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RESEARCH PAPER

The dual PPARα/γ agonist tesaglitazar blocks progression of pre-existing atherosclerosis in *APOE*3Leiden.CETP* transgenic mice

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Background and purpose: We have evaluated the effects of a peroxisome proliferator-activated receptor (PPAR)α/γ agonist on the progression of pre-existing atherosclerotic lesions in *APOE*3Leiden.cholesteryl ester transfer protein* (*E3L.CETP*) transgenic mice.
Experimental approach: *E3L.CETP* mice were fed a high-cholesterol diet for 11 weeks to induce atherosclerosis, followed by a low-cholesterol diet for 4 weeks to obtain a lower plasma total cholesterol level of ~10 mmol·L^{-1}. Mice were divided into three groups, which were either killed before (baseline) or after an 8 week treatment period with low-cholesterol diet without (control) or with the PPARα/γ agonist tesaglitazar (10 μg·kg^{-1}·day^{-1}). Atherosclerosis was assessed in the aortic root.
Key results: Treatment with tesaglitazar significantly reduced plasma triglycerides, total cholesterol, CETP mass and CETP activity, and increased high-density lipoprotein-cholesterol. At baseline, substantial atherosclerosis had developed. During the 8 week low-cholesterol diet, atherosclerosis progressed in the control group with respect to lesion area and severity, whereas tesaglitazar inhibited lesion progression during this period. Tesaglitazar reduced vessel wall inflammation, as reflected by decreased monocyte adhesion and macrophage area, and modified lesions to a more stabilized phenotype, with increased smooth muscle cell content in the cap and collagen content.
Conclusions and implications: Dual PPARα/γ agonism with tesaglitazar markedly improved the atherogenic triad by reducing triglycerides and very low-density lipoprotein-cholesterol and increasing high-density lipoprotein-cholesterol and additionally reduced cholesterol-induced vessel wall activation. These actions resulted in complete inhibition of progression and stabilization of pre-existing atherosclerotic lesions in *E3L.CETP* mice.
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Abbreviations: CET(P), cholesteryl ester transfer (protein); E3L, APOE*3Leiden; FPLC, fast-performance liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; PPAR, peroxisome proliferator-activated receptor; SAA, serum amyloid A; VLDL, very low-density lipoprotein

Introduction

A doubling of the global burden of diabetes within 25 years from now has been predicted (Wild *et al.*, 2004). Patients with obesity, insulin resistance or type 2 diabetes are prone to develop the atherogenic triad, as characterized by raised plasma triglycerides, reduced high-density lipoprotein-cholesterol (HDL-C) and a predominance of small dense low-density lipoprotein (LDL) (Grundy, 1998). Besides insulin resistance and high glucose levels, these lipid abnormalities are all associated with an increased risk of cardiovascular diseases (Rader, 2007). HMGCoA-reductase inhibitors (statins) effectively lower plasma LDL-cholesterol (LDL-C) but do not optimize the other lipid abnormalities associated with increased risk of cardiovascular disease. Therefore, additional therapies are required to further improve the atherogenic dyslipidemia typically associated with insulin resistance and type 2 diabetes.

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Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that control the expression of genes involved in both carbohydrate and lipid metabolism, and could be valuable as additional drug targets. Stimulation of PPARα by fibrates (i) inhibits hepatic triglyceride production; (ii) increases lipoprotein lipase-mediated triglyceride lipolysis; (iii) provides a higher affinity of remnants for the LDL receptor (LDLr); (iv) enhances human apoAI and apoAII synthesis (Staels *et al.*, 1998); and (v) reduces the expression of the cholesteryl ester transfer protein (CETP) (Van der Hoogt *et al.*, 2007). These concomitant effects of fibrates result in a significant (average 36%) decrease in triglyceride and a 10% increase of HDL-C in humans (Birjmohun *et al.*, 2005). Fibrates reduce atherosclerosis in mice independent of their cholesterol-lowering effect (Kooistra *et al.*, 2006), which may be related to their anti-inflammatory capacities (Libby and Plutzky, 2007). They also reduce cardiovascular events in humans, which is most evident in obese and insulin-resistant patients (Staels and Fruchart, 2005; Keating and Croom, 2007). Thus, PPARα agonism is beneficial by its effect on the lipoprotein profile and its inflammation-reducing properties. On the other hand, PPARγ agonists (glitazones) improve insulin sensitivity and induce glycaemic control in diabetic animals as well as in patients with type 2 diabetes (Staels and Fruchart, 2005). Independent of their metabolic action, they also reduce inflammatory markers such as C-reactive protein, matrix metalloproteinase-9, serum amyloid A (SAA), soluble CD40 ligand, monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor α (TNFα), which may contribute to their anti-atherogenic properties (Mohanty *et al.*, 2004; Libby and Plutzky, 2007).

Thus, compounds that stimulate both PPARα and PPARγ (dual PPARα/γ agonists; glitazars) should additively improve both lipid and glucose abnormalities in animal models and human subjects with insulin resistance and/or type 2 diabetes. For example, tesaglitazar markedly improved apolipoprotein levels in non-diabetic subjects with insulin resistance, as shown by an increase in apoAI and decreases in apoB and apoCIII levels (Schuster *et al.*, 2008). Furthermore tesaglitazar reduced plasma levels of triglycerides, apoB, total cholesterol, non-HDL-C and very low-density lipoprotein (VLDL)-C, and increased HDL-C in patients with type 2 diabetes (Goldstein *et al.*, 2006). In obese dyslipidemic patients, tesaglitazar improved dyslipidemia on top of atorvastatin and was more effective in glucose modulation than the PPARγ agonist pioglitazone (Tonstad *et al.*, 2007). However, its clinical development was discontinued in May 2006 following phase III clinical trial results as its benefit-risk profile did not provide a significant advantage over existing anti-diabetic therapies. End-point studies using tesaglitazar or other dual PPARα/γ agonists in humans have thus far not been performed. However, because of the proven benefits of the PPARα and PPARγ agonists and the convincing efficacy in animals and humans, dual PPARα/γ agonists are still of interest as treatments for metabolic disorders.

We have previously shown that PPARα/γ agonism with tesaglitazar improved the homeostatic model assessment for insulin resistance (HOMA-IR) index, lowered plasma total cholesterol and triglyceride levels and lowered inflammation markers in hyperlipidemic *APOE*3Leiden* (*E3L*) mice (Zadelaar *et al.*, 2006). These mice display a lipoprotein profile comparable to that of patients with dysbetalipoproteinemia [in which plasma total cholesterol and triglyceride are mainly confined to (V)LDL (Van Vlijmen *et al.*, 1994)] and respond to a variety of hypolipidemic drugs, as do humans (Zadelaar *et al.*, 2007). Tesaglitazar did not increase HDL in *E3L* mice, probably because mice naturally lack expression of CETP, which is an important factor for human HDL metabolism. In these mice, tesaglitazar reduced atherosclerosis development in a prevention design, in which pharmacological treatment was started before the onset of atherosclerosis and was applied in combination with an atherogenic, high-cholesterol diet (Zadelaar *et al.*, 2006).

The aim of the present study was to evaluate the effect of a dual PPARα/γ agonist on atherosclerosis development in a more clinically relevant design under conditions mimicking those of diabetic dyslipoproteinemia, in which pharmacological treatment with the PPARα/γ agonist tesaglitazar was started after atherosclerosis had been developed and after plasma cholesterol levels were lowered. To this end, we used our recently developed *E3L.CETP* transgenic mouse model (Westerterp *et al.*, 2006), a mouse model for human-like lipoprotein metabolism that has been shown to respond to both lipid-lowering interventions (Zadelaar *et al.*, 2007) and HDL-raising interventions (Van der Hoogt *et al.*, 2007; De Haan *et al.*, 2008a,b; Van der Hoorn *et al.*, 2008).

Methods

Mice and diets

All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Human *CETP* transgenic mice that express CETP under control of its natural flanking regions (strain 5203) (Jiang *et al.*, 1992) were obtained from Jackson laboratories (Bar Harbor, MC) and cross-bred with *E3L* mice (Van den Maagdenberg *et al.*, 1993) in our local animal facility at TNO to obtain heterozygous *E3L.CETP* mice (Westerterp *et al.*, 2006). Forty-seven female *E3L.CETP* mice (average 18 weeks of age) received a semi-synthetic high-cholesterol Western-type diet, containing 40.5% sucrose and 15% cocoa butter, supplemented with 0.3% ($w \cdot w^{-1}$) cholesterol for 11 weeks to induce hypercholesterolemia and atherosclerosis development. After 11 weeks, the diet was replaced by a low-cholesterol Western-type diet containing 0.1% ($w \cdot w^{-1}$) cholesterol for another 4 weeks to reduce total cholesterol towards a more modest level (~10 $mmol \cdot L^{-1}$). Thereafter, the animals were divided into three groups, after matching based on age, body weight and plasma total cholesterol and triglyceride levels. Subsequently, the mice were fed the low-cholesterol diet without ('control group'; $n = 16$) or with tesaglitazar ('tesaglitazar group', 0.25 $\mu mol \cdot kg \cdot diet^{-1}$ ~ 10 $\mu g \cdot kg$ body $weight^{-1} \cdot day^{-1}$; $n = 16$) to reduce total cholesterol levels by about 50% (dose determined in a pilot study). This intervention period was an additional 8 weeks. To quantify the extent of atherosclerosis at the start of the drug intervention, a third group of mice was killed immediately after matching, which was considered a reference for the

baseline atherosclerosis level ('baseline group', $n = 15$). The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study.

Plasma lipids and apolipoproteins

After a 4 h fasting period from 9:00 AM to 1:00 PM, blood was collected from the tail vein into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). Plasma was assayed for total cholesterol (No-236691, Roche Diagnostics, USA) and triglyceride (No-1488872, Roche Diagnostics, USA) and for murine apoAI and human apoE by using sandwich ELISAs as described previously (Westerterp *et al*., 2006). The distribution of cholesterol of the various lipoproteins was determined after separation of lipoproteins by fast-performance liquid chromatography (FPLC) using a Superose 6 column (Westerterp *et al*., 2006). HDL-C was also quantified in plasma after precipitation of apoB-containing lipoproteins. Thus, 10 μL heparin (LEO Pharma, The Netherlands) and 10 μL 0.2 mol·L^{-1} $MnCl_2$ were added to 20 μL plasma, and mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at 13 000× *g* at 4°C.

Plasma CETP mass and CET activity

Cholesteryl ester transfer protein mass was determined by using the DAIICHI CETP ELISA kit (Daiichi Pure Chemicals Co, Japan) according to manufacturer's instructions. Cholesteryl ester transfer (CET) activity was measured exactly as described and calculated as nmol·mL^{-1}·h^{-1} (Van der Hoogt *et al*., 2007).

Plasma inflammation markers

Serum amyloid A (Biosource International, Belgium), adiponectin, E-selectin, MCP-1 and vascular cellular adhesion molecule-1 (all R&D Systems Inc, USA) were determined by ELISA according to the manufacturers' instructions. Fibrinogen was measured with an in-house procedure as described previously (Delsing *et al*., 2003).

Histological assessment of atherosclerosis

The various mouse groups were killed either at the start of low-cholesterol treatment ('baseline group') or after the 8 week treatment period with low-cholesterol diet ('control group') or low-cholesterol diet supplemented with tesaglitazar ('tesaglitazar group'). The hearts were dissected, formalin-fixed and embedded in paraffin. Serial cross sections (5 μm) throughout the entire aortic valve area were used for histological analysis. In each mouse, four sections at intervals of 50 μm were used for quantitative and qualitative assessment of the atherosclerotic lesions after staining with haematoxylin-phloxin-saffron (HPS). For determination of the severity of atherosclerosis, the lesions were classified into five categories as described by Verschuren *et al*., (2005): (i) early fatty streak; (ii) regular fatty streak; (iii) mild lesion; (iv) moderate lesion; and (v) severe lesion. The percentages of all lesions found in the respective categories were calculated for each mouse. The total lesion area was calculated per cross section. In each segment used for qualitative assessment of the lesions, monocytes were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, USA), and the number of monocytes adhering to the endothelium was counted (Delsing *et al*., 2001). Macrophage area was measured after immunostaining with anti-mouse Mac-3 (BD Pharmingen, the Netherlands). Smooth muscle cell area was measured after immunostaining with mouse anti-human actin (DAKO, Denmark) that cross-reacts with murine actin. The collagen content of the lesions was quantified morphometrically after Sirius Red staining. Smooth muscle cell area in the cap was measured and calculated as percentage of the total lesion area. All analyses were performed by the same operator, who was unaware of the experimental group allocation.

Statistical analysis

Data are presented as means ± SD unless indicated otherwise. Statistical differences were assessed by using the non-parametrical Kruskal-Wallis test followed by Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

Results

Tesaglitazar decreases (V)LDL-C and increases HDL-C

To induce the development of atherosclerosis, *E3L.CETP* mice were fed a high-cholesterol diet for 11 weeks, resulting in a relatively high plasma total cholesterol level (19.9 ± 4.9 mmol·L^{-1}) and a moderate triglyceride level (2.3 ± 1.3 mmol·L^{-1}) (Figure 1). Thereafter, the mice were fed a low-cholesterol diet for 4 weeks to obtain more mild total cholesterol levels (10.8 ± 4.9 mmol·L^{-1}) without reducing triglyceride levels (2.2 ± 0.9 mmol·L^{-1}) at the start of the intervention period. One mouse group was subsequently killed ('baseline group'), and the other groups were treated with either low-cholesterol diet ('control group') or the low-cholesterol diet supplemented with tesaglitazar ('tesaglitazar group') for 8 weeks. As compared with the control group, tesaglitazar reduced total cholesterol by about half ($P < 0.001$; Figure 1A) and triglycerides to a greater extent ($P < 0.001$; Figure 1B). Lipoprotein fractionation by FPLC showed that the total cholesterol-lowering effect of tesaglitazar was accounted for by a large reduction in (V)LDL-C (~80%), whereas tesaglitazar increased HDL-C (~+45%) (Figure 2A). The increase in HDL-C was confirmed by direct measurement of HDL-C in plasma after precipitation of apoB-containing lipoproteins ($P < 0.01$; Figure 2B). No differences were observed in plasma concentrations of apoAI (1.4 ± 0.4 mg·mL^{-1} vs. 1.5 ± 0.5 mg·mL^{-1}) and human apoE (0.22 ± 0.08 mg·mL^{-1} vs. 0.21 ± 0.08 mg·mL^{-1}).

Tesaglitazar decreases CETP mass and CET activity

As the HDL-raising effect of tesaglitazar may have resulted from decreased CETP expression, plasma CETP mass and CET activity were determined after the 8 weeks low-cholesterol treatment (Figure 3). As compared with the control group, tesaglitazar lowered CETP mass ($P < 0.001$) and reduced CETP activity ($P < 0.001$).

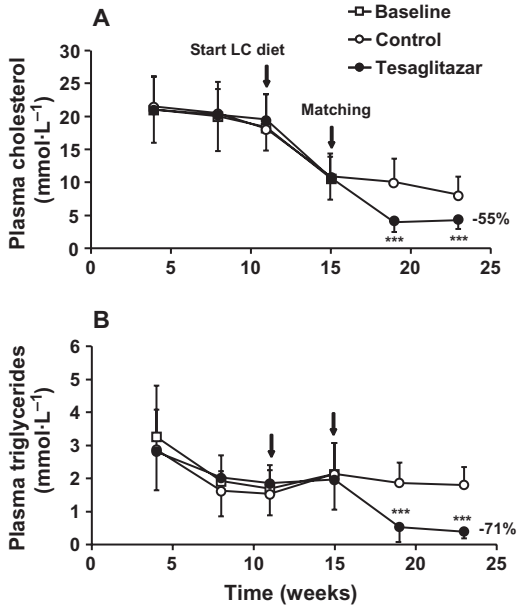

Figure 1 Effect of dietary cholesterol reduction and tesaglitazar on plasma cholesterol and triglyceride levels. *E3L.CETP* mice received a high-cholesterol (0.3% w·w^{-1}) diet for 11 weeks followed by a low-cholesterol (LC, 0.1% w·w^{-1}) diet for 4 weeks. Subsequently, the mice were matched and either killed (baseline) or continued to be fed the low-cholesterol diet without tesaglitazar (control) or with tesaglitazar for 8 weeks. At the indicated times, blood samples were taken and plasma was analysed for total cholesterol (A) or triglycerides (B). Values are means ± SD (n = 15–16 per group). ***P < 0.001 as compared with the control group. CETP, cholesteryl ester transfer protein; E3L, APOE*3Leiden.

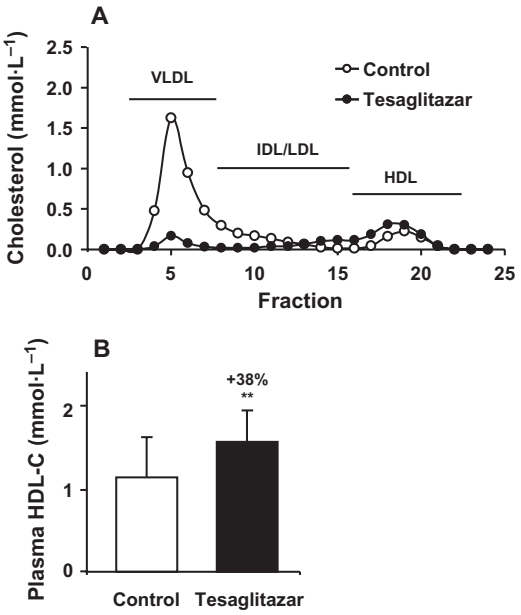

Figure 2 Effect of tesaglitazar on plasma lipoproteins. After matching, *E3L.CETP* mice were fed the low-cholesterol diet without (control) or with tesaglitazar for 8 weeks. The distribution of cholesterol over the individual lipoproteins in pooled plasma was determined after separation of lipoproteins by FPLC (A). Plasma HDL-C levels were also measured individually after precipitation of apoB-containing lipoproteins (B). Values are means ± SD (n = 16 per group). **P < 0.01 as compared with the control group. CETP, cholesteryl ester transfer protein; E3L, APOE*3Leiden; FPLC, fast-performance liquid chromatography; HDL-C, high-density lipoprotein-cholesterol; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

Tesaglitazar blocks progression of atherosclerosis development

Eleven weeks of feeding the high-cholesterol diet followed by 4 weeks on the low-cholesterol diet resulted in a lesion area per cross section of 136 ± 87 × 10^3 μm^2 as determined in the baseline group (Figure 4A). Prolonged feeding of the mice with the low-cholesterol diet without tesaglitazar for another 8 weeks increased the lesion area (P < 0.05), and this diet-induced increase in lesion area was fully blocked by tesaglitazar (no significance vs. baseline). These changes in lesion area were also reflected by changes in lesion severity. Whereas prolonged feeding of the low-cholesterol diet without tesaglitazar increased the percentage of severe lesions (type IV-V; P < 0.01) at the expense of mild (type I-II) and moderate (type III) lesions, the low-cholesterol diet with tesaglitazar did not result in an increase in type IV-V lesions or a reduction in type III lesions, compared with baseline (Figure 4B).

Tesaglitazar reduces inflammation in the arterial wall

To investigate the contribution of inflammation to the observed effects of tesaglitazar, we measured inflammation markers. At baseline, plasma markers for systemic inflammation such as adiponectin (5.0 ± 0.9 μg·mL^{-1}), SAA (1.0 ± 0.6 μg·mL^{-1}) and fibrinogen (1.8 ± 0.3 mg·mL^{-1}) were in the physiological range, as were the vascular inflammation markers E-selectin (43.5 ± 7.2 ng·mL^{-1}), vascular cellular adhesion molecule-1 (1.1 ± 0.3 μg·mL^{-1}) and the macrophage-derived chemokine MCP-1 (75.0 ± 20.1 pg·mL^{-1}). These markers in plasma were not differentially changed in the control group and the tesaglitazar group as compared with baseline levels, albeit that we observed a slight increase in E-selectin levels upon tesaglitazar treatment (51.0 ± 7.2 ng·mL^{-1}; P < 0.05).

Local inflammation in the vessel wall was histologically assessed by measuring the adhesion of monocytes to the endothelium (Figure 5A) and the macrophage content of the lesions (Figure 5B). As compared with baseline, the number of monocytes adhering to the endothelium and the macrophage content of the lesions were not affected in the control group. Tesaglitazar, however, potently reduced inflammation in the vessel wall as compared with baseline, as reflected by a 48% decrease in adhesion of monocytes (P < 0.05) and a strong reduction (85%) in macrophage content of the lesions (P < 0.001).

Tesaglitazar increases the smooth muscle cell and collagen content of the atherosclerotic lesions

To evaluate the effect of tesaglitazar on parameters reflecting lesion stability, we measured the collagen content (Figure 5C) and smooth muscle cell area in the cap (Figure 5D). As compared with baseline, the lesions in the control group were more severe and contained therefore more collagen (P < 0.001), but had a similar amount of smooth muscle cells in the cap per

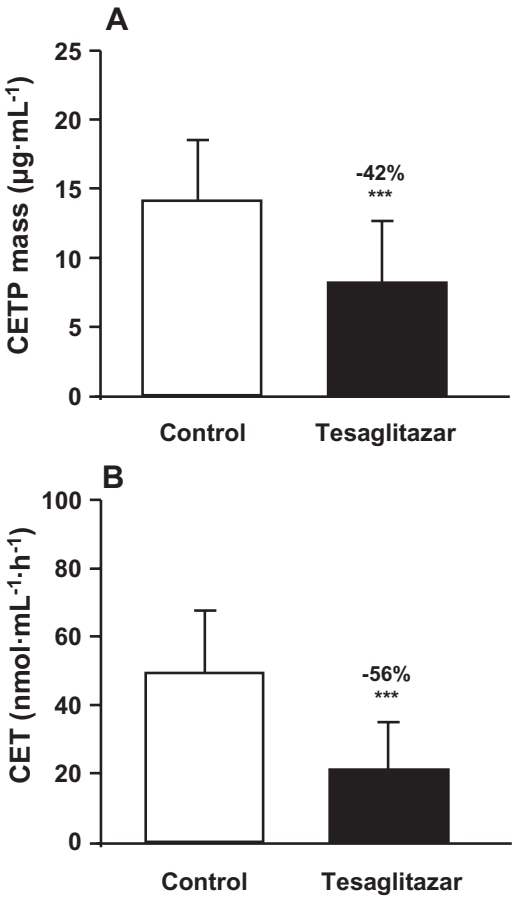

Figure 3 Effect of tesaglitazar on CETP mass and CET activity. After matching, *E3L.CETP* mice were fed the low-cholesterol diet without (control) or with tesaglitazar for 8 weeks. Plasma CETP mass (A) and CET activity (B) were determined. Values are means ± SD ($n = 16$ per group). ***$P < 0.001$ as compared with the control group. CET(P), cholesteryl ester transfer (protein); E3L, APOE*3Leiden.

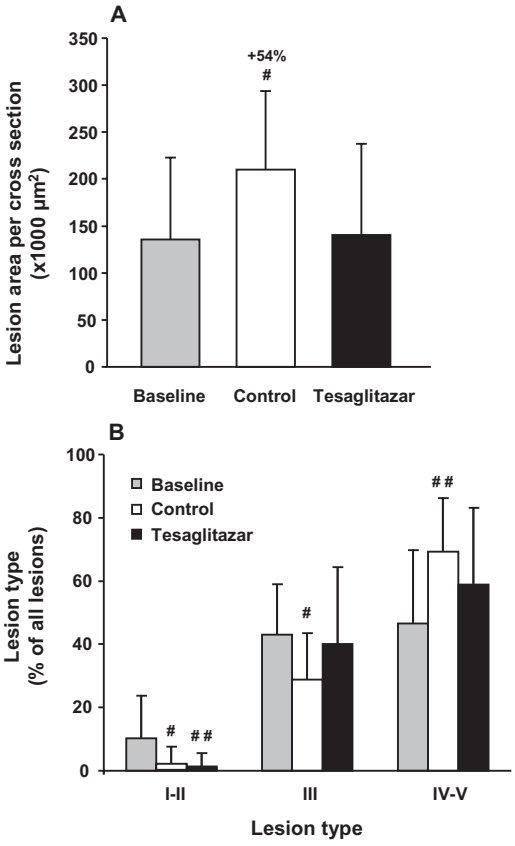

Figure 4 Effect of tesaglitazar on development of atherosclerosis. *E3L.CETP* mice received the high-cholesterol diet for 11 weeks, followed by the low-cholesterol diet for 4 weeks. Then, mice were killed at baseline, or mice were fed the same low-cholesterol diet without (control) or with tesaglitazar for another 8 weeks. In the valve area of the aortic root, lesion area was measured per cross section (A) and the severity of lesions was analysed (B). Values are means ± SD ($n = 15$–16 per group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared with the baseline group. CETP, cholesteryl ester transfer protein; E3L, APOE*3Leiden.

lesion area. Tesaglitazar not only blocked lesion development (Figure 4A), but also enhanced the induction of a more stable lesion phenotype as compared with control, reflected by a further increase in collagen content ($P < 0.001$ vs. baseline, $P < 0.05$ vs. control). The increase in smooth muscle cell area in the cap was 64%, compared with the baseline ($P < 0.05$), which was not different from the control group.

Discussion

In this study we showed that stimulating both PPARα and PPARγ with tesaglitazar, in addition to a lowered cholesterol, but not dietary cholesterol lowering alone, fully prevented the progression of pre-existing atherosclerosis in *E3L.CETP* transgenic mice. Tesaglitazar also reduced inflammation in the vessel wall as reflected by less monocyte adherence and less macrophage area in the lesions and induced a more stable lesion phenotype as indicated by an increased collagen content and smooth muscle cell area in the cap.

We previously showed that *E3L* mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels. Moreover, *E3L* mice show a human-like response to drug interventions aimed at treatment of cardiovascular disease, such as statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists (Delsing *et al.*, 2001; 2003; Kleemann *et al.*, 2003; Kooistra *et al.*, 2006; Van der Hoorn *et al.*, 2007) in terms of alterations in the lipoprotein profile and/or atherosclerosis development. To be more specific, all lipid-lowering treatments resulted in significant reductions of plasma triglycerides and (V)LDL-C, to a similar extent as in humans, thereby attenuating atherosclerosis development. This is in marked contrast with wild-type C57Bl/6 mice and conventional hyperlipidemic mice, such as apoE-deficient or LDLr-deficient mice, which show either an adverse response or no response to such interventions (Zadelaar *et al.*, 2007). In particular, administration of tesaglitazar to LDLr-deficient mice did not affect plasma lipid levels (Chira *et al.*, 2007), while in *E3L* mice, tesaglitazar lowered triglyceride and cholesterol within apoB-containing lipoproteins (Zadelaar *et al.*, 2006), comparable to its effects in humans (Goldstein *et al.*, 2006; Tonstad *et al.*, 2007; Schuster *et al.*, 2008).

Recently, we showed that introduction of the human *CETP* gene in *E3L* mice results in a mouse model that also displays

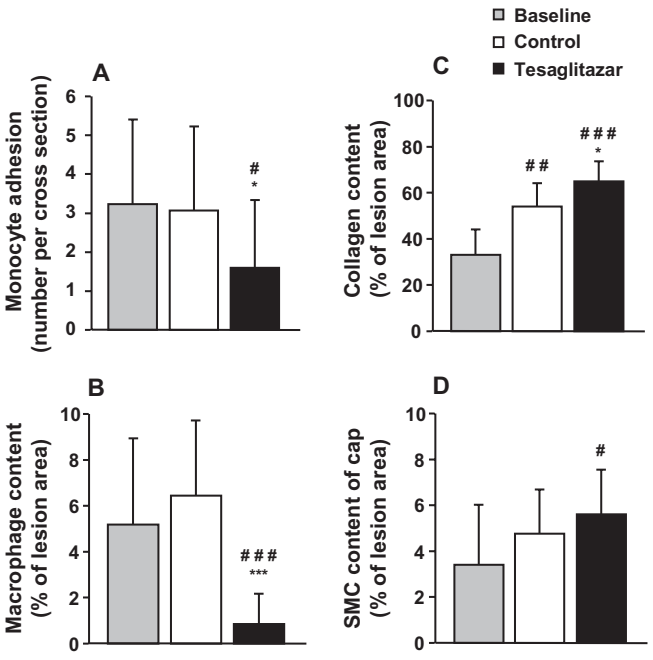

Figure 5 Effect of tesaglitazar on lesion composition. *E3L.CETP* mice received the high-cholesterol diet for 11 weeks, followed by the low-cholesterol diet for 4 weeks. Then, mice were killed at baseline, or mice were fed the same low-cholesterol diet without (control) or with tesaglitazar for 8 weeks. The number of monocytes adhering to the endothelium was counted (A). The macrophage content (B), the collagen content (C) and the smooth muscle cell (SMC) content in the cap (D) of the lesions were measured. Values are means ± SD (n = 15–16 per group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the baseline group; $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with the control group. CETP, cholesteryl ester transfer protein; E3L, APOE*3Leiden.

a human-like response with regard to therapies that raise HDL-C. Administration of fenofibrate, atorvastatin and niacin not only decreased triglycerides and (V)LDL-C as in *E3L* mice, but also increased HDL-C as in humans, demonstrating the significant role of CETP in HDL-C metabolism (Van der Hoogt *et al*., 2007; De Haan *et al*., 2008a; Van der Hoorn *et al*., 2008). Atorvastatin treatment either alone or in combination with the CETP inhibitor torcetrapib significantly reduced atherosclerosis development (De Haan *et al*., 2008b).

In the present study, we confirmed that treatment of *E3L.CETP* mice with tesaglitazar reduced (V)LDL-C and strongly reduced triglycerides, as observed previously in *E3L* mice. In addition, we demonstrated that tesaglitazar also raised HDL-C levels in *E3L.CETP* mice, as reported in clinical intervention trials (Goldstein *et al*., 2006; Tonstad *et al*., 2007). The presence of CETP thus plays a crucial role in the HDL-C-raising effect of tesaglitazar. In fact, tesaglitazar markedly decreased CETP mass in plasma. This is most probably due to the PPARα-agonistic effect of tesaglitazar, as we have recently observed that treatment of *E3L.CETP* mice with another PPARα agonist, fenofibrate, reduced hepatic CETP expression as well as the plasma CETP mass and CETP activity (Van der Hoogt *et al*., 2007). The observation that plasma CETP activity is reduced to a larger extent than CETP mass may relate to the large reduction in plasma triglycerides, as the capacity of apoB-containing lipoproteins to accept cholesteryl esters from HDL is closely correlated to its plasma level (Mann *et al*., 1991; Marzetta *et al*., 1993; Guerin *et al*., 1994a,b). The tesaglitazar-induced increase in HDL-C was not accompanied by a rise in apoAI, suggesting that tesaglitazar increases the particle size of HDL rather than the amount of HDL particles, which was indeed confirmed by FPLC profiling.

To establish the effect of tesaglitazar on atherosclerosis in a setting mimicking the clinical situation, we first induced atherosclerosis in *E3L.CETP* mice by feeding a high cholesterol-containing Western-type diet. Then, plasma cholesterol was lowered by limiting the dietary cholesterol before treatment with tesaglitazar was started. Interestingly, lowering cholesterol alone did not prevent the further development of atherosclerosis. More specifically, vessel wall inflammation was not attenuated by cholesterol lowering alone, and the atherosclerosis had actually progressed with respect to total lesion area and lesion severity. In contrast, tesaglitazar inhibited further atherosclerosis development and stabilized lesions. This can be ascribed to a further reduction of atherogenic (V)LDL-C, an increase in HDL-C as well as to local anti-inflammatory effects of tesaglitazar in the vessel wall, as atherosclerosis is a dynamic lipid- and inflammation-driven process.

As shown by the strikingly reduced adherence of monocytes and macrophage content of the lesion, tesaglitazar decreased the local vessel wall inflammation induced by the high-cholesterol diet, whereas cholesterol lowering alone did not. These observations confirm our previous observations in *E3L* mice, which showed that tesaglitazar reduced NF-κB-positive areas in the atherosclerotic lesions, indicating reduced local inflammation. This was accompanied by lower levels of intercellular adhesion molecule-1- and of MCP-1-positive areas (Zadelaar *et al*., 2006), which explains decreased monocyte adherence and transmigration, and thus decreased macrophage content of the lesions. Interestingly, similar features have been observed in *E3L* mice after treatment with the PPARα agonist fenofibrate (Kooistra *et al*., 2006). As the effect of PPARγ agonists in this model is unknown, we cannot discriminate between the individual contributions of the PPARα or PPARγ effects to the anti-atherogenic effect of tesaglitazar. Moreover, comparable anti-inflammatory effects regarding endothelium activation have been shown for both single PPARα and PPARγ agonists (Libby and Plutzky, 2007; Bouhlel *et al*., 2008). It recently has been shown that PPARγ activation may programme mononuclear precursor cells towards an M2 phenotype *in vivo*, leading to generation of a macrophage population with enhanced anti-inflammatory properties (Gordon and Rifkind, 1989; Bouhlel *et al*., 2007). The anti-inflammatory effect of tesaglitazar is local and not systemic, as tesaglitazar did not decrease the (normal) plasma levels of inflammation markers (SAA and fibrinogen) in either *E3L* or *E3L.CETP* mice.

Furthermore, tesaglitazar stabilized the lesions by increasing the smooth muscle cell content of the lesion cap as well as the collagen content of the lesion. Inflammation not only enhances lesion development and progression, but also destabilizes lesions by stimulating the release of metalloproteinases from macrophages, which are able to degrade collagen. Tesaglitazar may thus increase the collagen content as a conse-

quence of its reduction of macrophage accumulation in the lesion. In fact, stimulation of PPARγ by rosiglitazone in humans stabilized lesions in the carotid artery, which was ascribed to a reduced expression of metalloproteinases-3, 8 and 9 (Meisner *et al*., 2006). These observations indicate that, in established atherosclerotic lesions, the anti-inflammatory actions of dual PPARα/γ activation not only inhibit lesion progression but also induce a more stable lesion phenotype.

To date, all dual PPARα/γ agonists including tesaglitazar have been discontinued either at a preclinical stage or during clinical development and thus no dual PPARα/γ agonist has yet been approved for clinical use (Balakumar *et al*., 2007a). Long-term clinical effects can therefore only be extracted from clinical studies using either single PPARα or PPARγ agonists. The effect of PPARα agonist fibrates on cardiovascular morbidity and mortality was studied in primary [HHS (Frick *et al*., 1987) and FIELD (Keech *et al*., 2005)] and secondary [The BIP Study Group (2000), VA-HIT (Rubins *et al*., 1999) and FIELD (Keech *et al*., 2005)] cardiovascular prevention studies. The results from these trials suggest that fibrate therapy reduced cardiovascular disease and was most efficacious in overweight individuals with insulin resistance and chronic inflammation. Although PPARγ agonists are effective in the management of insulin resistance and type 2 diabetes in a number of prospective clinical trials, their effects on cardiovascular disease are still a matter for debate (Balakumar *et al*., 2007b; Lincoff *et al*., 2007; Nissen and Wolski, 2007). While pioglitazone reduced the incidence of myocardial infarction and acute coronary syndrome in patients with type 2 diabetes [PROactive study (Erdmann *et al*., 2007)], rosiglitazone has been noted to increase the risk of cardiovascular disease [ADOPT(Kahn *et al*., 2006) and DREAM (Gerstein *et al*., 2006) study]. The contradicting results of these clinical trials using different compounds to stimulate PPARγ might just reflect the complex and meticulous balance in PPAR pathways and mechanisms involved in either their beneficial or detrimental effects. Likewise, whereas the dual PPARα/γ agonist tesaglitazar has atheroprotective effects in *E3L.CETP* mice, as shown in the present study, and in other mouse models for hyperlipidemia and atherosclerosis (Zadelaar *et al*., 2006; Chira *et al*., 2007), the dual PPARα/γ agonist 'compound q' aggravated inflammation and atherosclerosis in apoE-deficient mice (Calkin *et al*., 2007).

In conclusion, we have shown that the PPARα/γ agonist tesaglitazar halted progression of pre-existing atherosclerosis and stabilized lesions in *E3L.CETP* mice, as related to reduction of (V)LDL-C, increase of HDL-C and of local anti-inflammatory properties in the vessel wall. Despite the clinical failure of tesaglitazar, these data clearly demonstrate a potential therapeutic role for PPARα/γ agonists in the treatment of diabetic cardiovascular complications.

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Conflicts of interest

EL and GC are employees of AstraZeneca, Mölndahl, Sweden.

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