Modulation of Vascular Inflammation In Vitro and In Vivo by Peroxisome Proliferator–Activated Receptor-γ Activators

Vincenzo Pasceri, MD; Henry D. Wu, MD; James T. Willerson, MD; Edward T.H. Yeh, MD

Background—Peroxisome proliferator–activated receptor-γ (PPARγ) is expressed in atherosclerotic plaques and in endothelial cells. The possible effects of PPARγ activators on endothelial activation and inflammatory response within the plaque are currently unknown.

Methods and Results—We tested the hypothesis that PPARγ activators inhibit vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) expression in cultured endothelial cells (evaluated by flow cytometry) and homing of monocyte/macrophages to atherosclerotic plaques in vivo. In endothelial cells, the PPARγ agonist troglitazone at 100 μmol/L and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) at 20 μmol/L markedly attenuated the tumor necrosis factor–induced expression of VCAM-1 and ICAM-1. A significant inhibition of VCAM-1 expression was also evident at 5 and 10 μmol/L 15d-PGJ2 and 20 μmol/L troglitazone. Expression of E-selectin and PECAM-1 was not altered. To confirm the biological relevance of these results, we assessed the effects of troglitazone on monocyte/macrophage homing to atherosclerotic plaques in apoE-deficient mice. A 7-day treatment with troglitazone (400 mg/kg) significantly reduced monocyte/macrophage homing to atherosclerotic plaques (236 ± 77 versus 177 ± 43 macrophages, P < 0.03); an even more striking inhibition was found at 3200 mg/kg troglitazone (344 ± 76 versus 172 ± 83 macrophages, P < 0.005).

Conclusions—PPARγ activators inhibit expression of VCAM-1 and ICAM-1 in activated endothelial cells and significantly reduce monocyte/macrophage homing to atherosclerotic plaques. These findings suggest that PPARγ activators, currently used in treatment of type II diabetes, may have beneficial effects in modulating inflammatory response in atherosclerosis. (Circulation. 2000;101:235-238.)

Key Words: cell adhesion molecules ▪ receptors ▪ atherosclerosis

Experimental and pathological studies have suggested that the peroxisome proliferator–activated receptor-γ (PPARγ) may have a role in the pathogenesis of atherosclerosis.1 PPARγ is a nuclear receptor highly expressed in several tissues, including adipose tissue, monocytes/macrophages, and smooth muscle cells.2 In atherosclerotic plaques, PPARγ is expressed by macrophage/foam cells, and activation of this receptor can inhibit macrophage activation.3,4 Although PPARγ is also expressed by endothelial cells,5 the possible effects of PPARγ activators in atherosclerotic plaques are currently unknown.

Expression of adhesion molecules by endothelial cells and adhesion of leukocytes to endothelial cells is an essential step in atherogenesis.6,7 We have recently shown, in an animal model of atherosclerosis, that monocyte/macrophage homing to atherosclerotic plaques depends on the expression of the adhesion molecules vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1).8 The aim of our study was to assess the effects of PPARγ activators on the expression of adhesion molecules on activated endothelial cells and on monocyte/macrophage homing to atherosclerotic plaques in vivo.

Methods

Cell Cultures
Human umbilical vein endothelial cells (HUVECs, Cascade Biology) were grown in M199 medium with 15% FBS, 0.2 mg/mL heparin, 0.1 mg/mL endothelial cell growth supplement (Biomedical Technologies), 2 mmol/L L-glutamine, and 1% penicillin/streptomycin. Cells were used at passage 2 to 4. Viability by trypan blue exclusion was >95% in each experiment. Apoptosis was assessed by detection of fragmented DNA at flow cytometry after staining with propidium iodide, according to a previously published protocol.9

Mouse RAW 264.7 cells were grown in DMEM with 10% FBS, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin.

Detection of Adhesion Molecules
HUVECs were pretreated with the PPARγ agonists troglitazone (Parke-Davis), ciglitazone (Biomol), 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2, from Calbiochem); with the PPARγ agonist fenofibrate (Sigma); or with vehicle (0.1% DMSO) at the concentrations indicated. After 2 hours, the cells were incubated with tumor necrosis factor–induced expression of VCAM-1 and ICAM-1. A significant inhibition of VCAM-1 expression was also evident at 5 and 10 μmol/L 15d-PGJ2 and 20 μmol/L troglitazone. Expression of E-selectin and PECAM-1 was not altered. To confirm the biological relevance of these results, we assessed the effects of troglitazone on monocyte/macrophage homing to atherosclerotic plaques in apoE-deficient mice. A 7-day treatment with troglitazone (400 mg/kg) significantly reduced monocyte/macrophage homing to atherosclerotic plaques (236 ± 77 versus 177 ± 43 macrophages, P < 0.03); an even more striking inhibition was found at 3200 mg/kg troglitazone (344 ± 76 versus 172 ± 83 macrophages, P < 0.005).

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factor (TNF-α) at 10 ng/mL for 12 hours. Cells were detached with 10 mmol/L EDTA in PBS (without trypsin) and stained with R-phycocerythrin–labeled monoclonal antibodies (Pharmingen) against VCAM-1 (CD106), ICAM-1 (CD54), or PECAM-1 (CD31) with FITC-labeled monoclonal antibodies (R and D) against E-selectin (CD62E) or with the appropriate isotype IgG (phycoerythrin or FITC) as control. Fluorescence intensity of 9000 cells for each sample was quantified by a FACScalibur analyzer (Becton-Dickinson). All experiments were performed in triplicate.

Monocyte/Macrophage Homing to Atherosclerotic Plaques

Monocyte/macrophage homing to the atherosclerotic plaques in vivo was assessed according to a previously published protocol, modified to use a RAW murine macrophage cell line rather than activated peritoneal macrophages. RAW 267.4 cells were incubated for 75 minutes with 2 μM fluorescent microspheres (Molecular Probes). Cells were then injected into the tail vein of apoE knockout mice (10 × 10⁶ cells per mouse). The mice were euthanized after 48 hours. Labeled cells adhering to or present inside the atheromatous plaque were quantified in 200 serial sections covering the first 1 mm of the ascending aorta. To validate RAW cells as monocyte/macrophage surrogates in our animal model, we performed an inhibition experiment by pretreating apoE-deficient mice with monoclonal antibody against integrin-α4 (R1-2, from Pharmingen) 6 hours before injection with RAW cells. Mice (6 to 8 in each group) were randomized to troglitazone administered by gavage (0.5-mL suspension in water 2 times per day) or to vehicle for 7 days before monocyte/macrophage injection and for the following 2 days. Two different doses of troglitazone (400 mg · kg⁻¹ · d⁻¹ or 3200 mg · kg⁻¹ · d⁻¹) were tested in 2 studies.

Statistics

Results are expressed as mean±SD. Monocyte/macrophage homing was analyzed with the Mann-Whitney U test to take into account the small sample size. A value of P<0.05 (2-tailed) was considered significant.

Results

Expression of Adhesion Molecules

HUVECs showed expression of PECAM-1, low expression of ICAM-1, and no detectable VCAM-1 or E-selectin (Figure 1). Treatment with 100 μmol/L of either troglitazone or ciglitazone and 20 μmol/L of 15d-PGJ2 did not induce apoptosis (apoptotic cells <2% in any condition) and did not change baseline expression of adhesion molecules (data not shown). As shown in Figure 1, incubation with TNF-α 10 ng/mL for 12 hours significantly increased the expression of ICAM-1 (A and B), VCAM-1 (C and D), and E-selectin (E and F), with no change in the expression of PECAM-1 (G and H). Pretreatment with troglitazone at 100 μmol/L and with 15d-PGJ2 20 μmol/L decreased the expression of both ICAM-1 (A and B) and VCAM-1 (C and D) almost to baseline levels. In addition, ciglitazone 100 μmol/L (L) decreased expression of VCAM-1 (but not ICAM-1), with an even stronger effect at 200 μmol/L (M). A significant reduction of VCAM-1 expression was also evident with 20 μmol/L of troglitazone (O) and with 5 or 10 μmol/L of 15d-PGJ2 (P and Q), although these doses did not significantly change expression of ICAM-1 (data not shown). Expression of E-selectin (E and F) or PECAM-1 (G and H) was not changed.

The PPARα agonist fenofibrate at 100 μmol/L was associated with a slight reduction of VCAM-1 expression after TNF-α stimulation (J) but did not change the expression of ICAM-1 (I) or E-selectin (data not shown). Other prostaglandins (PGE₂ 20 μmol/L and PGF₁α 20 μmol/L) did not change expression of adhesion molecules (data not shown).

Monocyte/Macrophage Homing

Labeled RAW cells migrated to atherosclerotic plaque, and anti-α4 antibodies marked inhibited this phenomenon (Figure 2). Pretreatment with troglitazone at 400 mg · kg⁻¹ · d⁻¹ for 7 days reduced homing of labeled monocyte/macrophages to atherosclerotic plaques in the aortic root significantly, by 25% (P=0.03, Figure 2). High-dose troglitazone (3200 mg · kg⁻¹ · d⁻¹) was associated with a 50% reduction of monocyte/macrophage homing (P=0.005). Troglitazone treatment did not change serum levels of total cholesterol, triglycerides, or glucose.

Discussion

The present study shows that PPARγ activators markedly decrease expression of adhesion molecules in activated human endothelial cells. Short-term treatment with the PPARγ activator troglitazone also significantly inhibits macrophage homing to atherosclerotic plaques.

PPARγ is expressed in atherosclerotic plaques, and activation of PPARγ inhibits macrophage activation. The antidiabetic drugs thiazolidinediones are specific activators of PPARγ, whereas the natural agonists are still largely unknown. However, natural polyunsaturated fatty acids can activate PPARγ, and 15d-PGJ2 is the most specific (Kᵣ=2.5 μmol/L). PPARγ is expressed by human endothelial cells, including HUVECs. Activation of this receptor can inhibit endothelial cell proliferation and may modulate PAI-1 expression, reduce endothelin-1 production, and induce apoptosis, in particular in serum-free conditions. Activation of PPARγ results in inhibition of the AP-1 and NF-κB pathways that regulate the expression of adhesion molecules by activated endothelial cells. We found that several PPARγ activators inhibit expression of ICAM-1 and VCAM-1 in activated HUVECs. However, as in several previous studies, the response to troglitazone and ciglitazone occurred at concentrations higher than their binding affinity for PPARγ, whereas the effects of 15d-PGJ2 were in a concentration range compatible with its Kᵣ. The reasons for this discrepancy are unclear, and activation of additional pathways cannot be excluded. However, in a recent study using endothelial cells in conditions similar to our experiments (with complete medium including serum), only high concentrations (100 μmol/L) of ciglitazone could activate a PPAR reporter. Thus, it appears that high concentrations of thiazolidinediones are necessary to activate the PPARγ receptor in our experimental conditions.

Although high concentrations of troglitazone and 15d-PGJ2 (but not of ciglitazone) may also activate PPARα, this is unlikely to explain our results, because 5 μmol/L 15d-PGJ2 and 100 μmol/L ciglitazone are not associated with any PPARα activation. Marx et al recently reported that PPARα activators reduced expression of VCAM-1 (but not ICAM-1 and

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Figure 1. Effects of PPARγ activators on TNF-α–induced expression of adhesion molecules in HUVECs. Data are shown as relative fluorescence intensity (on a logarithmic scale, x axis) and number of cells (y axis). The isotype control (broken line) is shown only for ICAM-1 and PECAM-1, because baseline expression of VCAM-1 and E-selectin was similar to isotype (<1% positive cells at baseline). Histograms show expression of adhesion molecules at baseline (dotted line), after 12-hour incubation with TNF-α 10 ng/mL (solid line), and after incubation with TNF+2-hour pretreatment with PPARγ activators troglitazone, 15d-PGJ2, or ciglitazone or with PPARα activator fenofibrate (shaded curve).
E-selectin) by activated human saphenous vein endothelial cells. The same study did not find any significant effect of PPARγ agonists (troglitazone or 15d-PGJ2 10 μmol/L) on the expression of adhesion molecules. Differences in the study design (2-hour pretreatment in our study versus 24-hour) may explain their negative results, because we found that inhibition of VCAM-1 expression by PPARγ agonists was much less evident with 24-hour pretreatment than with a 2-hour pretreatment (data not shown).

We reported previously that in apoE-deficient mice, homing of activated peritoneal macrophages to atherosclerotic plaques is reduced by pretreatment with monoclonal antibodies against ICAM-1 and α4 integrin (the natural ligand for VCAM-1). We modified this model to use a mouse macrophage cell line (RAW 267.4) that does not express significant levels of PPARγ. Troglitazone treatment significantly inhibits its monocyte/macrophage homing, with a 50% reduction at 2 hours with 24-hour pretreatment than with a 2-hour pretreatment (data not shown).

Figure 2. Homing of RAW cells to atherosclerotic plaques in apoE-deficient mice. First experiment: macrophage migration to atherosclerotic plaques was significantly inhibited by a specific monoclonal antibody against integrin α4 (271 ± 34 vs 53 ± 13 macrophages, P < 0.001). Second experiment: inhibition of macrophage migration by 7-day pretreatment with troglitazone 400 mg·kg-1·d-1 (236 ± 77 macrophages in placebo vs 177 ± 43 macrophages in treatment group, P = 0.03). Third experiment: inhibition of migration with troglitazone 3200 mg·kg-1·d-1 (344 ± 76 vs 172 ± 83 macrophages, P = 0.005). Bars indicate average values.

PPARγ activators are used in treatment of type II diabetes, and in a preliminary study, treatment with troglitazone was found to reduce carotid intimal-medial thickness, a marker of early stages of atherosclerosis. However, atherosclerosis is a composite phenomenon involving many different molecular pathways, and further studies are needed to assess the effects of PPARγ activators on the progression of atherosclerosis.

References

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