹; or PIO 10 mg · kg⁻¹ · d⁻¹+ATV 10 mg · kg⁻¹ · d⁻¹. In experiment 2, rats received water; PIO 10 mg · kg⁻¹ · d⁻¹+ATV 10 mg · kg⁻¹ · d⁻¹; PIO+ATV and valdecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor; PIO+ATV and zileuton, a selective 5-lipoxygenase inhibitor; or zileuton alone. There were 4 rats in each group. Hearts were harvested and analyzed for myocardial lipoxin-A₄ and 15(R)-epi-lipoxin-A₄ levels and for COX-2 and 5-lipoxygenase protein expression. ATV and PIO at 5 and 10 mg · kg⁻¹ · d⁻¹ significantly increased myocardial 15-epi-LXA₄ levels compared with the sham-treated group (0.5±0.2 ng/mg). Myocardial 15-epi-LXA₄ were significantly higher in the PIO+ATV group (1.29±0.02 ng/mg; \( P < 0.001 \) versus each other group). Both valdecoxib and zileuton abrogated the PIO+ATV increase in 15-epi-LXA₄, whereas zileuton alone had no effect. PIO, ATV, and their combination resulted in a small increase in myocardial lipoxin-A₄ levels, which was not statistically significant. ATV alone or in combination with PIO markedly augmented COX-2 expression. PIO had a much smaller effect on COX-2 expression. Myocardial expression of 5-lipoxygenase was not altered by PIO, ATV, or their combination.

Conclusions—Both PIO and ATV increase myocardial levels of 15-epi-LXA₄, a mediator with antiinflammatory properties. This finding may explain the antiinflammatory properties of both PIO and ATV. (Circulation. 2006;114:929-935.)

Key Words: aspirin ■ diabetes mellitus ■ hypercholesterolemia ■ inflammation ■ prostaglandins

Both 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins)³–⁶ and thiazolidinediones⁷–¹⁰ have antiinflammatory properties. Both have been shown to reduce serum markers of inflammation, including C-reactive protein. The mechanisms underlying their antiinflammatory effects have not been clarified.

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The possibility that the arachidonic acid metabolites lipoxin-A₄ ([5S,6R,15S]-trihydroxy-(7E,9E,11Z,13E)-eicosatetraenoic acid) and 15(R)-epi-lipoxin-A₄ ([5S,6R,15R]-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid) (15-epi-LXA₄) are the unifying mechanism was explored in this study. Lipoxin-A₄ is an arachidonic acid metabolite produced by transcellular metabolism involving 15-lipoxygenase, 5-lipoxygenase, and 12-lipoxygenase (Figure 1) that appears to mediate key events in endogenous antiinflammation and resolution.¹¹,¹² Recent studies have suggested that aspirin, in addition to blocking cyclooxygenase-1 (COX-1) and inhibiting the production of prostaglandin and thromboxane, modifies cyclooxygenase-2 (COX-2) by acetylation at the serine residue 530 near the active enzyme site. This modification restricts access of arachidonic acid to the COX-2 catalytic core, leading to incomplete reaction with preferential arachidonic acid conversion to 15-hydroxyeicosatetraenoic acid (15-R-HETE) rather than prostaglandin H₂ (the precursor of all prostaglandins).¹²–¹⁴ 15-R-HETE in turn is converted by 5-lipoxygenase to 15-epi-LXA₄, also called aspirin-triggered lipoxin.¹²,¹³ 15-epi-LXA₄ serves as a local antiinflammatory mediator involved in protean and diverse human diseases.
including airway inflammation and asthma, arthritis, graft versus host disease, and multiple cardiovascular, gastrointestinal, periodontal disease and kidney disease. It has been found that nonsteroidal antiinflammatory agents other than aspirin and selective COX-2 inhibitors do not share this property and are unable to generate 15-epi-LXA₄. Moreover, selective COX-2 inhibitors prevent 15-epi-LXA₄ generation by aspirin.¹⁷

We have shown that both atorvastatin (ATV) and pioglitazone (PIO) increase the expression and activity of both cytosolic phospholipase A₂ (cPLA₂) and COX-2 in the murine heart. cPLA₂ releases arachidonic acid from the cell membranes, which may then be metabolized to prostaglandins (including prostacyclin [PGI₂] and thromboxane) by 12-LO, 12-lipoxygenase, and to leukotrienes by 5-lipoxygenase, and to lipoxins by 15-lipoxygenase (Figure 1). However, when COX-2 is altered by aspirin, 15-epi-LXA₄ is formed. In the present study, we investigated whether ATV and PIO increase lipoxin-A₄ and 15-epi-LXA₄ production in the rat heart, a property that may explain their antiinflammatory effects.

**Methods**

**Animal Care**

All animals received humane care in compliance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Experiments were conducted on male Sprague-Dawley rats (Harlan, Houston, Tex). The protocol was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

**Drugs and Pretreatment**

**Experiment 1**

Rats received 3-day pretreatment with water alone (sham); PIO 2.5, or 10 mg · kg⁻¹ · d⁻¹; ATV 2.5, or 10 mg · kg⁻¹ · d⁻¹; or ATV 10 mg · kg⁻¹ · d⁻¹ and PIO 10 mg · kg⁻¹ · d⁻¹.

**Experiment 2**

Rats received sham; ATV 10 mg · kg⁻¹ · d⁻¹ and PIO 10 mg · kg⁻¹ · d⁻¹; ATV 10 mg · kg⁻¹ · d⁻¹; PIO 10 mg · kg⁻¹ · d⁻¹, and valdecoxib, a selective COX-2 inhibitor, 3 mg · kg⁻¹ · d⁻¹; and PIO 10 mg · kg⁻¹ · d⁻¹, PIO 10 mg · kg⁻¹ · d⁻¹, and zileuton, a selective 5-lipoxygenase inhibitor, 200 mg · kg⁻¹ · d⁻¹; or zileuton 200 mg · kg⁻¹ · d⁻¹. There were 4 rats in each group. PIO, ATV, and valdecoxib were administered by oral gavage once daily; zileuton was added to the drinking water. The sham-treated group received water by oral gavage once daily. On the fourth day, the rats were anesthetized, and the hearts were explanted and analyzed for myocardial 15-epi-LXA₄ and lipoxin-A₄ levels and for myocardial COX-2 and 5-lipoxygenase protein expression (experiment 1). Hearts from experiment 2 were assessed for myocardial levels of 15-epi-LXA₄.

**Immunoblotting**

The hearts were rapidly explanted, rinsed in cold phosphate-buffered saline (pH 7.4) containing 0.16 mg/mL heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at −70°C. Myocardial samples from the anterior left ventricular wall were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, Calif) and centrifuged at 14 000 rpm for 15 minutes at 4°C. The supernatant was collected, and the total protein concentration was determined with the Lowry protein assay (Pierce, Rockford, Ill). The protein samples with loading buffer were run in 4% to 20% Tri-HCl Ready Gel (Bio-Rad, Hercules, Calif) at a 100 V for 2 hours until the desired molecular weight bands were separated. After electrophoresis, the gel was equilibrated in transfer buffer (25 mmol/L Tris, 193 mmol/L glycine, 0.1% SDS, and 10% methanol), and the proteins were transferred to nitrocellulose membrane. The protein signals were quantified by an image-scanning densitometer (Image-J 1.33a, NIH), and the strength of each protein signal was normalized to the corresponding β-actin stain signal. Data are expressed as a ratio to the corresponding β-actin signal density.

**ELISA**

The hearts were rapidly explanted, rinsed in cold phosphate-buffered saline (pH 7.4) containing 0.16 mg/mL heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at −70°C. Myocardial samples from the anterior left ventricular wall were homogenized in ethanol (5 mL/g) and centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was diluted with water and acidified to pH 3.5 with 1N HCl. The sample was loaded into a C-18 Sep-Pak light column (Waters Corp, Milford, Mass) and washed with 1 mL water followed by 1 mL petroleum ether. The sample was eluted with 2 mL methyl formate. The methyl formate was evaporated with N₂, and the residue was dissolved in extraction buffer. We followed the manufacturer’s instruction (Oxford Biomedical Research, Oxford, Mich) for the lipoxin-A₄ and 15-epi-LXA₄ immunoassay kits.

**Immunohistochemical Study**

Immunofluorescent labeling was performed on paraffin sections (5 μm) of 4% formaldehyde-fixed rat cardiac tissue, as described previously. The primary antibodies were mouse anti-myosin immunoglobulin G (IgG), diluted to 1:2000; rabbit anti-COX-2 IgG, diluted to 1:1000; and rabbit anti–5-lipoxygenase IgG, diluted to 1:2000. The secondary antibodies were goat anti-mouse Alexa 488 (diluted 1:500; Molecular Probe, Eugene, Ore) and goat anti-rabbit Alexa 594 (diluted 1:500; Molecular Probe, Eugene, Ore). The specificity of mouse and rabbit primary antibodies was tested by substituting them with universal negative controls for mouse and rabbit IgG (DAKO Corp, Carinarteria, Calif). All slides were viewed under an Olympus BX51 microscope, and images were recorded by a D7P70 digital camera (Olympus Optical Co, Ltd, Tokyo, Japan).

**Materials**

Polyclonal anti-COX-2 and polyclonal anti–5-lipoxygenase antibodies were purchased from Cayman Chemicals (Ann Arbor, Mich), and monoclonal anti–β-actin antibodies and monoclonal anti-myosin antibodies were from Sigma (St Louis, Mo). Lipoxin-A₄ and 15-epi-LXA₄ were expressed as a ratio between the protein and the corresponding β-actin signal effects.
LXA₄ ELISA kits were purchased from Oxford Biomedical Research (Oxford, Mich). Pioglitazone was provided by Takeda Pharmaceuticals North America, Inc (Lincolnshire, Ill). We used crashed tablets for ATV and valdecoxib (Pfizer Pharmaceuticals, New York, NY) and zileuton (Critical Therapeutics, Inc, Lexington, Mass).

Statistical Analyses
Data are expressed as mean±SEM. Comparisons among groups were performed by 1-way ANOVA with Sidak correction for multiple comparisons (SPSS version 11.5.2.1; SPSS, Inc, Chicago, Ill). Values of P<0.05 were considered statistically significant.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
ATV and PIO significantly increased myocardial content of 15-epi-LXA₄. With both agents, the effect was dose dependent (Figure 2). Myocardial 15-epi-LXA₄ was significantly higher in the PIO+ATV group than in the other 3 groups. Both valdecoxib and zileuton completely abrogated the ATV+PIO increase in myocardial 15-epi-LXA₄, whereas zileuton alone had no effect (Figure 2D).

ATV, PIO, and their combination resulted in a small increase in myocardial lipoxin-A₄ levels, which was not statistically significant (Figure 3).

COX-2 expression was very low in the sham-treated rats. COX-2 expression was markedly increased in the ATV alone and PIO+ATV groups (Figure 4). In the same blot, we were not able to detect increased COX-2 expression in the PIO alone group. Therefore, we repeated the experiment for sham and PIO groups (n=4 in each group) with longer film exposure, showing a significant increase in the PIO alone group, although of a much smaller magnitude than seen with ATV (Figure 5). On the other hand, 5-lipoxygenase expression was not altered by ATV, PIO, or their combination (Figure 6).

Immunofluorescence showed enhanced expression of both COX-2 and 5-lipoxygenase by PIO+ATV in cells positive for 15-epi-LXA₄.
for myosin staining, suggesting that PIO and ATV augmented 15-epi-LXA₄ production in the myocardium (Figure 7). No immunoreactivity was detected in control slides with universal negative control methods.

**Discussion**

The main findings of the present study are that both ATV and PIO increased myocardial levels of 15-epi-LXA₄, an antiinflammatory lipid mediator that until now has been reported to be induced only by aspirin. This effect was dose dependent. We showed that 15-epi-LXA₄ is produced by both COX-2 and 5-lipoxygenase; inhibition of these enzymes with valdecoxib and zileuton, respectively, prevented the augmentation of 15-epi-LXA₄. Immunofluorescence staining showed that the cardiomyocytes augment COX-2 and 5-lipoxygenase expression with PIO and ATV treatment.

We have previously shown that 3-day pretreatment with ATV 10 mg · kg⁻¹ · d⁻¹ upregulates cPLA₂ and COX-2 expression and activity. In that study, we found that the ATV blood level 16 hours after the last dose of 10 mg · kg⁻¹ · d⁻¹ was 128.5 ± 9.2 ng Eq/mL (unpublished data). Data provided by Pfizer shows that after a 14-day treatment with oral ATV 80 mg/d, the maximal concentration of ATV in humans is 252 ng Eq/mL. The t₁/₂ of ATV in this experiment is 19.3 hours. Therefore, ATV blood levels achieved in the rat with a dose of 10 mg · kg⁻¹ · d⁻¹ are comparable to those seen in humans treated with 80 mg/d.

In the present study, we demonstrate that PIO upregulated COX-2 expression, although to a lesser extent than ATV. Recently, we found that a 3-day pretreatment with PIO 10 mg · kg⁻¹ · d⁻¹ augments COX-2 expression and activity, although to a lesser extent than ATV. PIO significantly upregulates cPLA₂ expression and activity. Both ATV and PIO increase myocardial PGI₂ production, as evidenced by an increase in the concentrations of myocardial 6-keto-PGF₁α (the stable metabolite of PGI₂). Kim et al and Atar et al have shown that inducible nitric oxide synthase (iNOS) activates COX-2 by S-nitrosylation. S-nitrosylation of COX-2 occurs on all its 13 cysteine residues; however, it seems that S-nitrosylation of Cys⁵²⁶ is responsible for COX-2 activation, at least as assessed by prostaglandin E₂ production. It is unclear how ATV and PIO alter COX-2 activity to produce 15-R-HETE in addition to prostaglandin H₂ (the precursor of prostaglandins). 15-R-HETE is converted by 5-lipoxygenase to 15-epi-LXA₄. We have shown that ATV upregulates iNOS expression and activity and that the ATV induction of 5-lipoxygenase with PIO.}

**Figure 3.** Myocardial lipoxin-A₄ levels.

**Figure 4.** A, Representative immunoblots of COX-2 and the corresponding β-actin. B, Densitometric analyses of COX-2/β-actin (arbitrary units).

**Figure 5.** A, Immunoblots of COX-2 and the corresponding β-actin with prolonged exposure. B, Densitometric analyses of COX-2/β-actin ratio (arbitrary units).

**Figure 6.** A, Representative immunoblots of 5-lipoxygenase and the corresponding β-actin. B, Densitometric analyses of 5-lipoxygenase/β-actin ratio (arbitrary units).
myocardial 6-keto-PGF$_{1\alpha}$ concentration is completely blunted with 1400W, a specific iNOS inhibitor. However, PIO does not induce iNOS in our model. Moreover, others have reported that thiazolidinediones suppress iNOS expression. Thus, alteration of COX-2 by S-nitrosylation of a cysteine residue(s) may explain the mechanism by which PIO alters COX-2.

It has been suggested that arachidonic acid can be converted to 15-R-HETE by cytochrome P-450 in the liver and in human lung adenocarcinoma cell line (A549) but not in rat epidermal microsomes. Valdecoxib, a selective COX-2 inhibitor, completely blocked the effect of ATV and PIO on 15-epi-LXA$_4$ formation (Figure 2), suggesting that the production of 15-R-HETE by P-450 in the heart is negligible. However, valdecoxib may suppress P-450 2C9 (CYP2C9). On the other hand, it has been reported that P-450 induced by aspirin generates both 15-R-HETE (40%, which is converted to 15-epi-LXA$_4$) and 15-S-HETE (60%, which is converted to lipoxin-A$_4$). Indeed, it was demonstrated that aspirin augmented the generation of both lipoxins and 15-epi-lipoxins in human lung adenocarcinoma cell line. In contrast, we have found a much larger increase in 15-epi-LXA$_4$ than in lipoxin-A$_4$, suggesting that the COX-2, not P-450, is the major source of 15-R-HETE in our model. There are conflicting data on whether PIO affects P-450 activity. Most investigators have reported that PIO does not affect P-450 expression and activity in the kidney. In vitro experiments showed that PIO induces CYP3A4 and CYP2B6 expression in primary human hepatocytes. However, PIO also has the potential to inhibit CYP2C8 activity. On the other hand, in humans, PIO at 45 mg/d for 14 days did not affect CYP3A4 activity. The mechanisms of augmented 15-R-HETE production by PIO and the role of P-450 should be further investigated.

Lipoxin-A$_4$ and 15-epi-LXA$_4$ inhibit chemotaxis, adherence, and transmigration of neutrophils. In addition, they inhibit neutrophil–epithelial and endothelial cell interactions. Stable analogs of lipoxins modulate expression of both L-selectin and CD11/CD18 on resting and immunosupulated leukocytes and inhibit neutrophil adhesion to human coronary artery endothelial cells by attenuating CD11/CD18 expression. They also inhibit eosinophil migration and chemotaxis and block cytotoxicity of natural killer cells. On the other hand, they stimulate monocyte chemotaxis and enhance macrophage phagocytosis of apoptotic leukocytes, leading to resolution of inflammation. 15-epi-LXA$_4$ mediates its antiinflammatory effect via activation of the lipoxin receptors (ALX). The same receptor also is activated by annexin-1. Annexin-1 has antiinflammatory properties and has been shown to reduce myocardial infarct size. In addition, 15-epi-LXA$_4$ binds and inhibits the leukotriene D$_4$ receptor (CysLT$_1$), thus antagonizing the proinflammatory effects of leukotrienes. Both aspirin and a stable analog of 15ALX$_4$ induce endothelial heme oxygenase-1. Heme oxygenase-1 protects the cells against oxidative injury and has antiinflammatory, antiapoptotic, and antiproliferative effects. Some investigators suggested that 15-epi-LXA$_4$ exerts its antiinflammatory effects by inducing endothelial NOS and iNOS; however, we have previously shown that concomitant treatment with valdecoxib 3 mg · kg$^{-1}$ · d$^{-1}$ does not blunt the upregulation of phosphorylated endothelial NOS and iNOS expression by ATV. Here, we show, however, that the same dose of valdecoxib abrogated the ATV induction of 15-epi-LXA$_4$, suggesting that the induction of nitric oxide production by ATV is not dependent on 15-epi-LXA$_4$. Some have suggested that 15-epi-LXA$_4$, probably through upregulation of nitric oxide production, causes vascular relaxation and modulates systemic blood pressure.

In conclusion, we have demonstrated that both PIO and ATV, and especially their combination, increase myocardial levels of 15-epi-LXA$_4$, a lipid mediator with antiinflammatory properties. Further studies are required to assess whether 15-epi-LXA$_4$, probably through upregulation of nitric oxide production, causes vascular relaxation and modulates systemic blood pressure.

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Disclosures
Dr Birnbaum has served on the Speakers Bureau of and received honoraria from Takeda, has been an expert witness for Wyeth, and has served as a consultant for Takeda. The other authors report no conflicts.

References


CLINICAL PERSPECTIVE

Statins have antiinflammatory and antiatherosclerotic effects that are not fully explained by their ability to lower serum cholesterol. Studies in humans have repeatedly shown that statins reduce serum markers of inflammation such as C-reactive protein. Recent studies have suggested that pioglitazone, a peroxisome proliferation–activated receptor-γ agonist used to treat diabetes mellitus, also reduces serum inflammatory markers. Both atorvastatin and pioglitazone have been shown to reduce cardiovascular events in clinical studies. It is still unclear how these drugs mediate their antiinflammatory and antiatherosclerotic effects. In the present study, we show that both pioglitazone and atorvastatin increase myocardial levels of 15-epi-lipoxin-A4 (15-epi-LXA4), an arachidonic acid metabolite with potent antiinflammatory properties. 15-epi-LXA4 is produced from 15-hydroxyeicosatetraenoic acid (15-R-HETE) by 5-lipoxygenase. Studies have suggested that 15-R-HETE is produced from arachidonic acid mainly by cyclooxygenase-2 acetylated by aspirin. It should be noted that in the liver cytochrome P-450 also can produce 15-R-HETE after treatment with aspirin. The findings of the present study demonstrate that the myocardium produces 15-epi-LXA4 without exposure to aspirin and that myocardial levels of 15-epi-LXA4 are increased by both pioglitazone and atorvastatin. The effect of both drugs on 15-epi-LXA4 levels is additive. Increasing 15-epi-LXA4 levels by upregulation and activation of cyclooxygenase-2 and 5-lipoxygenase may explain the mechanism of the antiinflammatory and antiatherosclerotic effects of both statins and thiazolidinediones. Further studies are needed to assess the importance of 15-epi-LXA4 in slowing the progression of atherosclerosis and preventing plaque inflammation in animal models and in humans.

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