PPAR-γ Activation Attenuates Angiotensin II-Induced Vascular Inflammation, Arterial LDL Accumulation and Endothelial Layer Permeability in Mice

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KEY WORDS: atherosclerosis, chemokines, inflammation, endothelial cell, lipoproteins

ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) have been shown to be important in modulating vascular inflammation and atherosclerosis. Angiotensin II (Ang II) is known to promote vascular inflammation and accelerate atherosclerosis. We demonstrated that these vascular changes induced by Ang II are associated with up-regulation of pro-inflammatory genes, as well as down-regulation of PPAR-γ expression. We hypothesized that a PPAR-γ agonist would have vasculoprotective effects in a mouse model of Ang II-induced atherosclerosis by: 1) attenuation of pro-inflammatory mediators, 2) attenuation of arterial low-density lipoprotein (LDL) accumulation, and 3) attenuation of endothelial layer permeability. To examine the effects of a PPAR-γ agonist on vascular inflammation, we administered a diet containing fenofibrate (40 mg/kg/day) for 5 weeks to male apolipoprotein E-deficient (apoE-KO) mice that were infused with Ang II (1.44 mg/kg/day). Fenofibrate reversed the pro-inflammatory genes induced by Ang II, and up-regulated PPAR-γ mRNA expression in the aorta. To determine whether fenofibrate could reverse the functional consequences of Ang II, arterial LDL accumulation and endothelial layer permeability from isolated mouse carotid arteries were assessed by quantitative fluorescence microscopy. Fenofibrate significantly attenuated both the rate of arterial LDL accumulation and

Table 1. Effects of Angiotensin II (Ang II) and Ang II + Fenofibrate on Gene Expression in the Aorta of Apolipoprotein E-deficient (apoE-KO) Mice*

	Ang II (n = 4)	Ang II + fenofibrate (n = 5)
PPAR-/GAPDH	3.47 ± 0.77	12.64 ± 2.02‡
MCP-1/vWF	6.74 ± 2.78	1.96 ± 0.5
M-CSF/vWF	17.59 ± 3.43	2.11 ± 0.19†
E-selectin/vWF	9.76 ± 0.51	3.68 ± 0.32§
ICAM-1/vWF	6.64 ± 2.79	2.85 ± 0.48
VCAM-1/vWF	7.03 ± 1.29	1.94 ± 0.33†

*PPAR- indicates peroxisome proliferator-activated receptor-alpha; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage-colony stimulating factor; E-selectin, endothelial-selectin; ICAM-1, intercellular adhesion molecule-1; and VCAM-1, vascular cell adhesion molecule-1. Values were normalized to either glyceraldehyde-3 phosphate dehydrogenase (GAPDH) or to von Willebrand Factor (vWF). Statistical analysis was performed using one-way ANOVA and data are expressed as mean ± SEM.
†$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$, significantly different from Ang II alone.

endothelial layer permeability induced by acute administration of Ang II. Our studies demonstrate that PPAR- activation has functional effects to reduce LDL accumulation and endothelial layer permeability in the arterial wall. The pleiotropic effects of PPAR- agonists on vascular wall inflammation certainly participate in the inhibition of atherosclerosis development.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent nuclear transcription factors that form a subfamily of the nuclear receptor superfamily. PPARs regulate target gene expression by binding to specific peroxisome proliferator response elements (PPREs) in enhancer sites of regulated genes. PPAR- regulates genes involved in the -oxidative degradation of fatty acids.[1,2] Fatty acids and their derivatives have been identified as natural ligands for PPAR-. PPAR- also is the primary target of numerous classes of synthetic ligands, including lipid-lowering fibrates. The Helsinki Heart Study was the first large clinical trial to show benefits from the use of a fibrate by which gemfibrozil reduced the incidence of coronary heart disease in men with dyslipidemia.[3] The Veterans Affairs HDL Cholesterol Intervention Trial demonstrated that gemfibrozil therapy resulted in a significant reduction in the risk of major cardiovascular events in patients with coronary disease.[4] Although attributed mainly to the hypolipidemic and HDL-raising actions of fibrate drugs, other actions of these PPAR- agonists on the vasculature may also have contributed to the benefits seen in these trials.

PPAR- is normally present at high levels in the liver, where its activation increases fatty acid oxidation and alters apolipoprotein expression. PPAR- also is expressed in the vascular wall in endothelial cells, smooth muscle cells, monocytes/macrophages, and foam cells.[5-8] There is strong evidence suggesting that PPAR- agonists have a potential modulatory role in the pathogenesis of atherosclerosis related to effects on vascular inflammation and metabolic risk factors. Possible mechanisms for the anti-atherogenic effects of PPAR- activators on the vessel wall include the inhibitory effects of PPAR- on foam cell formation, expression of cell adhesion molecules, cytokine and chemokine production, and the indirect effects of decreasing lipids.[4,6,9,10]

We and others have recently demonstrated that angiotensin II (Ang

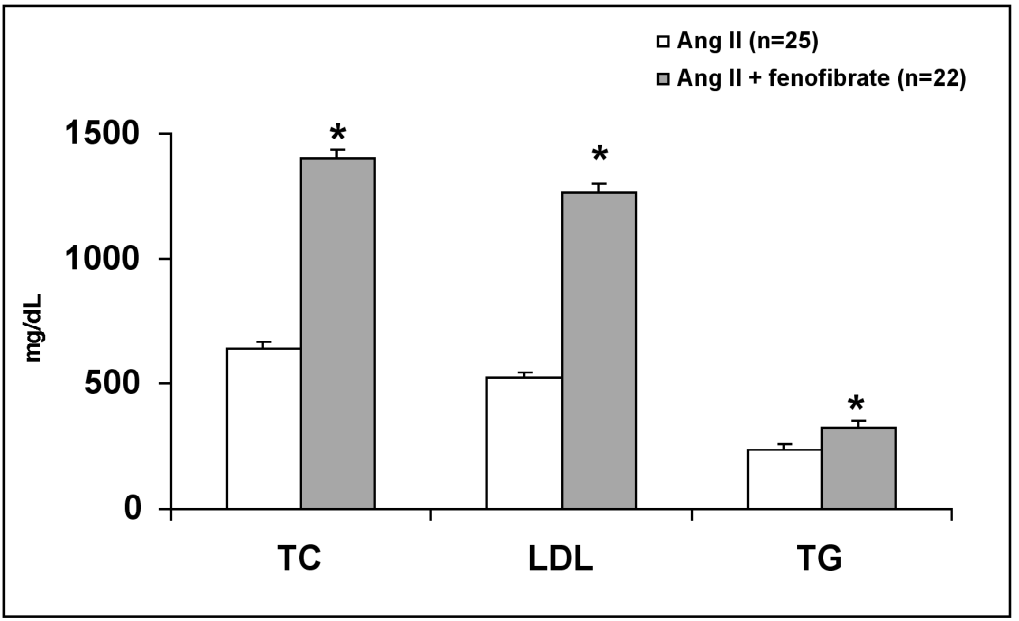

Figure 1. Serum lipoproteins in apolipoprotein E-deficient (apoE-KO) mice treated with angiotensin II (Ang II) and Ang II plus fenofibrate. Statistical analysis was performed using one-way ANOVA and data are expressed as mean ± SEM. *$P < 0.001$, significantly different from Ang II treatment alone.

II) promotes vascular inflammation and accelerates atherosclerosis.[14-16] These vascular changes were associated with activation of nuclear factor-kappa B (NF-κB)-mediated induction of chemokines and endothelial cell adhesion molecules, as well as down-regulation of PPAR-γ expression.[14] We postulated that the down-regulation of PPAR-γ by Ang II may diminish the anti-inflammatory potential of PPAR-γ, thus contributing to enhanced vascular inflammation. We hypothesized that a PPAR-γ agonist would have vasculoprotective effects by attenuation of pro-inflammatory mediators. Also, the reduction in vascular inflammation would attenuate arterial low-density lipoprotein (LDL) accumulation and endothelial layer permeability. To examine the effects of a PPAR-γ agonist on the vascular wall, we administered fenofibrate to male apolipoprotein E-deficient mice (apoE-KO) that were treated with Ang II to promote vascular inflammation and accelerate atherosclerosis. We measured the expression of potential target genes in the artery wall, arterial LDL accumulation, endothelial layer permeability, the effects on lipid and glucose metabolism, and the extent of atherosclerosis. Our data suggest that fenofibrate has direct beneficial vascular effects as demonstrated by anti-inflammatory changes in gene regulation and transcription factor activation in the aorta. In addition, our studies demonstrate that PPAR-γ activation has functional effects to reduce LDL accumulation and endothelial layer permeability in the arterial wall, potential mechanisms for the prevention of atherogenesis.

METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California at Davis and Berlex

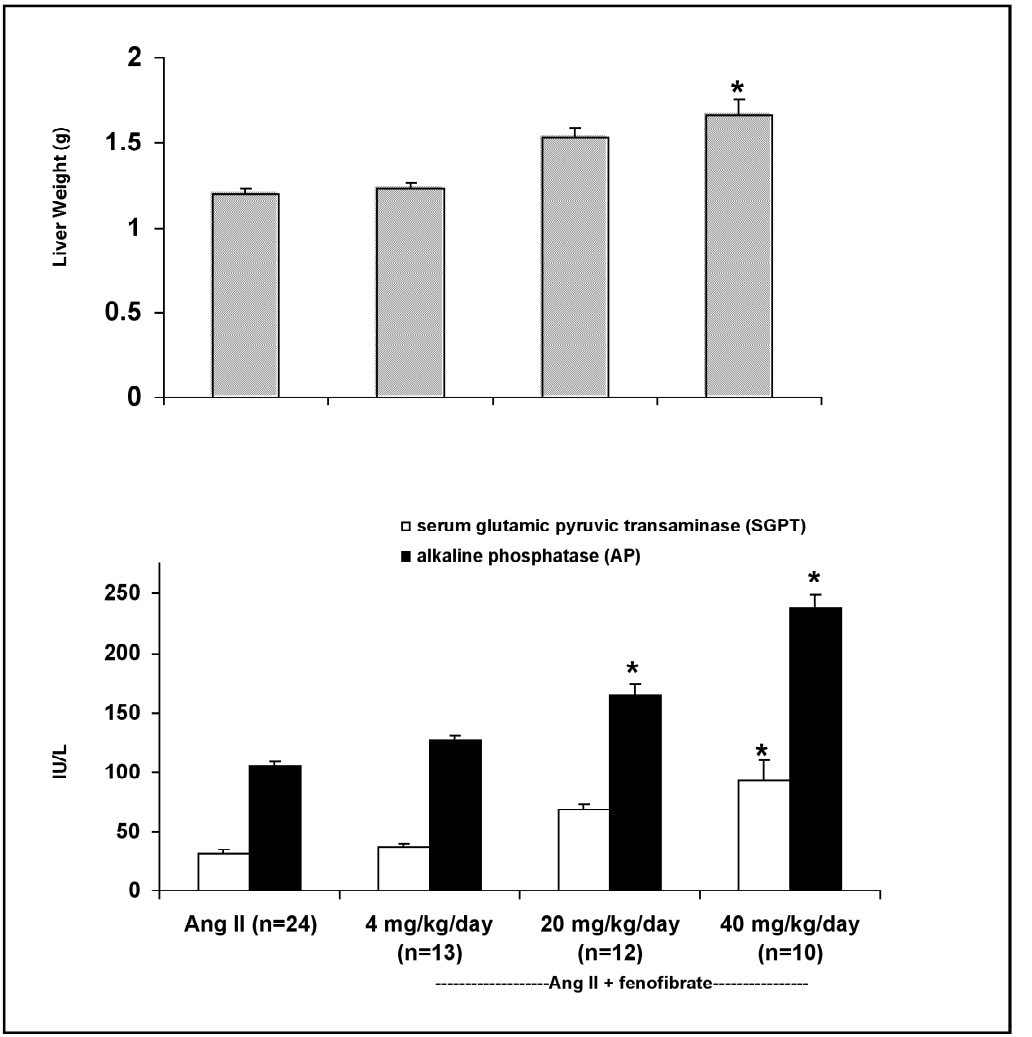

Figure 2. Liver weight and enzymes, serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase, in apolipoprotein E-deficient (apoE-KO) mice treated with angiotensin II (Ang II) and Ang II plus fenofibrate. Statistical analysis was performed using one-way ANOVA and data are expressed as mean ± SEM. *$P < 0.001$, significantly different from Ang II treatment alone.

Biosciences. Six-month old male apolipoprotein E-deficient (apoE-KO) mice were fed a diet containing either fenofibrate (4, 20, 40 mg/kg/day) or normal chow. After 7 days, all mice were subcutaneously implanted with osmotic minipumps (Alzet, Model 2004; ALZA, Palo Alto, Calif) containing Ang II (1.44 mg/kg/day; CalBiochem, La Jolla, Calif).[14,15,17] Both diet and Ang II infusion continued for an additional 30 days. Mice and feed were weighed daily. After 30 days, systolic blood pressure was measured in conscious mice using a tail-cuff system (Kent Scientific, Litchfield, Conn). Mice were trained to lie quietly in a restrainer placed on a warm pad for a period of at least 30 minutes for 1 to 4 days before the study. On the day of the study, the mice were placed in the temperature-controlled restrainer for 15 minutes. Blood pressure was then measured repeatedly and recorded on a data acquisition system (PowerLab, 16/s,

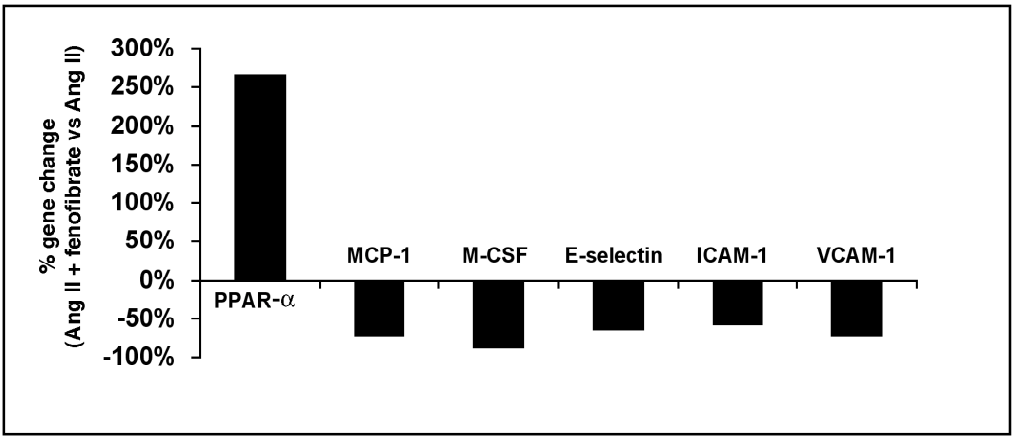

Figure 3. Change in gene expression of in the aortas of apolipoprotein E-deficient (apoE-KO) mice treated with angiotensin II (Ang II) plus fenofibrate compared to Ang II alone. Total RNA extracted from the aorta was subjected to quantitative RT-PCR using primers specific for peroxisome proliferator-activated receptor-alpha (PPAR-α), monocyte chemotactic protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF); endothelial-selectin (E-selectin); intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1).

ADInstruments, Australia). Systolic blood pressure was averaged from consecutive 5 measurements. Mice were euthanized by CO_2 asphyxiation.

Quantification of Atherosclerosis.
The left and right carotid arteries were dissected, cut open longitudinally, and pinned down individually on silicon-coated petri dishes. Atherosclerotic plaques were visible without staining. The images of the open luminal surface of both carotid arteries were recorded by a digital camera (Sony, Japan) mounted on a dissecting microscope. The plaque area was quantified using C-Imaging Systems (Compix, Cranberry Township, Pa) and expressed as a percentage of the total luminal surface area of the carotid arteries as previously described.[18-20]

Metabolic Studies
Fasting serum glucose was measured on a Beckman glucose analyzer 2 (Beckman, Brea, Calif) using the glucose oxidase method and fasting insulin levels were assayed by radioimmunoassay (Linco Research, St. Charles, Mich). Total cholesterol (TC), high density lipoprotein (HDL), and triglyceride (TG) were directly measured in fasting serum by standard clinical laboratory analysis (IDEXX, Sacramento, Calif). Low-density lipoprotein (LDL) values were calculated. Serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (AP) levels were also measured to assess potential liver toxicity (IDEXX).

RT-PCR-based Quantitative Gene Expression Analysis
Real-time detection of PCR was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, Calif). The differential displays of aortic mRNAs for PPAR-α, monocyte chemotactic protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF), endothelial-selectin (E-selectin), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were determined. Total RNA from the aorta was isolated using an RNA isolation

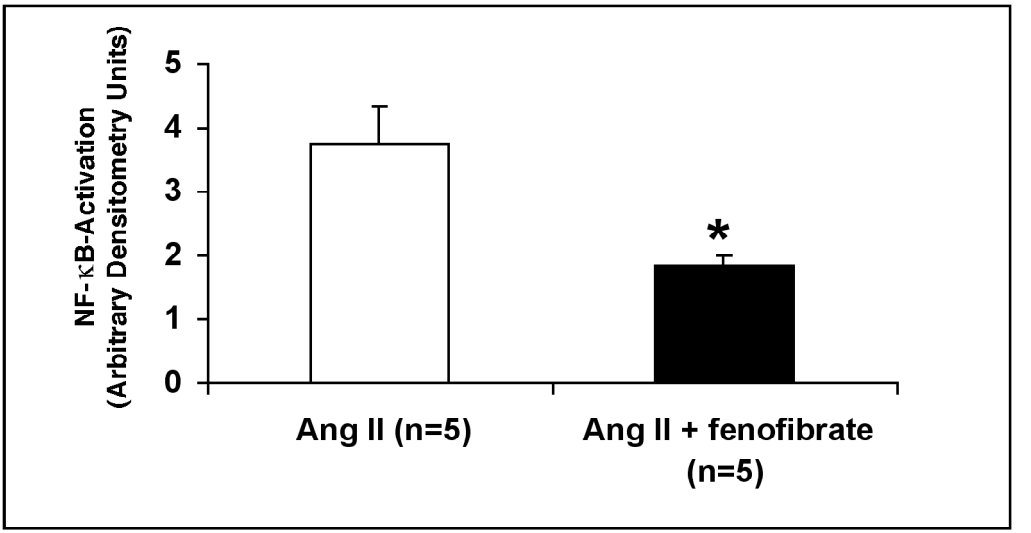

Figure 4. Induction of nuclear factor-kappa B (NF-□B) binding activity in the aortas of apolipoprotein E-deficient (apoE-KO) mice treated with angiotensin II (Ang II) and Ang II plus fenofibrate. Nuclear extracts prepared from the aortas of apoE-KO mice were used to bind the 3′-biotin-labeled NF-□B oligonucleotide and the appearance of the sequence-specific NF-□B binding activity was detected by electrophoretic mobility shift assay. NF-□B activation was quantified by densitometry and expressed as arbitrary densitometry units. Statistical analysis was performed using two-tailed Student *t* test and data are expressed as mean ± SEM. *$P < 0.05$, significantly different from Ang II treatment alone.

reagent (Rneasy kit, QIAGEN, Valencia, Calif). Total RNA was used to generate cDNA for oligo dT oligodeoxynucleotide primer (T12-18) following the protocol for SuperScript II Rnase H-Reverse Transcriptase (GibcoBRL, Rockville, Md). The following primers were designed using Primer Express software (Applied Biosystems) and synthesized by Operon (Alameda, Calif):

PPAR-□:
5'CCTCTTCCCAAAGCTCCTTCA3' (forward);
5'CTGCGTCGGACTCGGTCTT3' (reverse);
MCP-1:
5'CAGCCAGATGCAGTTAACGC3' (forward),
5'GCCTACTCATTGGGATCAT C T T G 3' (reverse);
M-CSF:
5'AGCATGGACAGGCAGGGAC3' (forward),
5'CTGCGTGCCTTTATGCCTTT3' (reverse);
E-selectin:
5'GGCAGACATATTGGCTTTATCCC3' (forward),
5'GATGGATCTCATGCTGGCTTC3' (reverse);
ICAM-1:
5'GAGTTTTACCAGCTATTTATTGAGTACCC3' (forward), 5'CTCTCACAGCATCTGCAGCAG3' (reverse);
VCAM-1:
5'TTAAAGTCTGTGGATGGCTCGTAC3' (forward),
5'CTTAATTGTCAGCCAACTTCAGTCTT3' (reverse);
GAPDH: 5'GCAACAGGGTGGTGGACCT3' (forward), 5'GGATAGGGCCTCTCTTGCTCA3' (reverse);
vWF: 5'AATGCCTTATTGGCGAGCAC3' (forward),
5'CACTGCTTGCTGTACACCAGAAA3' (reverse).

Equal amounts of cDNA were used in duplicates and amplified with the SYBR Green I Master Mix (Applied Biosystems). The thermal cycling parameters were as follows: thermal activation for 10 minutes at 95°C, and 40 cycles of PCR (melting for 15 minutes at 95°C and annealing/extension for 1 minute at 60°C). A standard curve was constructed with a dilution curve (1:5, 1:10, 1:20,

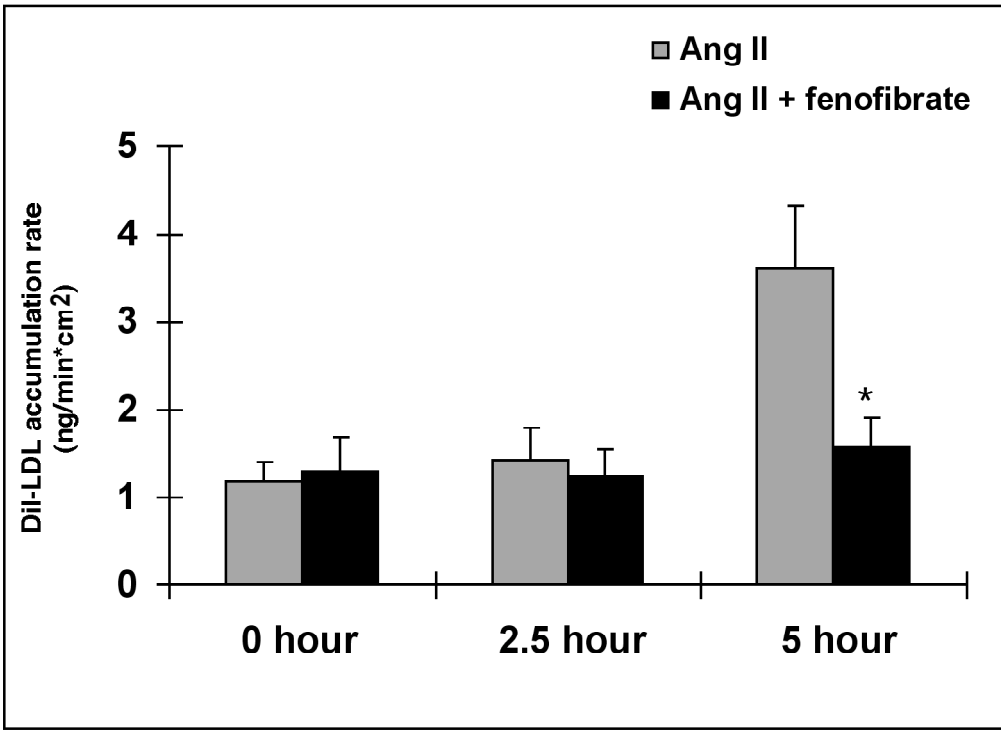

Figure 5. Effects of angiotensin II (Ang II) and Ang II plus fenofibrate on arterial low-density lipoprotein (LDL) accumulation. Carotid arteries from apolipoprotein E-deficient (apoE-KO) mice were perfused with angiotensin II (Ang II) or Ang II plus fenofibrate. Arterial LDL accumulation was determined by measuring 1,1′-dioctadecyl-1,3,3,3′,3′-tetramethyl-indocarbocyanine (DiI)-LDL accumulation in the artery wall. There was a significant decrease in the rate of arterial LDL accumulation (n = 6) in the fenofibrate-perfused vessels compared to Ang II alone after 5 hours. Statistical analysis was performed using one-way ANOVA and data are expressed as mean ± SEM. *$P < 0.05$, significantly different from Ang II treatment alone.

1:40, 1:80, 1:160, 1:320, 1:640) of total RNA from mouse aorta. A "no template control" was included with each PCR. Amplification efficiencies were validated and normalized against glyceraldehyde-3 phosphate dehydrogenase (GAPDH) or von Willebrand Factor (vWF). Correct PCR product size was confirmed by electrophoresis through a 1% agarose gel stained with ethidium bromide. Purity of the amplified PCR products was determined by a heat-dissociation protocol to enable detection of nonspecific amplification.

Assessment of NF-□B Activation by Electrophoretic Mobility Shift Assay

Nuclear extracts of aortic tissue were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, Ill). Tissues were homogenized on ice using a Tissue Tearor (Biospecs Products, Racine, Wis). Protein concentration was measured by a modified Lowry assay kit (Bio-Rad, Hercules, Calif) using bovine IgG as the standard. The NF-□B consensus oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'; Santa Cruz Biotechnology, Santa Cruz, Calif) was 3' end-labeled with biotin (Pierce). The binding reactions containing equal amounts of protein (10 μg) and 20 fmol of oligonucleotide were performed for 20 minutes in binding buffer (2.5% glycerol, 5 mmol/L $MgCl_2$, 50 ng/μL poly (dI-dC) 0.05% NP-40; Pierce). The reaction volumes were held constant to 20

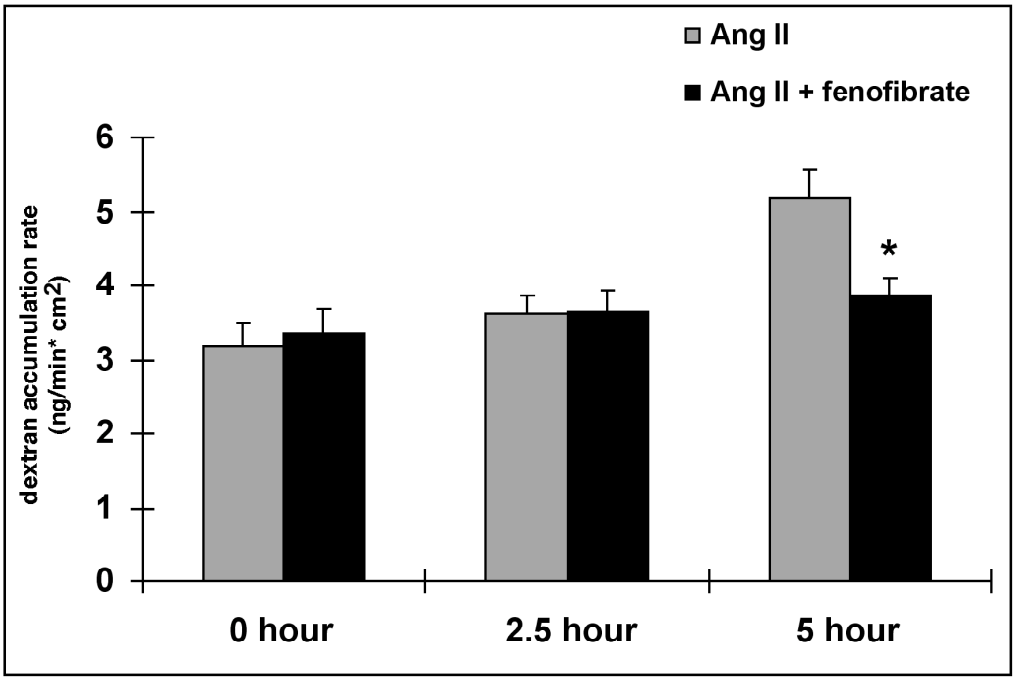

Figure 6. Effects of angiotensin II (Ang II) and Ang II plus fenofibrate on endothelial layer permeability. Carotid arteries from apolipoprotein E-deficient (apoE-KO) mice were perfused with angiotensin II (Ang II) or Ang II plus fenofibrate. Endothelial layer permeability was determined by measuring TRITC-dextran (4,400 MW) accumulation. There was a significant decrease in the rate of endothelial layer permeability (n = 7) in the fenofibrate-perfused vessels compared to Ang II alone after 5 hours. Statistical analysis was performed using one-way ANOVA and data are expressed as mean ± SEM. *$P < 0.05$, significantly different from Ang II treatment alone.

μL. The reaction products were separated in a 6% polyacrylamide gel, detected with streptavidin-horseradish peroxidase and analyzed by autoradiography. The NF-□B activation was quantified using the Kodak Electrophoresis Documentation & Analysis System 290 (Eastman Kodak, Rochester, NY). To demonstrate specificity of binding of the NF-□B oligonucleotide, a mutant NF-□B consensus oligonucleotide (Santa Cruz Biotechnology) was used as a negative control. Competition assays with 50 ng of unlabeled oligonucleotides were also used to confirm the specificity of this NF-□B binding assay.

Assessment of Transcription Factor Activation

Nuclear extracts of aortic tissue were isolated and quantified as described above. Using the Myria protein/DNA array kit (Panomics, Redwood City, Calif), a set of biotin-labeled DNA binding oligonucleotides were pre-incubated with aortic nuclear extracts in order to allow the formation of DNA/protein complexes. The DNA/protein complexes were then extracted and hybridized to the MYRIA array that contained 54 consensus-binding sequences corresponding to a specific eukaryotic transcription factor. Detection of signals was quantified using the Kodak Electrophoresis Documentation & Analysis System 290 (Eastman Kodak). Activated transcription factors were compared between treatment groups by normalization to biotinylated DNA spotted on each array. All experiments were performed in duplicates.

Fluorescent LDL Preparation

LDL was isolated and labeled as

described by Pitas et al.[21] Briefly, blood from fasting nonsmoking human males was obtained in vacutainers containing EDTA and centrifuged for 10 minutes at 2,800 rpm at 4˚C. The plasma was isolated and LDL (density = 1.01 to 1.06) and lipoprotein-deficient plasma were obtained by sequential density gradient ultracentrifugation. LDL was labeled with the fluorophore 1,1'-dioctadecyl-1,3,3,3',3'-tetramethyl-indocarbocyanine [DiI] (Molecular Probes, Eugene, Ore) and dialyzed in PBS at 4˚C for 48 hours. The spectral properties of DiI are 540 nm excitation maximum and 556 nm emission maximum.

Measurement of Arterial LDL Accumulation

Arterial LDL accumulation experiments from 6-month old male apoE-KO mice were evaluated by measuring DiI-LDL accumulation in either Ang II (10^{-6} M) or Ang II plus fenofibrate (200 µM) perfusate solution. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.1 mL of 50 mg/mL per 100 g body wt). The carotid arteries were isolated and adjacent tissues were carefully dissected away. The isolated arteries were placed in the perfusion apparatus and the rate of LDL accumulation in the artery wall was determined by quantitative fluorescence microscopy as previously described.[22-24] A control level of fluorescence intensity was established during perfusion of a nonfluorescent solution (1% BSA-Krebs-Henseleit buffer consisting of 116 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2.H_2O$, 1.2 mM $MgCl_2$, 1.2 mM NH_2PO_4, and 11mM glucose). At the end of a 5 minutes perfusion with solution containing DiI-LDL with Ang II or Ang II plus fenofibrate, the vessel lumen was cleared of DiI-LDL. Measurements of the rate of LDL accumulation were performed under control conditions and compared to treatment conditions. Initially, 3 control runs were performed on both left and right vessels from same mouse to obtain a control rate of accumulation. At 0 hours, vessels were treated with or without 200 µM fenofibrate. After 2.5 hours, 3 control runs were repeated on both vessels. Both vessels were then treated with 10^{-6} M Ang II. At 5.0 hours, 3 control runs were performed. All treatments were added directly to both the nonfluorescent and fluorescent solutions used to perfuse the arteries. Once the lumen was cleared of the fluorescent-labeled solution, any remaining fluorescence is a measure of the number of molecules of DiI-LDL that remains bound to the endothelial surface or in the vessel wall. It is during the washout phase that the analysis of LDL accumulation is performed. Measurement of accumulation involves analysis of washout data as two distinct processes: a rapid washout of the lumen fluorescence, followed by a slower vessel wall fluorescence washout. Calculation of fluorescence intensity (I_f) accumulation (the amount of fluorescent-labeled molecules in the artery wall) involves finding the intersection of tangents drawn to approximate these two processes. To determine accumulation rate, I_f accumulation is divided by the length of dye perfusion (5 min). Finally, an appropriate conversion factor is used to convert millivolts per minute to $ng \cdot min^{-1} \cdot cm^2$. This conversion factor comes from four measurements: 1) the surface area; 2) the lumen volume of the vessel in the photometric window; 3) the maximum If at time 0 (I_{f0}), which occurs at the beginning of dye perfusion; and 4) the concentration of fluorophore. Throughout the perfusion experiment, the vessel was perfused at a rate of 1.5 mL/min at 37˚C and pH 7.4.

Measurement of Arterial Permeability

Vascular permeability from 6-month old male apoE-KO mice was evaluated by

measuring tetramethylrhodamine isothiocyanate (TRITC)-dextran accumulation in either Ang II (10^{-6} M) or Ang II plus fenofibrate (200 μM) perfusate solution. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.1 mL of 50 mg/mL per 100 g body wt). The carotid arteries were isolated and adjacent tissues were carefully dissected away. As described above, 3 control runs established control levels of dextran (4,400 MW) accumulation with two sets of three runs performed with Ang II or Ang II plus fenofibrate perfusate solution in each carotid as described above. Each run consisted of 5 minutes of washout with the nonfluorescent solution. Both carotid arteries were examined in each animal.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed between two groups using two-tailed Student *t* test for unpaired values. One-way ANOVA (with a Bonferroni's post hoc test) was performed when comparing groups of three or more. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of a PPAR-α Agonist on Body Weight, Blood Pressure, Fasting Serum Chemistries and Atherosclerotic Lesions

PPAR-α agonist treatment with fenofibrate did not have a significant effect on body weight (27.4 ± 0.6 vs 27.5 ± 0.3 g), systolic blood pressure (202 ± 17 vs 160 ± 12 mm Hg), fasting serum glucose (92 ± 8 vs 107 ± 7 mg/dL) or insulin (0.43 ± 0.03 vs 0.54 ± 0.09 ng/dL) compared to Ang II treatment alone. However, Ang II plus fenofibrate treatment increased blood lipid levels. Total cholesterol (TC), low density lipoprotein (LDL) and triglyceride (TG) levels were significantly increased by Ang II plus fenofibrate compared to Ang II treatment alone (Figure 1). High density lipoprotein (HDL) levels, however, were not affected by a PPAR-α agonist treatment compared to Ang II treatment alone (68 ± 9 vs 65 ± 5 mg/dL).

A significant dose-dependent (4, 20, 40 mg/kg/day) increase in the liver weight was observed in the Ang II plus fenofibrate group compared to the Ang II treatment group (Figure 2). This dose-dependent increase in liver weight was accompanied by a dose-dependent increase in SGPT and AP suggesting potential liver toxicity (Figure 2). However, histological examination of the liver revealed no gross necrosis or inflammation. In addition, no significant fat was noted in the liver sections stained with oil red O (data not shown). In this milieu of increased lipids, liver weight and liver enzymes, there was no significant change in carotid plaque area observed with Ang II plus fenofibrate treatment (43.1 ± 2.6 vs 34.6 ± 3.6%) compared to Ang II treatment alone.

PPAR-α Agonist Activated Aortic Gene Expression of PPAR-α and Down-regulated Chemokines and Endothelial Cell Adhesion Molecules

To investigate the potential effects of a PPAR-α agonist on patterns of pro-inflammatory gene expression involved with the initiation and progression of the atherosclerotic process within the arterial wall, the differential displays of aortic mRNAs for PPAR-α, MCP-1, M-CSF, E-selectin, ICAM-1, and VCAM-1 were determined in apolipoprotein E-deficient (apoE-KO) mice treated with Ang II and Ang II plus fenofibrate. The mRNA level of PPAR-α was significantly up-regulated in the Ang II plus fenofibrate-treated group compared to the Ang II treatment alone (Table 1 and Figure 3). In addition, Ang II plus fenofibrate treatment significantly reduced both chemokine (M-CSF) and endothelial cell adhesion molecule

(E-selectin and VCAM-1) mRNA expression compared to the Ang II treatment alone (Table 1 and Figure 3). In addition, Ang II plus fenofibrate decreased MCP-1 and ICAM-1 expression by 71% and 57%, respectively, when compared to the Ang II treatment alone (Table 1 and Figure 3).

PPAR-□ Agonist Decreased Aortic Transcriptional Factor Activation of NF-□B, Egr-1 and Sp-1

In order to determine the molecular mechanisms underling the inducible expression of pro-inflammatory mediators in the vascular tissue, we first measured the nuclear translocation of NF-□B in the aorta. In this study, Ang II plus fenofibrate-treated nuclear extracts displayed a 51% decrease in the binding activity of NF-□B by electromobility shift assay with the use of a 3'-end biotin-labeled consensus probe for NF-□B, compared with nuclear extracts prepared from Ang II-treated aorta ($P < 0.05$) (Figure 4). As expected, reactions with the NF-□B mutant oligonucleotide resulted in no detectable binding activity and competition assays with unlabeled NF-□B oligonucleotides confirmed the specificity of this NF-□B DNA binding assay (data not shown).

We also analyzed Egr-1 and Sp-1 transcription factor binding activity. Nuclear extracts prepared from the aortas of apoE-KO mice were used to bind the 3'-biotin-labeled DNA binding oligonucleotides and the appearance of the sequence-specific Egr-1 and Sp-1 binding activity was detected on a DNA/protein array. Egr-1 and Sp-1 binding activity was quantified by densitometry and expressed as fold change. Ang II plus fenofibrate treatment resulted in a 2.4-fold decrease (1.2×10^6 vs 3.0×10^6 arbitrary densitometry units) in Egr-1 transcription factor binding activity in the aortas compared to the Ang II treatment group. Sp-1 transcription factor binding activity in the aortas of the Ang II plus fenofibrate-treated group was also decreased by 2.1-fold (1.2×10^6 vs 2.6×10^6 arbitrary densitometry units) compared compared to the Ang II-treated group.

PPAR-□ Agonist Attenuates Ang II-induced Vascular Injury

To address whether acute exposure of Ang II or Ang II plus fenofibrate affects arterial LDL accumulation and/or endothelial layer permeability, experiments were performed by perfusing carotid arteries taken from 6 month-old male apoE-KO mice. LDL accumulation was determined by measuring DiI-LDL accumulation in the artery wall, while endothelial layer permeability was determined by measuring TRITC-dextran (4,400 MW) accumulation in the artery wall. Three runs established control levels of either DiI-LDL or dextran accumulation with subsequent runs performed in the presence of Ang II or Ang II plus fenofibrate in each carotid artery. Ang II plus fenofibrate treatment significantly decreased LDL accumulation by approximately 57% after 5 hours of exposure compared to vessels perfused with Ang II alone ($P < 0.05$) (Figure 5). In addition, Ang II plus fenofibrate treatment significantly decreased endothelial layer permeability by approximately 25% after 5 hours of exposure compared to vessels perfused with Ang II alone ($P < 0.05$) (Figure 6).

DISCUSSION

This present study demonstrated that treatment with a PPAR-□ ligand, such as fenofibrate, had broad effects to attenuate the pro-inflammatory effects of Ang II in the aorta as demonstrated by: 1) up-regulation of PPAR-□ expression; 2) down-regulation of chemokines and endothelial cell adhesion molecules expression; and 3) reduction of multiple transcriptional factor activation, such as

NF-κB, Egr-1 and Sp-1. These molecular changes in the artery wall caused reduction of arterial LDL accumulation and endothelial layer permeability. Our data suggest that PPAR-γ ligands are novel regulators of gene expression in vascular cells that can modulate the functional inflammatory response.

As demonstrated in our previous study, the consequences of Ang II infusion was the up-regulation of a number of pro-inflammatory mediators and accelerated atherosclerosis.[14] Concomitant with the enhanced pro-inflammatory response, Ang II treatment also decreased expression of PPAR-γ in the aortic wall. In this study, treatment with fenofibrate attenuated production of chemokines and endothelial cell adhesion molecules induced by Ang II. The results in this study indicate that PPAR-γ expression itself is another site of interaction between PPAR-γ agonists and inflammatory mediators. It has been demonstrated that the human PPAR-γ gene can positively auto-regulate its own expression.[25] The induction of PPAR-γ expression may have important implications due of its ability to exert anti-inflammatory activities.

Several regulatory pathways can induce the expression of potentially pro-inflammatory genes, such as chemokines and endothelial cell adhesion molecules, which have been implicated in the progression of atherosclerosis.[26-31] These include NF-κB, Egr-1 and Sp-1 signaling pathways.[32,33] Many stimuli associated with the development of vascular disease, including Ang II, are capable of inducing these transcription factors.[34,35] Once activated, the transcription factors NF-κB, Egr-1 and Sp-1, bind to recognition elements in the promoter region of pro-inflammatory genes and act as a dominant regulator of transcription of these genes to induce inflammation.[36,37] Since activation of NF-κB, Egr-1 and Sp-1 are linked to enhanced expression of genes predominantly implicated in atherogenesis, we examined whether changes in pro-inflammatory gene expression detected with PPAR-γ agonist treatment were associated with changes in transcriptional binding activity of NF-κB, Egr-1 and Sp-1. Indeed, our study showed that aortic NF-κB, Egr-1 and Sp-1 transcriptional binding activity was dramatically decreased following PPAR-γ agonist treatment compared to Ang II treatment alone. Computer-assisted analysis of the human PPAR-γ promoter sequence identified various putative transcription factor binding sites for members of the Egr gene family, as well as potential sites for NF-κB.[25] These data suggest that decreased NF-κB, Egr-1 and Sp-1 nuclear translocation following PPAR-γ agonist treatment may be responsible for the decrease in Ang II-induced gene transcription of chemokines and endothelial cell adhesion molecules in the vascular wall. The reduction in expression of these pro-inflammatory mediators in the vascular wall observed with PPAR-γ activation may have direct anti-inflammatory and anti-atherogenic mechanisms. Moreover, the ability of PPAR-γ ligands to interfere with the vessel's inflammatory process in vivo holds promise in prevention and therapy of atherosclerosis.

PPAR-γ agonists are known to reduce vascular inflammation and atherosclerosis in mouse models of vascular disease.[38] A number of possible mechanisms for this have been proposed such as the inhibitory effects of PPAR-γ on foam cell formation, expression of cell adhesion molecules, cytokine and chemokine production, and the indirect effects of decreasing lipids.[4,6,9-13] Our studies support a new mechanism by which PPAR-γ agonists can mitigate atherosclerosis: by preventing increased endothelial layer permeability induced by Ang II treatment, resulting in decreased LDL accumulation in the

artery wall. A second mechanism involving LDL flux in the artery wall also is possible. It is likely that the inflammatory milieu present in the artery wall treated with Ang II provides the conditions for modification of LDL and binding of the LDL to the artery wall components, including lipid-filled macrophages, heparin sulfate proteoglycans, and lipoprotein lipase. Thus, by preventing artery wall inflammation, PPAR-γ agonists reduce entry of lipoproteins into the artery wall and, as a result of not binding lipoproteins to the intima of the artery wall, efflux of LDL from the artery wall is maintained. Thus, the pathophysiological mechanism that we propose here involves a reduction of LDL entry into the artery wall and facilitation of LDL efflux from the artery wall.

Previous metabolic studies have suggested that PPARs may have anti-atherogenic effects attributed to improvements in serum lipids.[39] In this study, mice treated with the specific PPAR-γ ligand exhibited a significant increase in serum lipoproteins. This effect has also been seen by other investigators.[38] Hypercholesterolemia is a major risk factor leading to the progression of atherosclerosis. Animals with hypercholesterolemia, resulting from either a high cholesterol diet[20,40-43] or from inherited defects in lipid metabolism,[20,44-46] develop atherosclerotic plaques in the vascular wall.[47,48] Despite the hyperlipidemia and absence of an anti-atherogenic effect observed in the PPAR-γ agonist-treated animals in our study, arterial inflammation, endothelial layer permeability and LDL accumulation were all decreased. While other investigators have been able to demonstrate a reduction in atheroma formation using PPAR-γ agonists,[38,49,50] the increase in advanced and complex lesions attributed to hyperlipidemia, increased age and Ang II treatment may have been too severe in our study to induce a reduction in atherosclerosis with PPAR-γ agonist treatment in our experimental animal model. Also, we studied carotid atherosclerosis, whereas previous studies examined coronary sinus and aortic atherosclerosis. Recent studies have demonstrated the existence of regional differences in atherosclerotic lesion susceptibility.[38,51-53] It is possible that the atherosclerotic lesions measured in the carotid arteries in this study may not have been as responsive to the in vivo PPAR-γ agonist treatment as compared to other aortic regions.

The decline in liver function observed with fenofibrate treatment also may have played a role in the increased plasma levels of pro-atherogenic lipoproteins. These findings may be specific to this animal model of disease. Species differences in response to peroxisome proliferators may be attributed to differences both in quantity of hepatic PPAR-γ and to the functionality and quality of the pathway components, such as the gene promoters that regulate peroxisome proliferator-mediated gene transcription. Further studies are needed to determine the paradoxical elevation of serum lipids in this mouse model PPAR-γ agonists.

In conclusion, despite severe hyperlipidemia this study provides evidence that a PPAR-γ agonist can attenuate pro-inflammatory mediators induced by Ang II in the aorta by decreasing transcription factors, chemokines and endothelial cell adhesion molecules associated with the development of atherosclerosis. Functionally, this study has also demonstrated that a PPAR-γ agonist can attenuate the rate of arterial LDL accumulation and endothelial layer permeability induced by Ang II. Further studies building on these new data should provide important insights into the overall benefits of activating PPAR-γ clinically for the treatment and prevention of vascular disease.

ACKNOWLEDGEMENTS

This work was supported by grants from the Biotechnology Strategic Targets for Alliances in Research, the Richard A. and Nora Eccles Harrison Endowed Chair in Diabetes Research, the American Federation for Aging Research and NHLBI HL55667. We are grateful to Jefferson Davis for his excellent technical assistance.

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