Troglitazone Inhibits Atherosclerosis in Apolipoprotein E–Knockout Mice

Pleiotropic Effects on CD36 Expression and HDL

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Abstract—Atherosclerotic coronary heart disease is a common complication of the insulin resistance syndrome that can occur with or without diabetes mellitus. Thiazolidinediones (TZDs), which are insulin-sensitizing antidiabetic agents, can modulate the development of atherosclerosis not only by changing the systemic metabolic conditions associated with insulin resistance but also by exerting direct effects on vascular wall cells that express peroxisome proliferator–activated receptor-γ (PPAR-γ), a nuclear receptor for TZDs. Here we show that troglitazone, a TZD, significantly inhibited fatty streak lesion formation in apolipoprotein E–knockout mice fed a high-fat diet (en face aortic surface lesion areas were 6.9±2.5% vs 12.7±4.7%, $P<0.05$; cross-sectional lesion areas were 191 974±102 911 μm^2 vs 351 738±175 597 μm^2, $P<0.05$; n=10). Troglitazone attenuated hyperinsulinemic hyperglycemia and increased high density lipoprotein cholesterol levels. In the aorta, troglitazone markedly increased the mRNA levels of CD36, a scavenger receptor for oxidized low density lipoprotein, presumably by upregulating its expression, at least in part, in the macrophage foam cells. These results indicate that troglitazone potently inhibits fatty streak lesion formation by modulating both metabolic extracellular environments and arterial wall cell functions. **(*Arterioscler Thromb Vasc Biol*. 2001;21:372-377.)**

Key Words: atherosclerosis ■ thiazolidinediones ■ lipoproteins ■ CD36 ■ peroxisome proliferator–activated receptor

Type 2 diabetes mellitus is a major risk factor contributing to coronary heart disease (CHD), the leading cause of morbidity and mortality in developed countries.[1] The mechanisms by which diabetes promotes atherosclerosis remain obscure. Evidence suggests that the metabolic derangement frequently associated with type 2 diabetes and the insulin resistance syndrome, such as hypertension and lipoprotein abnormalities, are responsible for the increased incidence of CHD in diabetic patients.[2,3] Prospective studies have shown that hyperinsulinemia is an independent predictor of the development not only of type 2 diabetes but also of CHD.[4] Stout et al[5] proposed that hyperinsulinemia directly promotes the development of atherosclerosis. However, overwhelming evidence supports the idea that hyperinsulinemia is merely a consequence of insulin resistance, which directly or indirectly enhances the development of atherosclerosis in type 2 diabetes.[6]

Thiazolidinediones (TZDs), which are novel, insulin-sensitizing agents, are increasingly used as oral hypoglycemic agents.[7] They improve insulin sensitivity and thereby reduce hyperinsulinemia as well as hyperglycemia.[8,9] Furthermore, troglitazone, a TZD, improves hypertension and hypertriglyceridemia, both of which represent constellations of the insulin resistance syndrome.[10,11]

In addition to these potential indirect effects of TZDs on the risk factors of atherosclerosis, increasing evidence suggests that TZDs exert potent, direct effects on arterial wall cells. Recently, Sinohara[12] et al reported that the development of intimal lesions after balloon catheter injury in the rat aorta was significantly inhibited by troglitazone. They argued that this inhibition was mediated by troglitazone's antimitogenic effects on vascular smooth muscle cells.[13] It has also been reported that TZDs inhibit monocyte inflammatory cytokines,[14] macrophage activation,[15] and the expression of cell adhesion molecules expressed by vascular endothelial cells.[16,17] Peroxisome proliferator–activated receptor-γ (PPAR-γ), the receptor for TZDs,[18] is expressed in arterial wall cells such as vascular smooth muscle cells and macrophages.[19] PPAR-γ may be involved in regulation of the uptake of oxidized LDL by modulating CD36, a scavenger

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Effect of Troglitazone on Body Weight, Blood Glucose, Plasma Insulin, Plasma Free Fatty Acid (FFA) Levels, and Plasma Lipids

	Body Weight, g	Blood Glucose, mg/dL	Plasma Insulin, pg/mL	Plasma FFA, μEg/L	TC, mg/dL	TG, mg/dL	HDL-C, mg/dL
Control (n=20)							
Before	18.8±3.4	128.4±34.0	641.5	935±12	909±415	67±37	21±6
After	30.5±4.5‡	139.0±18.9	1366.9	1319±257‡	1746±598‡	236±115‡	21±10
Troglitazone (n=15)							
Before	21.0±1.2	141.6±26.3	590.7	976±90	1043±403	83±26	26±3
After	30.4±3.8‡	112.8±40.9*†	658.4	1210±3312‡	1651±655†	319±181‡	36±11*†

TC indicates total cholesterol; TG, triglycerides; and HDL-C, HDL cholesterol.
*$P<0.05$ vs control group; †$P<0.05$ vs before; ‡$P<0.01$ vs before by ANOVA.

receptor, in macrophages.[20,21] On the basis of these results, TZDs may promote foam cell formation. However, it remains unknown whether TZDs have antiatherogenic effects.

To investigate the effects of troglitazone on fatty streak formation, the initial step of atherosclerosis, we treated apolipoprotein (apo) E–knockout mice, an animal model of atherosclerosis,[22] with troglitazone and examined the resultant atherosclerotic lesions.

Methods

Animals

ApoE-knockout mice were a gift from Dr N. Maeda (University of North Carolina, Chapel Hill).[22] These mice were back-crossed to C57BL6 mice 9 times at our facility to produce congenic animals. They were maintained on a 12-hour dark/12-hour light cycle and allowed ad libitum access to food and water. The diets used were (1) a Western-type diet (containing 0.15% cholesterol, 15% unsalted butter) and (2) a Western-type diet supplemented with 0.1% (wt/wt) troglitazone, which was a gift of Sankyo Pharmaceutical Ltd, Tokyo, Japan. Thirty-five male animals aged 5 weeks (20 as controls and 15 for the troglitazone experiments) were fed the diets for 2 months. C57BL6 mice were used for preparation of peritoneal macrophages.

Plasma Lipids, Glucose, and Insulin

After a 6-hour fast, blood was collected from the retro-orbital venous plexus.[23] Blood glucose levels were immediately measured on an Antsense II (Daikin). Plasma levels of total cholesterol (TC), triglyceride (TG), and free fatty acids (FFAs) were measured enzymatically by using Determiner TC 555, Determiner TG 555, and Determiner NEFA methods (Kyowa Medex), respectively. Pooled plasma was used for determining insulin levels with an insulin assay kit from Morinaga. Lipoprotein profiles were analyzed by high-performance liquid chromatography (HPLC) essentially as described, with some modifications.[23] In brief, 5 μL of plasma was applied to a combined column system composed of 2 TSK gel Lipopropacks (Tosoh) connected in tandem and eluted with the supplied buffer (TSK eluent LP-1, Tosoh) at a rate of 0.6 mL/min. TC concentrations in the effluents were monitored by using a kit (Determiner LTC). Areas under the elution curves for HDL peaks were determined. These values and plasma TC levels were used to calculate HDL cholesterol concentrations.

Measurement of Atherosclerotic Lesion Size

Mice were killed by cervical dislocation after blood collection. The en face surface and cross-sectional lesion areas in the aorta were determined as described previously.[23]

Cell Culture

Peritoneal macrophages were prepared as described previously.[23] In brief, 1 mL of thioglycolate broth was injected into the peritoneal cavities of mice aged 3 months. After 4 days, the peritoneal cavities were lavaged with 10 mL of ice-cold saline. The cells were washed 3 times with PBS and resuspended in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM), and 10^6 cells were plated in 60-mm dishes (Corning). After incubation at 37°C for 2 hours, nonadherent cells were removed by washing 3 times with prewarmed PBS. The adherent cells were incubated with HG-DMEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, JRL Biosciences) at 37°C in an atmosphere of 5% CO_2 and 95% air. After 16 hours, the media were replaced with fresh media containing 10% (vol/vol) FCS supplemented with the indicated concentrations of troglitazone. Troglitazone was solubilized in dimethylsulfoxide (DMSO), and equivalent concentrations of DMSO were used for controls. The final concentrations of DMSO were kept at 0.1% (vol/vol).

Northern Blot Analysis

Total RNA was prepared from the aortas by use of Trizol reagent (Life Technologies, GIBCO-BRL). Twenty micrograms of RNA was subjected to electrophoresis in a 1.0% (wt/vol) agarose gel containing formalin. After transfer, the nylon membrane (Hybond N, Amersham Pharmacia Biotech) was hybridized with ^{32}P-labeled probes for mouse CD36, lipoprotein lipase (LPL), PPAR-γ, and β-actin. cDNA polymerase chain reaction fragments were used as probes for CD36 and PPAR-γ. Human LPL cDNA probes were as previously described.[23]

Statistics

All experimental data are expressed as mean±SD. Mean values were compared by Student's *t* test and ANOVA. Spearman's coefficients of correlation were calculated.

Results

Plasma Glucose and Lipoproteins

Table 1 compares the body weight, blood glucose, and plasma concentrations of insulin, FFAs, and lipoprotein profiles. Fasting blood glucose levels were significantly decreased by 18.8% in the troglitazone-treated group compared with controls ($P<0.05$). The plasma insulin level was decreased by 52% in the troglitazone-treated group. On the other hand, there were no significant differences in either body weight or plasma FFA levels between the 2 groups. Similarly, no significant differences in plasma TC and TG levels were observed between the 2 groups. HPLC analyses revealed a selective increase in cholesterol content in a peak corresponding to HDL (Figure 1). On average, HDL cholesterol levels were increased by 74% in the troglitazone-treated group compared with controls (36±11 vs 21±10 mg/dL, $P<0.05$).

Atherosclerotic Lesions in the Aorta and Aortic Sinus

En face surface atherosclerotic lesions of the troglitazone-treated group were grossly smaller than those of the control group (Figures 2A and 2B). These lesions were mainly distributed on the aortic arches and the areas surrounding the branching points of the arteries. The en face surface aortic

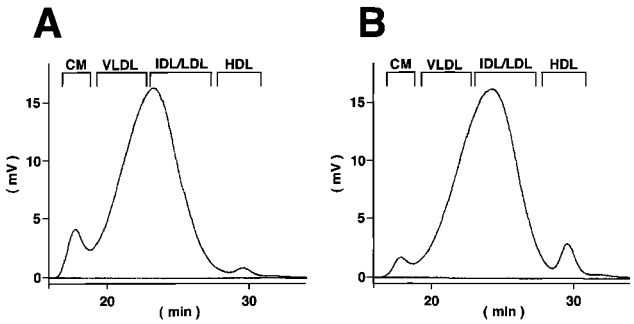

Figure 1. Comparison of plasma lipoprotein profiles between control (A) and troglitazone-treated (B) apoE-knockout mice. ApoE-knockout mice were fed a Western-type diet with or without 0.1% troglitazone for 2 months. Pooled plasma was subjected to HPLC. Cholesterol contents in the eluates were monitored. Lipoproteins isolated by standard ultracentrifugation were used to calibrate the elution positions of chylomicron (CM), VLDL, IDL/LDL, and HDL, as indicated above the elution pattern. Note that the HDL peak was significantly elevated in the treated group compared with the control group without changing the retention time.

lesion area of the troglitazone group was decreased by 45% compared with the control group (6.9% vs 12.7%, $P<0.05$) (Figure 3A).

Microscopic examination determined that the lesions in both groups were mainly of the intermediate type, consisting of several layers of foam cells with some intracellular lipid accumulation but without a typical lipid core (Figures 2C and 2D). Typical atheromas with well-developed, lipid-rich cores and foam cell infiltration but without fibrous tissue proliferation were observed in some sections of the control group. The cross-sectional lesion areas of the troglitazone-treated group were significantly smaller (by 45%) than those of the control group (191 974±102 911 vs 351 738±175 597 μm^2, $P<0.05$) (Figure 3B).

To determine the factors mediating the antiatherosclerotic effects of troglitazone, we performed correlational analyses between lesion sizes and plasma lipid levels. When analyzed as a whole, only HDL cholesterol levels were negatively correlated with the en face lesion sizes ($R=-0.54$, $P<0.05$). When analyzed in each group, however, there was no correlation between them.

Induction of CD36 Expression in the Aorta

Figure 4 shows the results of Northern blot analysis of CD36, PPAR-γ, and β-actin in the aorta. The mRNA levels of CD36 were markedly increased in the troglitazone-treated group compared with the control group (7-fold), while those of PPARγ were not significantly changed.

Induction of CD36 Expression in Macrophages in Culture

To determine whether troglitazone induced the expression of CD36 in vitro, peritoneal macrophages were incubated with various concentrations of troglitazone for 12 hours, and then the mRNA expression levels of CD36, LPL, PPAR-γ, and β-actin were estimated by Northern blot analysis (Figure 5). Incubation with troglitazone induced a 2.5-fold increase in CD36 mRNA levels in a dose-dependent manner, whereas it increased the LPL mRNA levels by only 50% at 12 hours. Later, at 48 hours, the mRNA levels of LPL and PPAR-γ were also significantly induced by 20 μmol/L troglitazone (Figure 6).

Discussion

In the present study, we have demonstrated that troglitazone significantly inhibits the development of atherosclerotic foam cell lesions in apoE-knockout mice fed a high-fat diet. The antiatherogenic effects of troglitazone appear to be mediated, at least in part, by favorable changes in the extracellular environment: suppression of hyperinsulinemic hyperglycemia and an increase in HDL cholesterol levels. Troglitazone also strongly increased the mRNA expression of CD36 in the aorta, which is conceivably proatherogenic, according to reported observations that targeted disruption of the CD36 gene was protective against atherosclerosis.[24] Therefore, the CD36-increasing effects of troglitazone might be overwhelmed by the antiatherogenic effects of other factors, such as glucose and lipoproteins metabolism. The possibility remains that the other effects, which include anti-inflammation, inhibition of cell adhesion, and antioxidation, accounted for the antiatherogenic effects of troglitazone.

Troglitazone increased HDL cholesterol levels in apoE-knockout mice fed a high-fat diet (Table 1), an effect that is likely antiatherogenic. It is well established that HDL cholesterol is negatively correlated with CHD in humans.[25] In

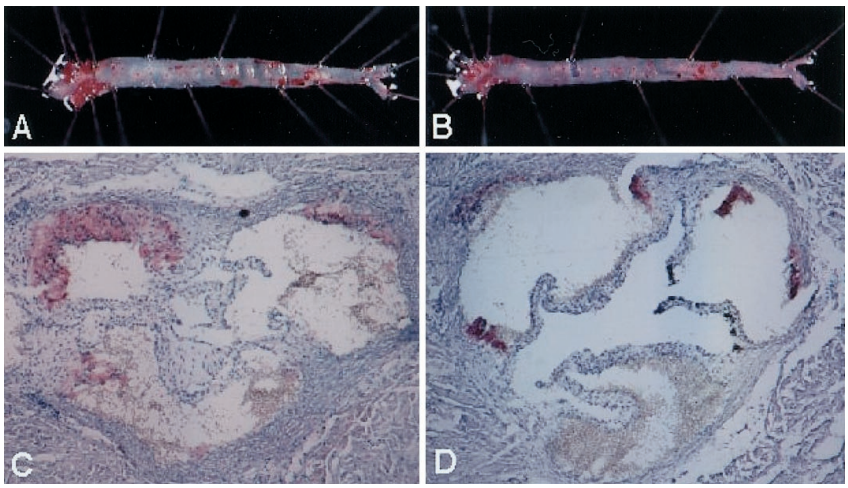

Figure 2. Morphology of en face surface lesions (A, B) and cross-sectional lesions (C, D) in control (A, C) and toglitazone-treated (B, D) apoE-knockout mice. ApoE-knockout mice were fed a Western-type diet with or without 0.1% troglitazone for 2 months. A representative aorta was stained for lipids with Sudan IV. Cross sections of the aortic sinus were stained with oil red O and counterstained with hematoxylin (×20).

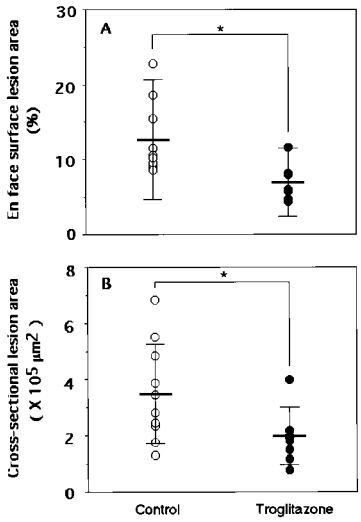

Figure 3. Comparison of the size of en face surface (A) and cross-sectional (B) lesions between the control and troglitazone-treated groups. ApoE-knockout mice were fed a Western-type diet with or without 0.1% troglitazone for 2 months (n=10). Short lines indicate the mean±SD * $P<0.05$ by Student's t test.

apoE-knockout mice that have extremely low levels of HDL cholesterol, subtle changes in this lipoprotein fraction may have profound effects on the development of atherosclerosis. For example, apoE-knockout mice that overexpress apo A-I, which results in increased HDL levels, are resistant to atherosclerosis.[26] Because the apo A-I gene contains a peroxisome proliferator–responsive element, it is possible that the apo A-I gene is positively regulated by PPAR-γ in addition to PPAR-α agonists.[27] Furthermore, activation of PPAR-γ upregulates LPL gene expression.[28,29] The increased LPL may stimulate lipolysis of TG-rich lipoproteins, thereby contributing to the elevation of plasma HDL cholesterol levels in apoE-knockout mice, as is the case with LPL-transgenic mice.[23]

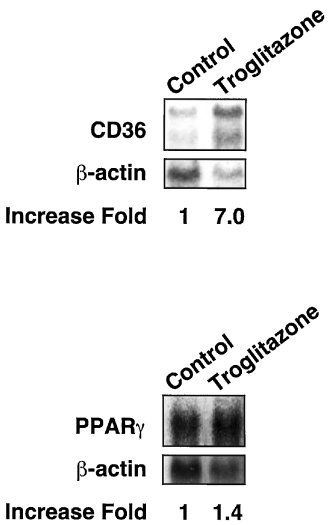

Figure 4. Northern blot analysis of CD36 and PPAR-γ in the aorta. Total RNA was extracted from the aorta. Twenty micrograms of pooled samples was subjected to Northern blot analysis. β-Actin was used as a loading control. Relative intensities of the bands for CD36 or PPAR-γ to those for β-actin are indicated below each panel. CD36 expression was elevated by 7-fold, whereas the PPAR-γ expression was slightly elevated by 1.4-fold in response to treatment with troglitazone.

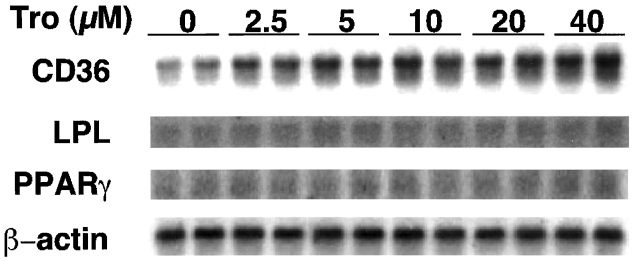

Figure 5. The mRNA expression of CD36, LPL, and PPAR-γ in peritoneal macrophages incubated with increasing concentrations of troglitazone. Peritoneal macrophages were harvested after thioglycolate elicitation. Sixteen hours after preparation, the cells were incubated with HG-DMEM containing 10% (vol/vol) FCS supplemented with the indicated concentrations of troglitazone for 12 hours. Twenty micrograms total RNA as subjected to Northern blot analysis. β-Actin was used as a loading control.

In addition to favorable changes in plasma lipoprotein metabolism, attenuation of hyperinsulinemic hyperglycemia, a hallmark of the insulin resistance syndrome, may underlie the antiatherogenic effects of troglitazone. Indeed, insulin has mitogenic effects on vascular smooth muscle cells.[30] However, it also causes vascular dilation[31] and inhibition of platelet aggregation.[32] Therefore, it is reasonable to conclude that the effects of insulin on atherosclerosis are neutral as a whole.

In contrast to changes in the extracellular environment, upregulation of CD36 mRNA in the aorta might promote atherosclerosis. CD36 is a membrane-bound molecule with diverse functions.[33] In addition to functioning as a receptor for fatty acids, CD36 mediates the uptake of oxidized LDL by macrophages as a sort of scavenger receptor.[34,35] Although lesion size was markedly reduced in troglitazone-treated mice, the overall mRNA levels of CD36 were increased in response to treatment with troglitazone (Figure 4). Accordingly, CD36 expression in each cell should be significantly enhanced by treatment with troglitazone. In the current study, we did not precisely define which cells expressed CD36. Macrophage foam cells and endothelial cells are likely candidates. In support of this possibility, troglitazone markedly increased the mRNA expression of CD36 of macrophages in culture (Figure 5). Because CD36 is highly ex-

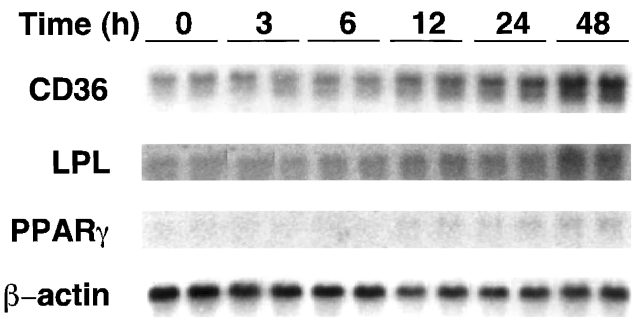

Figure 6. Time courses of mRNA expression of CD36, LPL, and PPAR-γ in peritoneal macrophages incubated with 20 μmol/L troglitazone. Peritoneal macrophages were harvested after thioglycolate elicitation. Sixteen hours after preparation, the cells were incubated with HG-DMEM containing 10% (vol/vol) FCS supplemented with 20 μmol/L troglitazone for the indicated periods. Twenty micrograms of total RNA was subjected to Northern blot analysis. β-Actin was used as a loading control.

pressed in human atherosclerotic lesions[36] and its expression is enhanced by high cholesterol levels,[37] it has been speculated that CD36 is atherogenic. Indeed, disruption of CD36 is reportedly protective against atherosclerosis under conditions of apoE deficiency.[24] Therefore, we may speculate that the increased expression of CD36 stimulates the uptake of oxidized LDL, thereby promoting foam cell formation and atherosclerosis. However, troglitazone exerted apparently contradictory effects in the current experiments: protection against atherosclerosis and enhanced expression of CD36. It is possible that the antiatherogenic effects of troglitazone on other factors, such as induction of LPL and cell adhesion, overcome the proatherogenic CD36-inducing effects. Alternatively, the induction of CD36 may be protective against atherosclerosis under certain conditions. For example, it is plausible that efficient removal of denatured lipoproteins deposited in the extracellular matrixes reduces atherogenic inflammatory reactions. Further studies are needed to clarify this issue.

Currently, it is widely accepted that PPAR agonists have anti-inflammatory effects.[38] In particular, it has been reported that TZDs decrease the expression of cell adhesion molecules expressed by vascular endothelial cells, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, thereby suppressing the homing of inflammatory leukocytes, including monocytes.[16,17] Therefore, it is conceivable that these anti-inflammatory effects of TZDs predominate over the induced expression of CD36 in macrophage foam cells. Furthermore, it is also possible that the antiatherosclerotic effects of troglitazone are, at least in part, accounted for by the effects of antioxidation due to its structural similarity to α-tocopherol.[39]

In conclusion, TZDs were shown to have protective effects against atherosclerosis in a mouse model of atherosclerosis. These effects may be mediated by the pleiotropic actions of troglitazone. Thus, TZDs are promising therapeutic agents that can be used for the prevention and/or treatment of atherosclerosis.

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