Pioglitazone Inhibits LOX-1 Expression in Human Coronary Artery Endothelial Cells by Reducing Intracellular Superoxide Radical Generation

Jawahar L. Mehta, Bo Hu, Jiawei Chen, Dayuan Li

Objective—LOX-1, a novel lectin-like receptor for oxidized LDL (ox-LDL), is expressed in response to ox-LDL, angiotensin II (Ang II), tumor necrosis factor (TNF)-α, and other stress stimuli. It is highly expressed in atherosclerotic tissues. Peroxisome proliferator–activated receptor (PPAR)-γ ligands, such as pioglitazone, exert antiatherosclerotic effects. This study examined the regulation of LOX-1 expression in human coronary artery endothelial cells (HCAECs) by pioglitazone.

Methods and Results—Fourth generation HCAECs were treated with ox-LDL, Ang II, or TNF-α with or without pioglitazone pretreatment. All 3 stimuli upregulated LOX-1 expression (mRNA and protein). Pioglitazone, in a concentration-dependent manner, reduced LOX-1 expression ($P<0.01$ versus ox-LDL, Ang II, or TNF-α alone). Ox-LDL, Ang II, and TNF-α each enhanced intracellular superoxide radical generation, and pioglitazone pretreatment reduced superoxide generation ($P<0.01$ versus ox-LDL, Ang II, or TNF-α). Furthermore, all 3 stimuli upregulated the expression of the transcription factors nuclear factor-κB and activator protein-1 (determined by electrophoretic mobility shift assay), and pioglitazone pretreatment reduced this expression ($P<0.01$ versus ox-LDL, Ang II, or TNF-α). To determine the biological significance of pioglitazone-mediated downregulation of LOX-1, we studied monocyte adhesion to ox-LDL–treated HCAECs. Pioglitazone reduced the adhesion of monocytes to activated HCAECs in a fashion similar to that produced by antisense to LOX-1 mRNA.

Conclusions—These observations suggest that the PPAR-γ ligand pioglitazone reduces intracellular superoxide radical generation and subsequently reduces the expression of transcription factors, expression of the LOX-1 gene, and monocyte adhesion to activated endothelium. The salutary effect of PPAR-γ ligands in atherogenesis may involve the inhibition of LOX-1 and the adhesion of monocytes to endothelium. **(*Arterioscler Thromb Vasc Biol*. 2003;23:2203-2208.)**

Key Words: angiotensin ■ atherosclerosis ■ oxidized LDL ■ peroxisome proliferator–activated receptor-γ ■ tumor necrosis factor-α

Endothelial dysfunction elicited by oxidized LDL (ox-LDL) plays a critical role in the pathogenesis of atherosclerosis.[1] Ox-LDL changes the secretory activities of the endothelium and causes it to become dysfunctional.[2] Recent studies have demonstrated that atherosclerotic tissues express large amounts of ox-LDL[3] and all the constituents of the renin-angiotensin system, such as ACE and angiotensin II (Ang II) type I receptors (AT_1Rs).[4,5] Other work has demonstrated that there is an enhanced expression of cytokines, such as tumor necrosis factor (TNF)-α, in the atherosclerotic plaque.[6] Ox-LDL, Ang II, and TNF-α all induce the expression of adhesion molecules on the endothelial cells, reduce constitutive NO synthase, and facilitate inflammation, a key process in atherogenesis.

Scavenger receptors on macrophages and smooth muscle cells are believed to mediate the biological effect of ox-LDL and Ang II. Recent studies show that LOX-1, a novel lectin-like receptor for ox-LDL, facilitates the uptake of ox-LDL and mediates several of its biological effects.[7] LOX-1 mediates ox-LDL–induced apoptosis in endothelial cells and phagocytosis of aged and apoptotic cells. The expression of LOX-1 gene is upregulated by ox-LDL, Ang II, inflammatory cytokines, and shear stress.[7] Other studies from our laboratory have shown a cross talk between ox-LDL and Ang II in the sense that ox-LDL upregulates the expression of ACE and AT_1Rs.[8,9] Recent studies have shown that LOX-1 expression is also upregulated in atherosclerotic tissues from rabbits and humans.[10–12] The expression of LOX-1[13] and AT_1R[14] is also increased in ischemic/reperfused tissues in the rat.

Peroxisome proliferator–activated receptors (PPARs) are members of the nuclear receptor family that, on ligand

Received August 4, 2003; revision accepted August 26, 2003.

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000094411.98127.5F**

activation, can modulate gene transcription.[15] There are 3 types of PPARs: α, γ, and δ. PPAR-γ ligand activation has been shown to affect glucose and lipid metabolism, and thiazolidine diones, such as rosiglitazone and pioglitazone, are pharmacological PPAR-γ ligands that are used in the treatment of type II diabetes. A number of studies have demonstrated that these agents exert potent antioxidant and anti-inflammatory effects that result in the protection of myocardium from ischemia/reperfusion injury in a nondiabetic setting.[16,17] These agents have also been shown to have a potent antiatherosclerotic effect.[18]

On the basis of the fact that PPAR-γ ligands exert antioxidant effects and that oxidant species enhance the expression of LOX-1,[19] we tested the hypothesis that pioglitazone may decrease LOX-1 expression in human coronary artery endothelial cells (HCAECs) elicited by a number of stimuli.

Methods

Cell Culture

The methodology for the culture of HCAECs has been described earlier.[8,9] The initial batch of HCAECs was purchased from Clonetics Corp. The endothelial cells were pure on the basis of morphology and staining for factor VIII–related antigen and acetylated LDL. These cells were 100% negative for α-actin smooth muscle expression.

Study Design

In preliminary studies, HCAECs were incubated with ox-LDL (10, 20, 40, and 80 μg/mL), Ang II (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L), and TNF-α (1 and 10 ng/mL) for 1, 3, 6, or 24 hours to determine the expression of LOX-1. The concentration and time point for maximal effect of ox-LDL, Ang II, and TNF-α were used in subsequent experiments. In parallel experiments, cells were pretreated with pioglitazone (1 and 10 mmol/L, Takeda Pharmaceuticals North America, Inc) for 60 minutes before incubation with ox-LDL, Ang II, and TNF-α. The harvested cells were used to measure the expression of LOX-1.

Concentrations of all reagents and the duration of incubation were chosen on the basis of previous studies.[8,9,20,21]

Preparation of Lipoproteins

Native LDL and ox-LDL were prepared as described earlier.[8,9] The thiobarbituric acid–reactive substance content of ox-LDL and native LDL was 10.2 ± 0.53 and 0.79 ± 0.26 nmol/100 μg protein, respectively ($P<0.01$). Ox-LDL was extensively dialyzed against Tris-saline. Ox-LDL was kept in 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 2 mmol/L EDTA at pH 7.4 and was used within 10 days of preparation. The level of endotoxin measured by the E-Toxate kit (Sigma) was consistently <0.005 endotoxin units/mL (lowest detection limit).

Measurement of Superoxide Radical Formation

HCAECs were treated with pioglitazone and then exposed to ox-LDL, Ang II, or TNF-α for 24 hours at 37°C, and then suspended in Krebs-Ringer buffer (pH 7.4) containing 10 μmol/L coelenterazine. In parallel experiments, cells were exposed to buffer or pioglitazone alone. The chemiluminescence of coelenterazine was detected on a scintillation counter (LS 7000, Beckman Coulter Inc) in out-of-coincidence mode with a single active photomultiplier tube. The data on superoxide anion generation was expressed as counts per minute per milligram protein, as described previously.[22]

RT-PCR for LOX-1 mRNA Expression

The method for LOX-1 mRNA expression was the same as that described earlier by us.[12,13] In brief, 1.5 μL of the reverse transcription (RT) material of each sample of total RNA was amplified with Taq DNA polymerase (Promega) by using a primer pair specific to human endothelial receptor (forward primer, 5′-TTACTCTCCATGGTGGTGCC-3′; reverse primer, 5′-AGCTTCTTCTGCTTGTTGCC-3′). The polymerase chain reaction (PCR) product was 193 bp. For PCR, 35 cycles were used at 94°C for 40 seconds, 55°C for 1 minute, and 72°C for 1 minute. RT-PCR–amplified samples were visualized on 1.5% agarose gels by using ethidium bromide. Human β-actin was amplified as a reference for quantification of LOX-1 mRNA. Relative intensities of the bands of interest were analyzed by an NSF-300G scanner (Microtek) and scan analysis software (Biosoft) and expressed as the ratio to the β-actin mRNA band.

Western Analysis for LOX-1 Protein in HCAECs

The method for LOX-1 protein expression was same as that described earlier.[12,13] The primary antibody to LOX-1 was a gift from Dr T. Sawamura, Osaka, Japan. The second antibody was purchased from Amersham Life Science.

Electrophoretic Mobility Shift Assay

Isolation of the nuclear fraction was accomplished according to the previously published procedure.[23] Oligonucleotides containing the consensus sequence for activator protein (AP)-1 and nuclear factor (NF)-κB were end-labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase and purified by use of Chroma Spin-10 columns (BD Biosciences). The labeled oligonucleotides were incubated with the nuclear fractions for 30 minutes at room temperature in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L dithiothreitol, 250 mmol/L NaCl, and 0.25 mg/mL poly(dI-dC). The products were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel by using 0.5× TBE (45 mmol/L Tris-borate and 1 mmol/L EDTA) as the running buffer. The gels were dried and exposed to a radiographic film.

Adhesion of Monocytes to Endothelial Cells

The method for isolation of human blood monocytes and their adhesion to HCAECs have been described previously.[24] The HCAECs were activated with ox-LDL (40 μg/mL), Ang II (10^{-7} mol/L), and TNF-α (10 ng/mL). In parallel experiments, HCAECs were pretreated with a specific antisense (or sense) to LOX-1 mRNA (0.5 mmol/L) for 24 hours before treatment with ox-LDL (40 μg/mL). Monocyte adhesion was quantified in LOX-1 antisense (LOX-1-AS)– and LOX-1 sense (LOX-1-S)–treated cells, as described.[24]

Data Analysis

All data represent the mean of 6 separately performed experiments. Data are presented as mean±SD. Data were analyzed by ANOVA, followed by the post hoc Scheffé F test. A value of $P<0.05$ was considered to be statistically significant.

Results

Ox-LDL, Expression of LOX-1, and Effect of Pioglitazone

Incubation of HCAECs with ox-LDL, Ang II, or TNF-α increased the expression of LOX-1 in a concentration- and time-dependent fashion as described earlier.[7] The increase in protein synthesis paralleled the increase in mRNA. In all subsequent experiments, HCAECs were incubated with ox-LDL (60 μg/mL), Ang II (10^{-7} mol/L), or TNF-α (10 ng/mL).

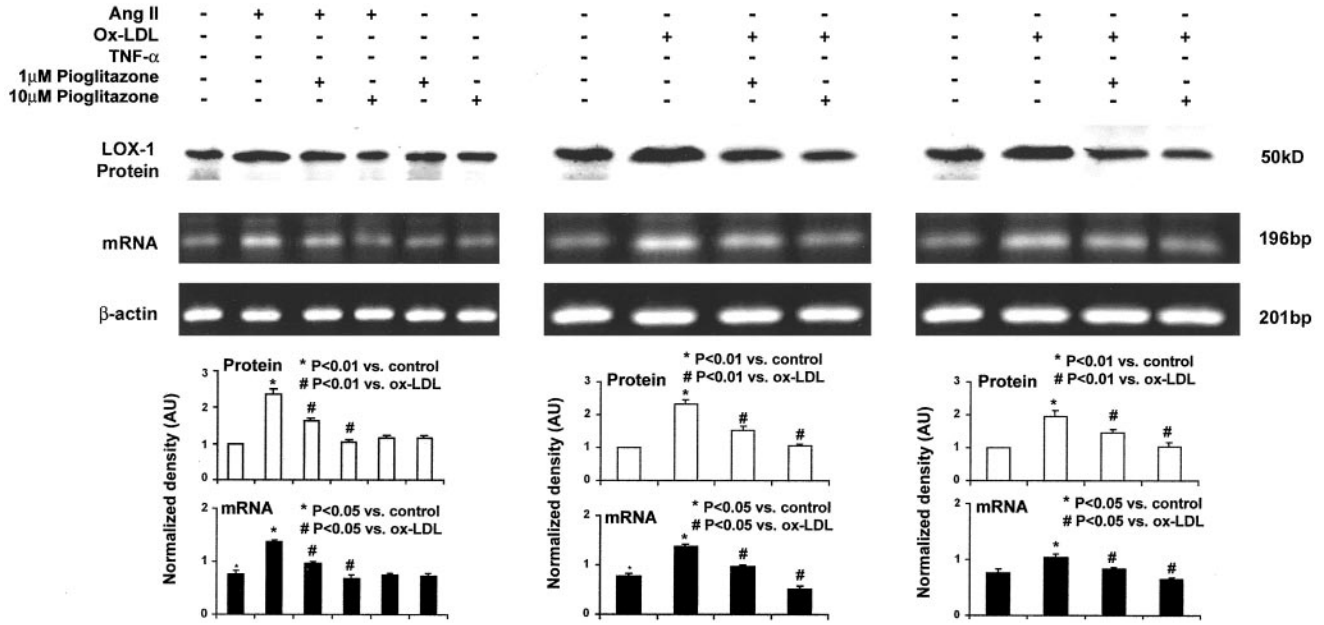

Figure 1. Pioglitazone and LOX-1 expression. Incubation of HCAECs with ox-LDL (60 μg/mL), Ang II (10^{-7} mol/L), or TNF-α (10 ng/mL) increased the expression of LOX-1. The increase in protein synthesis paralleled the increase in mRNA. Pretreatment of cells with pioglitazone markedly decreased the expression of LOX-1 (mRNA and protein) regardless of the stimulus, ie, ox-LDL, Ang II, or TNF-α. The 10 μmol/L concentration was more effective than the 1 μmol/L concentration of pioglitazone in this regard. Pioglitazone alone had no effect on the basal expression of LOX-1. AU indicates arbitrary units. Top panels are representative of 6 separate experiments. Bottom panels show summary of data (mean±SD) from these 6 experiments.

Pretreatment of cells with pioglitazone markedly decreased the expression of LOX-1 (mRNA and protein) in a concentration-dependent manner. The reduction in LOX-1 expression of LOX-1 was observed regardless of the stimulus, ie, ox-LDL, Ang II, or TNF-α. The 10 μmol/L concentration of pioglitazone was more effective than the 1 μmol/L concentration in this regard. Pioglitazone alone had no effect on the basal expression of LOX-1. Representative experiments and the summarized data from 6 independent experiments are shown in Figure 1.

Superoxide Radical Generation in Endothelial Cells and Effect of Pioglitazone

Previous studies have shown that LOX-1 expression and activation are associated with the generation of reactive oxygen species (ROS)[19] and that PPAR-γ ligands exert a modest antioxidant effect.[15] Therefore, we conducted experiments to examine superoxide radical generation in response to ox-LDL, Ang II, or TNF-α and its modulation by pioglitazone in HCAECs. As shown in Figure 2, treatment of cells with ox-LDL, Ang II, or TNF-α resulted in more than doubling of superoxide anion generation ($P<0.01$ versus baseline). Pretreatment of cells with pioglitazone reduced superoxide radical generation ($P<0.01$ versus superoxide generation in cells treated with ox-LDL, Ang II, or TNF-α alone; Figure 2).

Again, the 10 μmol/L concentration of pioglitazone was more effective than the 1 μmol/L concentration (Figure 2). The effect of superoxide radical generation paralleled the effect on LOX-1 expression. Pioglitazone (1 and 10 μm) had no effect on basal levels of superoxide radical formation.

Effect of Pioglitazone on Monocyte Adhesion

To determine the biological significance of the effect of pioglitazone on LOX-1 expression, we evaluated monocyte adhesion to the activated endothelium. As shown in Figure 3, ox-LDL, Ang II, and TNF-α each caused a significant increase in monocyte adhesion to HCAECs. Pretreatment with pioglitazone decreased the number of adherent monocytes. In parallel experiments, we pretreated HCAECs with LOX-1-AS or LOX-1-S. Pretreatment with LOX-1-AS de-

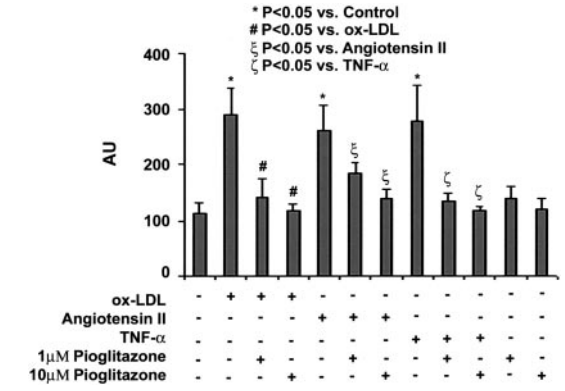

Figure 2. Pioglitazone and superoxide radical generation. Incubation of HCAECs (10^4) with ox-LDL (60 μg/mL), Ang II (10^{-7} mol/L), or TNF-α (10 ng/mL) increased the generation of superoxide radicals. Pretreatment of cells with pioglitazone markedly decreased superoxide radical generation regardless of the stimulus, ie, ox-LDL, Ang II, or TNF-α. The 10 μmol/L concentration was more effective than the 1 μmol/L concentration of pioglitazone in this regard. Pioglitazone alone had no effect on the basal expression of LOX-1. Graph is the summary of data (mean±SD) from these 6 experiments.

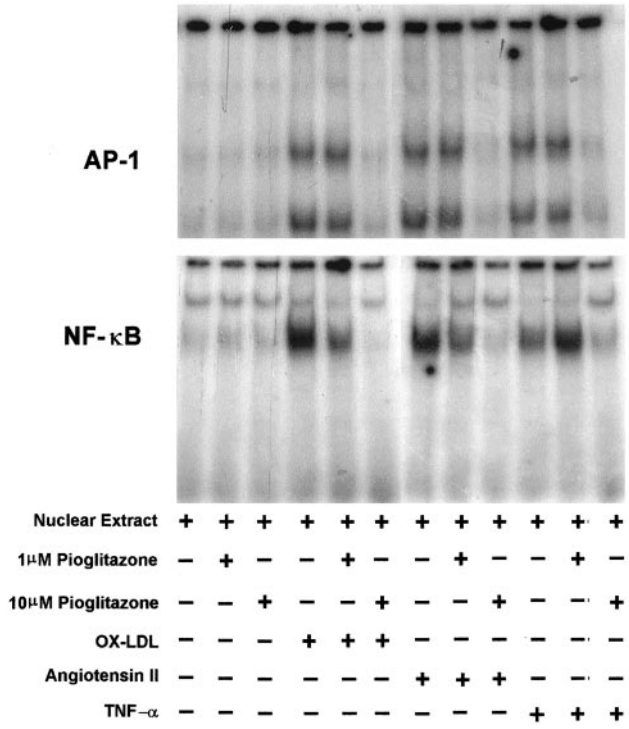

Figure 3. Monocyte adhesion to endothelial cells. Treatment of cells with ox-LDL, TNF-α, or Ang II markedly increased the adhesion of monocytes to HCAECs. Pretreatment of cells with pioglitazone reduced monocyte adhesion in response to all 3 stimuli. In parallel experiments, pretreatment of cells with LOX-1-AS reduced monocyte adhesion, whereas LOX-1-S had no effect. Notice the similarity of data on monocyte adhesion in response to pioglitazone and LOX-1-AS. Data (mean±SD) are based on 3 experiments.

creased the number of adhesion monocytes, whereas LOX-1-S had no effect. The reduction in monocyte adhesion by pioglitazone was qualitatively and quantitatively similar to that in LOX-1-AS–pretreated cells.

Intracellular Mechanism

To determine the intracellular mechanism of LOX-1 expression, we explored the role of transcription factors NF-κB and AP-1. We found that ox-LDL, Ang II, and TNF-α each activated redox-sensitive transcription factors NF-κB and AP-1. Pioglitazone attenuated this effect of ox-LDL, Ang II, and TNF-α (Figure 4).

Discussion

Atherosclerosis is characterized by the accumulation of ox-LDL, particularly in the rupture-prone region.[3] In addition, atherosclerotic tissues have been shown to express various components of the renin-angiotensin system[4,5] and a variety of proinflammatory cytokines, such as TNF-α.[6] Recent studies have demonstrated that atherosclerotic tissues of animals and humans exhibit intense LOX-1 expression.[10–12] Perhaps ox-LDL, Ang II, and TNF-α act in concert in the initiation and propagation of atherosclerosis. Accordingly, we used these 3 different mediators to assess LOX-1 expression in HCAECs in the present study. In accordance with previous data,[23,25,26] we observed that ox-LDL, Ang II, and TNF-α,

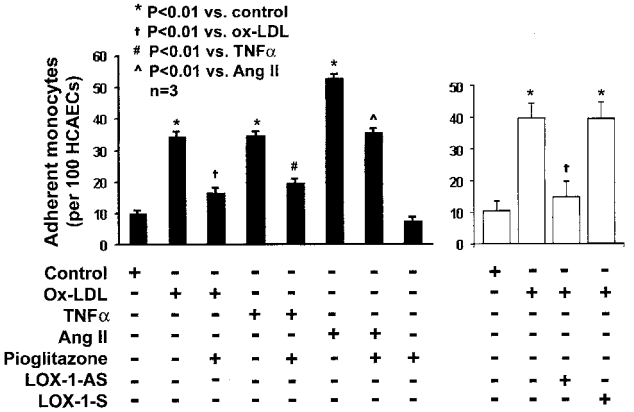

Figure 4. Pioglitazone and expression of transcription factors NF-κB and AP-1 by electrophoretic mobility shift assay. Incubation of HCAECs with ox-LDL (60 μg/mL), Ang II (10^{-7} mol/L), or TNF-α (10 ng/mL) increased the expression of transcription factors NF-κB and AP-1. Pretreatment of cells with pioglitazone markedly decreased the expression of NF-κB and AP-1. The 10 μmol/L concentration was more effective than the 1 μmol/L concentration of pioglitazone in this regard. Pioglitazone alone had no effect on the basal expression of LOX-1. This experiment is representative of 6 independent experiments.

each in a concentration- and time-dependent fashion, increased LOX-1 mRNA and protein expression. Importantly, we observed that pioglitazone almost completely blocked LOX-1 expression in response to these 3 different unrelated stimuli.

Atherosclerotic tissues have been shown to generate a large amount of ROS,[27] and antioxidants appear to diminish the extent of atherosclerosis, particularly in the animal models.[28,29] Some human studies also demonstrate the potentially beneficial effects of antioxidants on the progression of atherosclerosis.[30] Ox-LDL, Ang II, and TNF-α have each been shown to enhance superoxide radical generation in studies in vitro and in vivo. The present study again demonstrates that these stimuli result in a marked 2- to 3-fold increase in superoxide radical generation in HCAECs. Furthermore, pioglitazone, in a concentration-dependent fashion, decreased superoxide radical generation in all instances in HCAECs. Notably, pioglitazone did not affect the basal levels of superoxide generation in these cells.

Cominacini et al[19] showed that LOX activation is associated with an enhanced release of ROS and a reduction in cellular concentration of NO. Furthermore, oxidative stress stimulates LOX-1 expression.[7] We believe that the inhibitory effect of pioglitazone on superoxide radical generation may relate to the inhibition of transcription factors, such as NF-κB and AP-1, in HCAECs. PPAR-γ ligands have been shown to decrease the expression of redox-sensitive transcription factors, such as NF-κB, and it appears intuitive to attribute their inhibitory effects on transcription factors to the suppression of ROS.[19] Recent reports[31] have also attributed tissue protection with PPAR-γ ligands in diabetic and nondiabetic rat hearts to the inhibition of AP-1 in conjunction with Jun NH_2-terminal kinase phosphorylation. Therefore, one can hypothesize that PPAR-γ activation with pioglitazone either inhibits several transcription factors simultaneously or has a

more upstream effect on all redox-sensitive transcription factors, causing a uniform decline in their activity, even though the 2 hypotheses do not seem to be mutually exclusive. In a recent study in a rat model of myocardial ischemia/reperfusion,[17] we observed a reduction in p67phox NADPH oxidase and NF-κB with another PPAR-γ ligand, rosiglitazone. Collectively, we believe that PPAR-γ ligands, such as pioglitazone, primarily reduce ROS levels and subsequently inhibit several transcription factors simultaneously, thereby causing a significant decrease in the expression of genes such as LOX-1.

ROS, particularly the superoxide ions, are potent chemoattractants for inflammatory cells.[32] Ang II via AT_1R activation has been shown to enhance NADH/NADPH oxidase activity.[27] Ox-LDL and TNF-α are also potent proinflammatory mediators. We suggest that the LOX-1 inhibitory effect of pioglitazone shown in the present study may also translate into strong anti-inflammatory properties of this compound. Yue et al[16] have recently demonstrated a reduction in monocyte chemoattractant protein-1 and intracellular adhesion molecule-1 expression in Lewis rats treated with PPAR-γ ligands and exposed to ischemia/reperfusion. Shiomi et al[33] have shown that treatment with pioglitazone can reduce mRNA for TNF-α and monocyte chemoattractant protein-1 in mice with acute myocardial infarction.

We examined the biological significance of LOX-1 inhibition by pioglitazone by examining monocyte adhesion to HCAECs in response to ox-LDL, Ang II, or TNF-α. Pretreatment of cells with pioglitazone significantly decreased monocyte adhesion to HCAECs regardless of the stimulus used. We observed that this effect of pioglitazone was qualitatively similar to that of LOX-1 antisense.[34] PPAR-γ ligands, including pioglitazone, have previously been shown to decrease leukocyte deposition onto the activated endothelium in ischemic/reperfused tissues,[16,17,31] and this effect has been ascribed to a decrease in redox-sensitive transcription factors and the expression of adhesion molecules.[17,35] In recent studies,[13,36] we have shown that LOX-1 is upregulated in ischemia/reperfusion injury, and a specific monoclonal antibody to LOX-1 reduces ischemia/reperfusion injury in the rat. The present study provides a direct link between pioglitazone and LOX-1 inhibition, resulting in a decrease in monocyte adhesion to activated HCAECs.

Two other studies have examined the effect of PPAR ligands on LOX-1 expression. Chiba et al[37] have shown that 15d-PGJ_2, a PPAR-γ ligand, but not the PPAR-α ligands WY14643 and fenofibric acid, inhibits TNF-α–induced LOX-1 expression in bovine aortic endothelial cells. Actually, Hayashida et al[38] have shown that PPAR-α ligands enhance LOX-1 expression in vascular endothelial cells. Our studies conducted in human coronary endothelial cells extend these observations by exploring the intracellular mechanism of pioglitazone in LOX-1 gene transcription.

In essence, the present study has demonstrated that the PPAR-γ ligand pioglitazone inhibits intracellular superoxide radical generation and, subsequently, expression of the redox-sensitive transcription factor. This results in the downregulation of LOX-1 in response to a number of proinflammatory and proatherosclerotic stimuli, such as ox-LDL, Ang II, and TNF-α. These observations point to a potential mechanism for the antiatherosclerotic and tissue-protective effects of PPAR-γ ligands

Acknowledgments

This study was supported by a Scientist Development Grant and a Merit Review Award from the VA Central Office and a grant from Takeda Pharmaceuticals North America, Inc, Lincolnshire, Ill.

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