PPARα, but not PPARγ, Activators Decrease Macrophage-Laden Atherosclerotic Lesions in a Nondiabetic Mouse Model of Mixed Dyslipidemia

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Objective—Peroxisome proliferator-activated receptor (PPAR) α and γ are nuclear receptors that may modulate atherogenesis, not only by correcting metabolic disorders predisposing to atherosclerosis but also by directly acting at the level of the vascular wall. The accumulation of lipid-laden macrophages in the arterial wall is an early pivotal event participating in the initiation and promotion of atherosclerotic lesion formation. Because PPARα and γ modulate macrophage gene expression and cellular function, it has been suggested that their ligands may modulate atherosclerosis development via direct effects on macrophages. In this report, we investigated the effect of a PPARα ligand (fenofibrate) and 2 PPARγ ligands (rosiglitazone and pioglitazone) on atherogenesis in a dyslipidemic nondiabetic murine model that develops essentially macrophage-laden lesions.

Methods and Results—Mice were fed a Western diet supplemented or not with fenofibrate (100 mpk), rosiglitazone (10 mpk), or pioglitazone (40 mpk) for 10 weeks. Atherosclerotic lesions together with metabolic parameters were measured after treatment. Fenofibrate treatment significantly improved lipoprotein metabolism toward a less atherogenic phenotype but did not affect insulin sensitivity. Contrarily, rosiglitazone and pioglitazone improved glucose homeostasis, whereas they did not improve lipoprotein metabolism. Fenofibrate treatment significantly decreased the accumulation of lipids and macrophages in the aortic sinus. However, surprisingly, neither rosiglitazone nor pioglitazone had an effect on lesion lipid accumulation or macrophage content.

Conclusion—These results indicate that in a dyslipidemic nondiabetic murine model, PPARα, but not PPARγ, activators protect against macrophage foam cell formation. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: atherosclerosis — foam cells — peroxisome proliferator-activated receptors α and γ — ligands — murine model

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors regulating the expression of genes that control lipid and glucose homeostasis, thus modulating the major metabolic disorders predisposing to atherosclerosis. Moreover, PPARs exert additional anti-inflammatory and lipid-modulating effects in the arterial wall, therefore being interesting molecular targets for the treatment of atherosclerosis. PPARs are the targets of 2 classes of drugs currently used in clinical practice: fibrates (fenofibrate, clofibrate, ciprofibrate, and gemfibrozil) are PPARα agonists, whereas thiazolidinediones (TZDs) (rosiglitazone and pioglitazone) are potent PPARγ activators. Although the beneficial effects of fibrate treatment on coronary events and atherogenesis in humans are well-documented through epidemiological and clinical intervention studies, results of outcome trials with PPARγ agonists in humans are still awaited to answer whether treatment with TZDs translates into a therapeutic benefit in atherosclerotic cardiovascular disease. The majority of clinical studies performed to date assessed the effects of TZDs in diabetic patients and most suggested vascular protective effects of PPARγ ligands as determined by improved insulin-sensitivity, decreased vascular and systemic markers of inflammation, reduced carotid wall thickness and neointimal tissue proliferation, and, depending on the TZD, corrected dyslipoproteinemia. Interestingly, even in nondiabetic patients with coronary artery disease, rosiglitazone treatment induced beneficial effects on the vascular endothelium, exerted antiplatelet activity, and reduced carotid intima-media thickness progression. All these reports argue for potent antiatherogenic effects of TZDs, raising the possibility of their use in clinical conditions other than diabetes. Consistent with this therapeutic potential of TZDs against atherosclerosis in humans, results from experimental animal studies showed that PPARγ agonists...
reduce the development of atherosclerosis\textsuperscript{11–15} and limit its complications.\textsuperscript{16–20}

In the present study, we investigated the effect of a PPAR\(\alpha\) ligand (fenofibrate) and 2 different PPAR\(\gamma\) ligands (rosiglitazone and pioglitazone) on atherogenesis in a dyslipidemic non-diabetic murine model that develops essentially macrophage-laden atherosclerotic lesions.

**Materials and Methods**

**Animals and Diets**

Homozygous human apolipoprotein (apo) E2 knock-in mice (E2-KI mice) were used in the study. These mice express human apoE2 instead of mouse apoE.\textsuperscript{21} At weaning, male mice were fed a Western diet containing (wt/wt) 0.2\% cholesterol and 21\% fat (UAR, Epinay sur Orge, France) supplemented or not (control group) with fenofibrate, rosiglitazone, or pioglitazone (n=10 per group) for 10 weeks. Based on food consumption monitoring, the daily drug delivery was \(\approx 100, 10,\) and 40 mg/kg of body weight for fenofibrate, rosiglitazone, and pioglitazone, respectively. These doses were chosen based on their previously reported effects on atheroprotection or metabolic parameters in mice.\textsuperscript{12,14,22–24} The mice had ad libitum access to water and were weighed once per week. Blood was obtained after a 6-hour fasting period (8AM to 2 PM) by retroorbital puncture under isoflurane-induced anesthesia or, depending on the analysis, by tail-cutting on conscious mice. This study was conducted according to the Guidelines for the Care and Use of Experimental Animals.

**Biochemical Analyses**

At the end of the study, blood samples were drawn and glucose levels were determined using Glucotrend 2 (Roche Diagnostic, Meylan, France). Plasma was then separated for analyses. Insulin levels were determined using a solid phase 2-site enzyme immunoassay (Mercodia AB, Uppsala, Sweden). The homeostasis model of insulin resistance index was used as a measure of insulin resistance, in which homeostasis model of insulin resistance index=fasting plasma insulin (\(\mu\)U/mL) \(\times\) fasting blood glucose (mmol/L)/22.5.\textsuperscript{25} Plasma levels of total cholesterol (TC), triglycerides (TGs), and high-density lipoprotein (HDL) cholesterol (HDL-C) were measured using commercially available kits (Boehringer-Mannheim, Germany). Non-HDL cholesterol (N-HDL-C) was obtained by subtraction of HDL-C from TC. Lipoprotein cholesterol distribution profiles were analyzed in pooled plasma samples from each group by gel filtration chromatography using a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech), as previously detailed.\textsuperscript{26} Cholesterol concentration was determined in the eluted fractions as described.

**Analysis of Atherosclerotic Lesions**

After mice were euthanized by cervical dislocation, the heart of each animal was fixed with 4\% phosphate-buffered paraformaldehyde (pH 7.0) and serial 10-\(\mu\)m-thick sections were cut between the valves and the aortic arch for quantitative analysis of lipid deposition by Oil red-O.\textsuperscript{26} The sections were immunohistochemically stained with rat monoclonal anti-mouse macrophage (MOMA-2; Biosource International; 1:100 dilution) or mouse monoclonal anti-\(\alpha\)-actin antibodies (Sigma-Aldrich, France; 1:100 dilution) for smooth muscle cell (SMC) labeling, followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase. Immunostains were visualized using the DAB substrate-chromogen system (DAKO Corporation, Carpinteria, Calif). Images were captured by use of a JVC 3-charge–coupled device video camera. Sections were analyzed using the computer-assisted Quips Image analysis system (Leica Mikroskop and System GmbH, Wetzlar, Germany).

**Statistical Analysis**

Values are reported as mean\(\pm\)SD. The significance of differences between means was determined by ANOVA comparison of control and treated groups. Significant differences were then subjected to post-hoc analysis by using the Sheffe test. A value of \(P<0.05\) was considered as statistically significant.

**Results**

Male E2-KI mice were fed a Western diet with or without fenofibrate, rosiglitazone, or pioglitazone for 10 weeks. Both treated groups gained comparable weight as the control group, indicating that none of the compounds induced toxic effects (data not shown).

Fenofibrate, but not Rosiglitazone or Pioglitazone, Improves the Dyslipoproteinemia in E2-KI Mice

Compared with wild-type littermates, E2-KI mice fed a standard chow diet are dyslipidemic and display elevated plasma TC concentrations with low HDL-C/TC and high N-HDL-C/TC ratios, associated with increased TG levels (data not shown).

Fenofibrate-treated E2-KI mice exhibited a reduction in plasma TC concentrations \((P<0.05)\). Both N-HDL-C \((P<0.01)\) and TG \((P<0.01)\) levels decreased, whereas HDL-C increased \((P<0.001)\). In contrast, treatment with rosiglitazone or pioglitazone caused no significant changes in plasma lipid levels and only a slight decrease in both TG and TC (because of changes in N-HDL-C) levels were observed.
Rosiglitazone and Pioglitazone, but not Fenofibrate, Improve Glucose Homeostasis in E2-KI Mice

Chow-fed E2-KI mice present comparable fasting plasma glucose and insulin concentrations as compared with wild-type littersmates suggesting unaltered glucose homeostasis and insulin-sensitivity in E2-KI mice (data not shown). These levels were unaffected by the Western diet and after 10 weeks, plasma glucose and insulin levels remained stable in the control group (135 ± 19 versus 156 ± 35 mg/dL for glucose; 0.490 ± 0.217 versus 0.448 ± 0.211 μg/L for insulin; means ± SD, respectively, before and after diet).

Whereas no significant changes in fasting glucose or insulin levels were detected after fenofibrate treatment as compared with the control animals, a marked reduction in insulin levels (P < 0.01) were observed in rosiglitazone- or pioglitazone-treated mice (Figure 2). Compared with control or fenofibrate-treated mice (5.49 ± 0.08 and 5.69 ± 0.16, respectively), rosiglitazone and pioglitazone also significantly reduced homeostasis model of insulin resistance index (3.15 ± 0.21 and 3.74 ± 0.03, respectively) (P < 0.01).

Fenofibrate, but not Rosiglitazone and Pioglitazone, Decreases Atherosclerosis Progression in E2-KI Mice

The effect of fenofibrate treatment on atherosclerosis was assessed in E2-KI mice by measuring oil-red-O–stained surfaces at the aortic sinus. Fenofibrate-treated mice showed significantly decreased atherosclerotic lesion surfaces (~90%) when compared with control mice (0.0140 ± 0.0123 versus 0.0719 ± 0.0189 mm²; P < 0.001) (Figure 3A). In marked contrast, rosiglitazone and pioglitazone had no effect on atherosclerotic lesion size (0.0666 ± 0.0229 and 0.0626 ± 0.0257 mm², respectively) (Figure 3A).

Representative photomicrographs of atherosclerotic lesions show a decrease of lipid-stained surfaces at the aortic sinus from fenofibrate-treated E2-KI mice as compared with control or rosiglitazone or pioglitazone-treated animals (Figure 3B, a, c, e, g). Moreover, as revealed by MOMA-2–specific staining of the lesions (Figure 3B, b, d, f, h), macrophages colocalized with oil-red-O–stained-areas, pointing to a critical role for macrophages in atherogenesis on the E2-KI mice. Interestingly, rosiglitazone or pioglitazone treatment did not
lead to altered macrophage content within the atherosclerotic lesions. In contrast, few SMCs were present in the atherosclerotic lesions of E2-KI mice and SMC staining was not different between control and treated mice.

Discussion

In a nondiabetic murine model (E2-KI mice) that displays mixed dyslipidemia and develops atherosclerotic lesions consisting mainly of foam cells, we report that PPARγ activators have no effect on the promotion of atherogenesis. Conversely, a PPARα ligand delays the development of atherosclerosis in the mice.

Atherosclerosis results from a cascade of events that involve interactions between the vessel wall and blood components. The atherosclerotic lesion development starts as a result of endothelial injury and dysfunction triggered by risk factors for atherosclerosis. This injury facilitates recruitment of circulating lipoproteins into the artery wall, which are oxidized and consequently induce a chronic inflammatory response characterized by the appearance of peripheral blood cells, including monocytes. The local production of cytokines further attracts and enhances migration of the monocyte/macrophages into the vascular wall which then engulf the oxidized lipoprotein particles and transform into foam cells, constituting as such the fatty streaks. Foam cell formation is accompanied by macrophage activation characterized by induced expression of genes coding for inflammatory cytokines and growth factors. These growth factors cause smooth muscle cells to migrate from the media into the intima, where they proliferate, resulting in intimal hyperplasia and formation of fibroproliferative lesions, characteristic of advanced atherosclerotic plaques. Thrombotic occlusion caused by deposition of platelets and clot formation may ensue, leading, finally, in cases of rupture, to clinical events such as myocardial infarction or stroke.

Previous studies from our laboratory and others have documented that PPARs activation inhibits the development of atherosclerosis in apoE-deficient mice expressing a human apoA-I transgene, and LDL receptor-deficient mice, respectively. However, in both previous studies, it seemed that PPARα ligands exert their antiatherosclerotic effects via mechanisms unrelated to their plasma lipid-lowering activity. This observation is in line with a number of clinical studies reporting that the inhibition of atheromatous plaque progression as well as the decreased incidence of cardiovascular events on fibrate treatment occurs via effects possibly independent or in addition to their systemic action. Nevertheless, it is well known that controlling metabolic risk factors for atherosclerosis, such as dyslipidemia, improves endothelial dysfunction, an early pivotal event in the atherosclerosis process. It is interesting to point out that the beneficial effects of fenofibrate on lipid and lipoprotein metabolism in the E2-KI mice in the present study most likely participate in the favorable effect of the drug on atherosclerosis. Fenofibrate treatment decreased the atherogenic lipoprotein particles (VLDL, IDL, and LDL), and increased the protective HDL levels. The preclinical development of PPARα agonists, which would be expected to act on lipid metabolism and hence atherosclerosis, includes the analysis of their effects in appropriate murine models. However, the development of a reliable experimental model is not easy, because, ideally, the regulatory pathways in the studied models need to be similar to those in humans. Considering the different susceptibilities of mice strains to develop atherosclerosis, considering the species differences in affinity of synthetic PPARα agonists, considering the species differences in regulatory pathways, testing hypolipidemic drugs in animal models may not necessarily generate predictive results for the human situation, but may even lead to erratic conclusions concerning their potential atheroprotective activities. From this study, it seems that in contrast to other mouse models, E2-KI mice appear to be a relevant experimental model to test the efficacy of PPARα agonists on dyslipidemia and atherosclerosis.

Unexpectedly, our results demonstrate that treatment with 2 different PPARγ ligands do not protect E2-KI mice from atherosclerosis and foam cell accumulation. Previous studies performed in different mouse models (LDL receptor-deficient or apoE-deficient mice) demonstrated an atheroprotective effect of PPARγ activation by TZDs. From these studies, it could be plausibly concluded that the overall beneficial action of the TZDs occurred independently from systemic lipid changes and, possibly, from the insulin sensitizer activity of the compounds. In our present study, no significant changes in lipoprotein metabolism were observed in rosiglitazone-treated or pioglitazone-treated mice, and although glucose homeostasis was improved in the mice, this improvement was not sufficient to induce a delay in atherosclerosis development in this mouse model with normal glucose homeostasis. It is interesting to remark that at the doses used in our study, pioglitazone has no hypolipidemic effect. Because E2-KI mice respond well to PPARα agonists, it appears that unlike suggested by in vitro experiments, pioglitazone exerts very little if any PPARα activity, at least under the experimental conditions tested here.

PPARα and PPARγ are expressed in all cell types of the arterial wall, including endothelial cells, monocytes/macrophages, and vascular SMCs. Potentially direct antiatherosclerotic parietal effects of PPAR agonists have been observed in in vitro experiments, in vivo animal studies, as well as in clinical studies in humans. Besides their activities on macrophage lipid homeostasis with direct consequences for atherosclerosis development, PPARα and PPARγ modulate the earliest step of the atherosclerotic lesion by inhibiting the expression of certain cytokines involved in the recruitment of monocytes/macrophages by endothelial cells. Through these properties, PPARα and PPARγ may exert cardioprotective activities by decreasing foam cell formation. Our results show that PPARα activation has a beneficial effect on lipid-laden macrophage accumulation in the aortic sinus and it is plausible that the pleiotropic action of fenofibrate has contributed together with its hypolipidemic activity to the atheroprotective benefit seen in the mice. By contrast, the lack of effect of PPARγ activation on macrophage accumulation in mice contrasts with previous in vivo animal studies showing protective effects. Although the exact reasons are unclear, several explanation can be evoked. Atherosclerosis in the E2-KI mice, in contrast to other mouse models, is characterized by the almost exclusive presence of macro-
phages. It is possible that in the context of severe, uncorrected dyslipidemia, PPARγ activation in macrophages is insufficient to reverse the pro-atherogenic program. Moreover, a substantial controversy exists on the role of PPARγ in macrophage cholesterol metabolism in mice. In vitro, PPARγ ligands may induce macrophage foam cell formation through CD36 induction and increased uptake of oxidized LDL. However, in vivo rosiglitazone treatment in obese insulin-resistant ob/ob mice resulted in a decrease of peritoneal macrophage CD36 protein content likely caused by the insulin-sensitizing activity of the compound. However, LDL receptor-deficient mice that display only mild insulin resistance showed an increase in CD36 protein after rosiglitazone treatment in vivo. The effects of rosiglitazone on foam cell formation and atherogenesis could therefore differ depending on the degree of insulin resistance. E2-KI mice are nondiabetic and are not insulin resistant; therefore, the impact of PPARγ agonists on macrophages may not be sufficient to improve atherosclerosis in this mouse model. Thus, whether these observations can be extended to the human situation is unclear. Previous results on human and murine macrophages have demonstrated significant species differences in the control of cholesterol homeostasis by PPARγ ligands. For instance, whereas PPARγ activation results in the induction of the cholesterol efflux transporter ABCA1 in human macrophages, this effect is much less pronounced in murine macrophages. Finally, it is possible that the atheroprotective effects of PPARγ are mediated via other cell types that do not participate in E2-KI mice atherogenesis such as SMCs. In this respect, it is interesting to note that TZD treatment has been shown to improve endothelial function and decrease intima-media thickness also in nondiabetic patients. Such activities may be mediated, in part, via their effects on the proliferation and migration of vascular SMCs and the anti-inflammatory activities in these and other cell types involved in atherogenesis. Moreover, PPARα and PPARγ ligands may influence thrombogenesis and inhibit proteins implicated in plaque rupture resulting in plaque stabilization. Unfortunately, no murine models are currently available to test such effects. Therefore, we cannot exclude that determining the effects of TZDs on advanced atherosclerosis may prove to be a stronger predictor of their potential clinical benefit and testing their effects in other atherogenesis models and in humans is therefore warranted.

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References


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