Dual PPARα/γ Agonist Tesaglitazar Reduces Atherosclerosis in Insulin-Resistant and Hypercholesterolemic ApoE*3Leiden Mice

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Objective—We investigated whether the dual PPARα/γ agonist tesaglitazar has anti-atherogenic effects in ApoE*3Leiden mice with reduced insulin sensitivity.

Methods and Results—ApoE*3Leiden transgenic mice were fed a high-fat (HF) insulin-resistance–inducing diet. One group received a high-cholesterol (HC) supplement (1% wt/wt; HC group). A second group received the same HC supplement along with tesaglitazar (T) 0.5 μmol/kg diet (T group). A third (control) group received a low-cholesterol (LC) supplement (0.1% wt/wt; LC group). Tesaglitazar decreased plasma cholesterol by 20% compared with the HC group; cholesterol levels were similar in the T and LC groups. Compared with the HC group, tesaglitazar caused a 92% reduction in atherosclerosis, whereas a 56% reduction was seen in the cholesterol-matched LC group. Furthermore, tesaglitazar treatment significantly reduced lesion number beyond that expected from cholesterol lowering and induced a shift to less severe lesions. Concomitantly, tesaglitazar reduced macrophage-rich and collagen areas. In addition, tesaglitazar reduced inflammatory markers, including plasma SAA levels, the number of adhering monocytes, and nuclear factor κB-activity in the vessel wall.

Conclusions—Tesaglitazar has anti-atherosclerotic effects in the mouse model that go beyond plasma cholesterol lowering, possibly caused by a combination of altered lipoprotein profiles and anti-inflammatory vascular effects. (Arterioscler Thromb Vasc Biol. 2006;26:2560-2566.)

Key Words: atherosclerosis ■ cholesterol ■ inflammation ■ inhibitors
In the clinical setting, several studies with PPARα agonists have shown significant reduction in cardiovascular disease risk, especially in subjects with insulin resistance.23–25 In the recently completed FIELD trial, fenofibrate treatment significantly reduced the need for coronary revascularization, but despite this the primary end point for the study was not met.26 PPARγ agonists have also been shown to reduce the progression of intima-media thickening in patients with coronary artery disease,27 and recent evidence suggests that pioglitazone reduces the incidence of myocardial infarction and stroke in patients with type 2 diabetes and pre-existing cardiovascular disease, although again the primary end point of this study was not met.28

Dual PPARα/γ agonists (such as tesaglitazar, which until recently was in phase III clinical development) have been shown to improve both glucose and lipid abnormalities in animal models and human subjects with insulin resistance and/or type 2 diabetes.29–31 Based on their effects in animal models and humans, it has been proposed that dual PPARα/γ agonists may have additional benefits beyond their effect on the associated dyslipidemia in reducing components of insulin resistance that contribute to atherosclerosis and cardiovascular disease.31,32 In this study, we examined whether tesaglitazar could reduce atherosclerosis using ApoE*3-Leiden transgenic mice made insulin resistant and dyslipidemic with a high-fat diet.

When fed a high-cholesterol diet, ApoE*3-Leiden transgenic mice develop a human-like lipoprotein profile, which includes elevated plasma levels of very-low-density lipoprotein, intermediate density lipoprotein, and low-density lipoprotein (LDL), and leads to the development of atherosclerosis. In addition, when fed a high-calorie, high-fat, high-cholesterol diet, these mice develop insulin resistance.33 Depending on their plasma cholesterol levels, ApoE*3-Leiden mice develop atherosclerotic lesions that have comparable characteristics to human lesions.34–36 Because plasma cholesterol levels in ApoE*3-Leiden transgenic mice can be titrated to any level by adjusting dietary cholesterol intake, we were able to study the effects of tesaglitazar on atherogenesis, independent of its total plasma cholesterol-lowering effect.

Methods

Animals

Female heterozygous ApoE*3-Leiden transgenic mice (3 to 4 months of age), characterized by an enzyme-linked immunosorbent assay for human apoE,37 were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were provided by TNO-Biomedical Research (Leiden, The Netherlands).

Diet

During a run-in period of 3 weeks, animals received a high-fat/high-cholesterol diet, containing 23% (wt/wt) bovine lard and 1% (wt/wt) cholesterol (HC diet).38 After the run-in period, mice were matched for age and cholesterol level into 3 groups of 17 mice each. One group maintained the HC diet (HC group). The tesaglitazar-treated group received the same HC diet, but the diet was supplemented with tesaglitazar (0.5 μmol/kg diet), equaling 20 μg/kg body weight per day (T group). Tesaglitazar [(S)-2-Ethoxy-3-[4-[2-(4-methylsulphoxyloxyphenyl) ethoxy]phenyl propanoic acid], with an EC50 value for activating mouse PPARα of 32 μmol/L and for PPARγ of 0.25 μmol/L,39 was provided by AstraZeneca R&D, Mölndal, Sweden. The tesaglitazar dose used was selected from a pilot experiment, in which we aimed at only 20% cholesterol reduction to ensure atherosclerosis development within a reasonable time period. The low-cholesterol (LC) group received the same diet as the HC group, but containing 0.1% (wt/wt) instead of 1% cholesterol to titrate the plasma cholesterol level to that of the T group, as deduced from previous experiments in our laboratory. The LC group served as the cholesterol-matched control for the T group. The 3 mice groups were treated for 28 weeks. All animals had free access to food and water. Body weight and food intake were monitored, and blood samples were taken after a 4-hour fast by tail bleeding throughout the study.

Analysis of Plasma

Commercially available kits were used to measure total plasma cholesterol (No. 1489437; Roche Diagnostics) and triglyceride levels (No. 337-B; Sigma Diagnostics). Cholesterol exposure was calculated as the area under the curve of cholesterol levels versus time in weeks. Lipoprotein distribution was determined by fast performance liquid chromatographic (fast protein liquid [FPLC]) size fractionation (Pharmacia).35 Glucose and insulin levels were determined after euthanization at week 28. Plasma glucose was measured using commercial reagents (No. 2319 and 2320; Instruchemie) and plasma insulin was measured using a mouse-specific enzyme-linked immunosorbent assay (10 to 1150–01; Alpco). Homeostasis Model Assessment Insulin Resistance (HOMA-IR), a surrogate measure of insulin resistance, was calculated as the product of fasting insulin (μU/mL) and glucose (mmol/L) concentrations divided by 22.5.36 Plasma fibrinogen (homemade mouse kit)38 and serum amyloid A (SAA; Biosource) were measured using specific enzyme-linked immunosorbent assays.

Analysis of Atherosclerosis

After 28 weeks, animals were euthanized and the hearts were harvested, fixed, and embedded in paraffin.39 Serial 5-μm cross-sections of the entire aortic valve area (AVA) were prepared and stained with hematoxylin-phloxin-saffron (HPS) for histological analysis, and with Sirius Red to quantify the collagen area. Atherosclerotic lesions were categorized into types I to V, as described previously (see supplemental Figure I, available online at http://atvb.ahajournals.org). Cross-sectional lesion areas were quantified using Leica Qwin morphometric software.39 Four sections of each specimen, containing 3 valve segments each, were analyzed at 40-μm intervals to determine the average lesion number (numbers were counted and totalled per specimen and finally meaned per group [HC/T/LC]), type, and area.40 In addition, descending aortas were isolated and snap-frozen until further analysis. During later analysis, vessels were cleaned of adherent fat, and then stained for lipids using Oil red O for “en face” morphometry of the atherosclerotic lesion area (Leica Qwin morphometric software). All analyses were performed blind, without previous knowledge of feeding regime or treatment.

The number of monocytes adhering to atherosclerotic plaques may give an indication of endothelial activation, and thereby of the inflammatory status of the plaque. Macrophages were detected using AIA31240 anti-serum (1:3000; Accurate Chemical and Scientific). The inflammatory status of plaques was further examined by estimating the local presence of NFκB (a major regulatory component of inflammatory reactions) in the plaque. NFκB was detected using mouse anti-human p65-NFκB (F-6, 1:100; Santa Cruz Biotechnology). Endothelial and macrophage NFκB-positive cells (cytoplasm and nuclei) were counted per segment. ICAM-1 and MCP-1 were measured using rat anti-mouse CD54 (CBL1316, 1:150; Chemicon International) and goat anti-mouse MCP-1 (M18, 1:300; Santa Cruz Biotechnology), respectively.
Statistical Methods
Nonparametric Mann–Whitney U tests were used to analyze treatment differences, unless stated otherwise. P<0.05 (2-sided) was considered significant. Frequency data for lesion categorization were compared using the Fisher exact test. All data are presented as mean±SD.

Results
Plasma Lipids and Lipoprotein Profiles
Body weight (Figure 1A) and food intake (data not shown) did not differ between the 3 treatment groups during the study periods. As compared with the HC group, plasma cholesterol levels were reduced in the tesaglitazar-treated (T) group (Figure 1B). As required by the experimental design, the total plasma cholesterol levels were similar in the LC and T groups.

Lipoprotein profiles of the mice showed that tesaglitazar decreased very-low-density lipoprotein cholesterol levels. Additionally, after tesaglitazar treatment, a lipoprotein fraction appeared with a size between LDL and HDL lipoproteins (Figure 1C). Western blot analysis revealed that this lipoprotein fraction was poor in apoAI and apoB but rich in apoE (data not shown).

As derived from the area under the curve of Figure 1B, the HC group had significantly increased exposure to cholesterol compared with the respective T and LC groups (Figure 1D). There was no significant difference in cholesterol exposure between T and LC control groups. Triglyceride levels were significantly lower in T groups compared with HC and LC groups (Figure 1E) (P<0.05).

In the T group, plasma tesaglitazar levels reached 38.6±11.4 nmol/L. The HOMA-IR index indicated insulin resistance in HC mice at 28 weeks. HOMA-IR was significantly lower in both the T and LC groups compared with the HC group (4.3±3.2 versus 3.4±2.7 versus 11.1±6.8, respectively; P<0.05).

Atherosclerosis
Cross-sections of the AVA showed that tesaglitazar reduced atherosclerosis compared with the respective HC groups and cholesterol-matched LC groups (Figure 2A). Treatment with tesaglitazar significantly reduced total lesion area by 92% compared with the HC group, and by 83% compared with the cholesterol-matched LC control group (P<0.05). Oil-red-O–stained en-face preparations of the aortic arch and thoracic aorta show a similar trend, but because of the large standard deviation did not reach statistical difference (supplemental Figure II).

In the same cross-sections we counted the number of lesions. The average number of lesions per animal did not differ significantly between the HC and LC control groups (Figure 2B). However, treatment with tesaglitazar significantly reduced the average number of lesions by 73% compared with the HC group and by 67% compared with the
cholesterol-matched LC group ($P<0.05$). When lesions were categorized as either mild or severe, there was a significant shift ($P<0.05$) from severe to mild lesions in tesaglitazar-treated animals (Figure 2C).

To further characterize the atherosclerotic lesions, we measured macrophage and collagen areas in cross-sections serial to those used for morphometry (Figure 3). The macrophage-positive area was larger in the HC group compared with the tesaglitazar and LC groups (Figure 3A, 3B). Moreover, the macrophage-positive area was significantly smaller in the T group than in the cholesterol-matched LC group. The collagen-positive areas followed a similar trend (Figure 3A, 3C). When expressed as a percentage of the total lesion area, collagen decreased and macrophages increased in accordance with the shift to less severe lesions.

**Inflammatory Markers**

SAA levels were significantly reduced ($P<0.05$) in T and LC groups compared with HC group ($50.5\%$ and $23\%$, respectively; Figure 4A), tesaglitazar being more efficient than LC in this respect. Fibrinogen levels were unaffected (data not shown). There were fewer adhering monocytes in the tesaglitazar-treated group compared with the HC group (Figure 4B). There were no differences between the tesaglitazar-treated group and the cholesterol-matched LC control group in this respect.

P65-NFκB staining was found in the cytosol and nuclei of both endothelial cells and macrophages (Figure 5A). SMCs remained unstained. Positively stained endothelial cells were observed on plaques. When observed on normal vessel walls, the positively stained cells were in close proximity to the shoulder regions of plaques. Cytosolic and nuclear P65-NFκB expression were quantified in endothelial cells and macrophages (Figure 5B, 5C). In the endothelium, cytосочной P65-NFκB expression was increased in the tesaglitazar-treated and LC-treated group as compared with the HC-treated group. There were no differences between the tesaglitazar-treated group and the LC control group in this respect (Figure 5B). Macrophage NFκB expression was reduced in the cytosol and nucleus of the tesaglitazar and LC group compared with the HC-treated group. However, tesaglitazar further decreased the number of cytosolic NFκB expressing cells when compared with the LC-treated group (Figure 5C). HC- and LC-treated mice had NFκB expression in endothelial cells and macrophages of almost all segments; however, in the tesaglitazar-treated group, only $17/45$ segments showed little endothelial NFκB positive staining and $1/45$ segments showed macrophage endothelial NFκB-positive staining. We calculated the relative nuclear NFκB expression, using only the mice that showed NFκB expression in their aortic segments. In the endothelial cells and macrophages of the HC-treated group $10.2\%$ and $12.7\%$ of the NFκB positive staining was nuclear, ie, active NFκB, respectively. In the LC-treated group $7.6\%$ in the endothelium and $3.8\%$ in the macrophages was active NFκB.

ICAM-1 staining was found on endothelial cells. Although staining was present in segments with and without lesions, the endothelial lining of ICAM-1 expression appeared less intense in segments without lesions (ie, the tesaglitazar treated group) (Figure 5D).

MCP-1 staining was found in macrophages/foam cells and endothelial cells. Because the lesions in the tesaglitazar treated group were smaller less MCP-1–positive staining was observed in this group, compared with the HC and LC groups (Figure 5E).

**Discussion**

This study showed that tesaglitazar has atherosclerosis-reducing capacities in ApoE3*Leiden transgenic mice that cannot be attributed solely to its reduction of plasma total cholesterol. The mechanism by which tesaglitazar reduced atherogenesis in these mice could therefore involve changes in lipoprotein composition, and thereby quality, in combination with direct actions on the pro-inflammatory tissue response of vascular cells.
The hyperlipidemic ApoE*3Leiden mice used here have a lipoprotein profile that is more similar to the human profile than those of either apoE<sup>−/−</sup> or LDLr<sup>−/−</sup> mice. In agreement with previous studies with ApoE*3Leiden mice, we were able to titrate plasma cholesterol levels by adjusting dietary cholesterol intake. Previous studies have also shown that these mice respond to hypolipidemic drugs; treatment with statins reduces plasma cholesterol<sup>42,43</sup> and treatment with a PPAR<sub>α</sub> agonist reduces both plasma cholesterol and triglyceride levels (unpublished data). In addition, we showed in an earlier dose-finding study that ApoE*3Leiden mice respond to the dual PPAR<sub>α/γ</sub> agonist tesaglitazar. In the present study, we aimed for mild cholesterol-lowering with tesaglitazar to investigate direct anti-atherosclerotic effects on the vascular wall. At a dose of tesaglitazar 0.5 μmol/kg diet (or 20 μg/kg body weight/d), a moderate decrease in plasma cholesterol ≈20% was achieved.

The lipoprotein profiles of the mice suggested that treatment with tesaglitazar resulted in the formation of an additional particle, sized between the LDL and HDL fractions. Western blotting characterized the particle as poor in apoAI and apoB but rich in apoE (data not shown). Similar lipoprotein profiles have been observed after treatment of ApoE*3Leiden mice with the PPAR<sub>α</sub> agonist fenofibrate (unpublished data). Because cholesteryl ester transfer protein is not expressed in mice, these particles could represent large apoE-rich HDL<sup>44</sup>. Furthermore, the appearance of these large apoE-rich particles during tesaglitazar treatment was associated with a decrease in atherosclerosis, suggesting that they may have favorable anti-atherosclerotic properties. However, it remains unclear whether the accumulation of these particles is clinically relevant or a mouse-specific effect.

To examine the effects of tesaglitazar that might contribute to a reduction in atherosclerosis additional to the observed lipoprotein changes, we analyzed the levels of anti-inflammatory markers SAA and fibrinogen in plasma, and examined adhering monocytes and vascular NFκB expression in atherosclerotic plaques. We found a decrease in plasma SAA levels for tesaglitazar-treated mice, but no change in plasma fibrinogen levels. In tesaglitazar-treated mice, fewer monocytes adhered to the endothelium over plaques, coinciding with a decreased NFκB expression at the same location. Previous studies have shown evidence for anti-inflammatory activities of PPAR<sub>α</sub> and PPAR<sub>γ</sub> agonists, caused by upregulation of IκB, leading to decreased NFκB/C-EBPβ complexes and suppression of C-reactive protein synthesis.<sup>45</sup> Our model provides further evidence of the anti-inflammatory effects of tesaglitazar, including reduced total lesion area as a result of decreased relative macrophage and collagen areas. Both effects contributed to the observed decrease in plaque severity in drug-treated animals. These anti-inflammatory effects were more pronounced in the tesaglitazar groups than in the LC groups, and were thus not caused by cholesterol-lowering per se. Instead of HF feeding conditions, we performed the same study under low-fat (LF) feeding conditions. The effects of tesaglitazar under LF conditions on cholesterol-lowering, atherosclerosis, and inflammation were largely the same, though less prominent, as under HF conditions.

In cell-based reporter gene assays with only the ligand-binding domain of the transcription factors, tesaglitazar appeared to be >100-fold more efficacious for mouse PPAR<sub>γ</sub> than for mouse PPAR<sub>α</sub>.<sup>31</sup> However, we do not know in the intact animal if changes observed in lipid metabolism, insulin sensitivity, and apparent anti-inflammatory markers can be ascribed to primary individual or combined activation of the 2 transcription factors or if they are secondary to improvement on fatty acid metabolism and modification of endogenous ligands.<sup>10</sup>

In summary, the dual PPAR<sub>α/γ</sub> agonist tesaglitazar showed significant anti-atherogenic effects in this mouse model with moderate insulin resistance. These effects could result from tesaglitazar-induced effects in the lipoprotein profiles and from direct anti-inflammatory and anti-atherosclerotic actions in the vascular wall.

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Disclosures

None.

References


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Type I

Early fatty streak: up to 10 foam cells in the intima.

Type II

Regular fatty streak: over 10 foam cells present in the intima.

Type III

Mild plaque: extension of foam cells into the media and covered by a fibrotic cap.

Type IV

Moderate plaque: a more progressive lesion; fibrosis of the media, without loss of architecture.

Type V

Severe plaque: The media is severely damaged, elastic lamina are broken
Often visible: cholesterol clefts/crystals, mineralisation (calcium) and necrosis

Supplemental figure I. Representative pictures of type I-V atherosclerotic lesions in apoE3-Leiden mice and categorization into mild and severe phenotypes.
Supplemental figure II. En face prepared Oil red O stained aortic arch and thoracic aorta (A) and en face total lesion area (B) of apoE3*Leiden mice on a HC, T or LC diet.