Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 52, No. 10, 2008
ISSN 0735-1097/08/$34.00
doi:10.1016/j.jacc.2008.04.055

EXPEDITED PUBLICATIONS

The Peroxisome Proliferator-Activated Receptor-γ Agonist Pioglitazone Represses Inflammation in a Peroxisome Proliferator-Activated Receptor-α–Dependent Manner In Vitro and In Vivo in Mice

Gabriela Orasanu, MD,*‡ Ouliana Ziouzenkova, PHD,*‡ Pallavi R. Devchand, PHD,*‡ Vedika Nehra, MS,*‡ Osama Hamdy, MD,†‡ Edward S. Horton, MD,†‡ Jorge Plutzky, MD*‡

Boston, Massachusetts

Objectives Our aim was to investigate if the peroxisome proliferator-activated receptor (PPAR)-γ agonist pioglitazone modulates inflammation through PPARα mechanisms.

Background The thiazolidinediones (TZDs) pioglitazone and rosiglitazone are insulin-sensitizing PPARγ agonists used to treat type 2 diabetes (T2DM). Despite evidence for TZDs limiting inflammation and atherosclerosis, questions exist regarding differential responses to TZDs. In a double-blinded, placebo-controlled 16-week trial among recently diagnosed T2DM subjects (n = 34), pioglitazone-treated subjects manifested lower triglycerides and lacked the increase in soluble vascular cell adhesion molecules (sVCAM)-1 evident in the placebo group. Previously we reported PPARα but not PPARγ agonists could repress VCAM-1 expression. Since both triglyceride-lowering and VCAM-1 repression characterize PPARα activation, we studied pioglitazone's effects via PPARα.

Methods Pioglitazone effects on known PPARα responses—ligand binding domain activation and PPARα target gene expression—were tested in vitro and in vivo, including in wild-type and PPARα-deficient cells and mice, and compared with the effects of other PPARγ (rosiglitazone) and PPARα (WY14643) agonists.

Results Pioglitazone repressed endothelial TNFα-induced VCAM-1 messenger ribonucleic acid expression and promoter activity, and induced hepatic IκBα in a manner dependent on both pioglitazone exposure and PPARα expression. Pioglitazone also activated the PPARα ligand binding domain and induced PPARα target gene expression, with in vitro effects that were most pronounced in endothelial cells. In vivo, pioglitazone administration modulated sVCAM-1 levels and IκBα expression in wild-type but not PPARα-deficient mice.

Conclusions Pioglitazone regulates inflammatory target genes in hepatic (IκBα) and endothelial (VCAM-1) settings in a PPARα-dependent manner. These data offer novel mechanisms that may underlie distinct TZD responses. (J Am Coll Cardiol 2008;52:869–81) © 2008 by the American College of Cardiology Foundation

The increased risk for atherosclerotic complications evident in individuals with type 2 diabetes mellitus (T2DM) has driven interest in the cardiovascular effects of antidiabetic therapies both in use and under development (1,2). The number of insulin resistance-associated abnormalities that also promote atherosclerosis focused attention on the cardiovascular effects of insulin-sensitizing agents (3). In this context, thiazolidinediones (TZDs) held significant promise as insulin sensitizers that lower glucose and reportedly limited atherosclerosis and inflammation in vitro and in vivo in both mice and humans (4). Recently, the TZDs pioglitazone and rosiglitazone have been scrutinized for their possible distinct effects, including those on the cardiovascular system (5).

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TZDs bind to and activate PPARγ, a ligand-activated transcription factor that regulates key metabolic pathways,

From the *Cardiovascular Division, Brigham and Women's Hospital; †Clinical Research Center, Joslin Diabetes Center; and ‡Harvard Medical School, Boston, Massachusetts. Dr. Plutzky has received grant support from the National Institutes of Health (R01 HL071745). The clinical trial presented here was supported by Takeda Pharmaceuticals (Dr. Horton, primary investigator). The investigators have received prior funding from GlaxoSmithKline and Takeda Pharmaceuticals, although not specifically for the support of the pre-clinical murine studies included here.

Manuscript received March 10, 2008; revised manuscript received April 22, 2008, accepted April 29, 2008.

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Abbreviations and Acronyms

ACO = acyl-CoA oxidase
BAEC = bovine aortic endothelial cells
EC = endothelial cell
FPG = fasting plasma glucose
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
HbA_{1c} = hemoglobin A_{1c}
HDL = high-density lipoprotein
HSVEC = human endothelial cells isolated from saphenous vein
LBD = ligand binding domain
LDL = low-density lipoprotein
LPL = lipoprotein lipase
LPS = lipopolysacharide
PPAR = peroxisome proliferator-activated receptor
TG = triglyceride(s)
TNF = tumor necrosis factor
TZD = thiazolidinedione
T2DM = type 2 diabetes mellitus
VCAM = vascular cell adhesion molecule
2h-OGTT = 2-h plasma glucose ≥200 mg/dl during oral glucose tolerance testing

including adipogenesis and insulin sensitivity (6,7). Pioglitazone and rosiglitazone are approved as "highly selective" peroxisome proliferator-activated receptor (PPAR)-γ agonists (8). PPARγ is also expressed in vascular and inflammatory cells where its activation can regulate target genes relevant to atherosclerosis (9). Although pioglitazone and rosiglitazone target the same PPAR isoform (4), recent human TZD data have raised the possibility of variable responses between these agents as well as with other PPARγ agonists. In several studies, including one head-to-head clinical trial, pioglitazone and rosiglitazone have had variable as well as divergent effects on triglycerides (TG) (10,11). Meta-analysis data have suggested possible adverse cardiovascular outcomes with rosiglitazone (12–14), although not without controversy (15,16); similar studies with pioglitazone have not shown cardiovascular safety signals, including one prospective clinical trial (13,17). Novel dual PPARα/γ agonists in development have been abandoned for various adverse effects, including cardiovascular responses (12), raising concerns about dual PPARα/γ therapeutics (18,19). Clearly, additional mechanistic insight into how specific PPAR agonists exert their effects is needed.

During a small 16-week study on inflammatory markers in patients with recently diagnosed T2DM randomized to pioglitazone or placebo, we noted that levels of soluble vascular cell adhesion molecule (sVCAM)-1, an early player in atherogenesis, increased in the placebo group but not in the group on pioglitazone (20). Previously we reported that PPARγ activation had no effect on VCAM-1 expression in vitro, although those studies did not include pioglitazone (21). In contrast, PPARα agonists decrease VCAM-1 in a PPARα-dependent manner (22). Although limited in nature, this clinical data raised the hypothesis that pioglitazone might repress inflammation, including VCAM-1 expression, in part via PPARα. Although previously raised as a possibility, we studied pioglitazone effects on known PPARα responses in more definitive models, including PPARα-deficient cells and mice (23). We provide here the first evidence that pioglitazone represses key endothelial and hepatic inflammatory responses in vitro and in vivo in mice in a PPARα-dependent manner.

Methods

Human studies. SUBJECTS. Individuals meeting the American Diabetes Association criteria for T2DM—fasting plasma glucose (FPG) ≥126 mg/dl with a second confirmatory measurement or a 2-h plasma glucose ≥200 mg/dl during oral glucose tolerance testing (2h-OGTT)—were enrolled in a randomized, prospective, double-blinded, placebo-controlled clinical trial (n = 34). The study cohort included subjects with newly diagnosed T2DM or confirmed T2DM on a nonpharmacological dietary intervention for at least 4 weeks before the first study visit; all subjects were either drug-naïve or off any antidiabetic medication for at least 4 weeks. Exclusion criteria included prior TZD or insulin treatment, hemoglobin A_{1c} (HbA_{1c}) ≥10%; FPG ≥260 mg/dl; history of myocardial infarction, unstable angina, cerebral vascular accident, transient ischemic attack, coronary artery bypass graft, or percutaneous transluminal coronary angioplasty; New York Heart Association functional class III or IV congestive heart failure; diastolic blood pressure >100 mm Hg and/or systolic blood pressure >160 mm Hg; total cholesterol >300 mg/dl and/or TG >600 mg/dl; serum creatinine >1.5 mg/dl; current use of systemic corticosteroids, immunosuppressants, or androgens; any severe acute or chronic disease; other medical condition possibly interfering with study participation or assessment of the trial investigational products.

PROTOCOL. Participants, recruited from the Joslin Diabetes Center outpatient clinics or local media advertisement, were randomized 1:1 to either pioglitazone 30 mg tablet or a matching placebo once a day for the first 4 weeks, with a subsequent increase to pioglitazone 45 mg tablet or matched placebo once a day for the next 12 weeks (total 16 week intervention period). Both groups were evaluated at baseline and study conclusion. Patients were instructed on an isocaloric diet (50% carbohydrates, 20% protein, 30% fat) and to maintain their usual physical activity.

LABORATORY EVALUATIONS. All measurements were performed at the Clinical Research Center, Joslin Diabetes Center (24). Plasma sVCAM-1 concentrations in mice and humans were determined in duplicate blinded samples using ELISA (Quantikine, R&D Systems, Minneapolis, Minnesota). Intra-assay variation was less than 10%; the sVCAM-1 detection limit was 0.6 ng/ml. To analyze sVCAM-1 responses in different subgroups, national guideline cutpoint values were used: above and below FPG 126 mg/dl, 2h-OGTT 200 mg/dl, HbA_{1c} 7%, TG 150 mg/dl, low-density lipoprotein (LDL) cholesterol 100 mg/dl, high-density lipoprotein (HDL) cholesterol 40 mg/dl (25,26).

Reagents. Pioglitazone hydrochloride (pioglitazone HCl) was a gift from Takeda Pharmaceuticals North America

(Lincolnshire, Illinois); WY14643 (Biomol, Plymouth Meeting, Pennsylvania); rosiglitazone (BRL49653, GlaxoSmithkline, Research Triangle Park, North Carolina). All media (BioWhittaker, Walkersville, Maryland) contained fungizone, penicillin, streptomycin, and plasma as indicated. Human and murine TNFα were purchased from R&D Systems; Escherichia coli O111:B4 lipopolysacharide (LPS) and 2, 2, 2-tribromoethanol (Sigma-Aldrich, St. Louis, Missouri).

Cell culture. Human endothelial cells isolated from saphenous veins (HSVECs) were cultured in M199 medium, endothelial cell (EC) growth factor, and 5% fetal calf serum (21). Bovine aortic endothelial cells (BAECs) were grown in Dulbecco modified Eagle medium (10% fetal bovine serum, glutamine, penicillin, streptomycin, and fungizone) (27). Tumor necrosis factor (TNF)α stimulations were done at 10 ng/ml. PPARα$^{+/+}$ (129S1/SvImJ) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). PPARα$^{-/-}$ mice (129S4/SvJae) were a generous gift from F. Gonzalez (National Institutes of Health). Murine ECs from 1-month-old PPARα$^{+/+}$ and -α$^{-/-}$ mouse hearts were isolated using double selection with intercellular adhesion molecule 2 and platelet EC adhesion molecule 1 antibodies (BD PharMingen, San Diego, California) bound to Dynabeads (Dynal, Lake Success, New York) as before (28).

Plasmids. Human GAL4-PPARα- or -γ–ligand binding domain (LBD) constructs (pSG5 vector, S. Kliewer, University of Texas Southwestern, Dallas, Texas) were used for transactivation assays. The VCAM-1 promoter construct (755 upstream base pair [bp], T. Collins, Children's Hospital, Boston, Massachusetts) contains the major regulatory elements (AP-1, NF-κB, PPAR) (21). cDNA probes for Northern blotting included human VCAM-1 (2.1kb Kpn/Sphl fragment, G. Garcia-Cardena, Brigham and Women's Hospital, Boston, Massachusetts); mouse full-length PPARα (2kb cDNA fragment); human acyl-CoA-oxidase (ACO; ATCC, Manassas, Virginia); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ATCC).

Transient transfection assays. Standard GAL4-PPAR–LBD assays were performed as before (24-well plates, 2.5 × 10^4 BAECs/well, passage 2 to 5, FuGENE 6 (Roche Diagnostics, Indianapolis, Indiana) (27) in Dulbecco modified Eagle medium (1% delipidated fetal calf serum) using human GAL4-PPARα or -γ-LBD, pUASx4-TK-luc, and β-galactosidase (pcDNA-β-Gal) constructs before stimulation (24 h later) with the compounds indicated for 16 h. For VCAM-1 promoter studies, BAECs were plated in 1% Nutridoma SP (Roche Diagnostics), transfected with the VCAM-1 promoter construct (24 h), and then pre-treated with pioglitazone (3 to 30 μM) or WY14643 (25 to 225 μM, 3 h) before TNFα stimulation (12 h). Responses were normalized to co-transfected β-galactosidase (pcDNA3) activity using chlorophenol red-β-D-galactopyranoside substrate (Roche Diagnostics) as before (27). For PPARα reconstitution experiments, murine PPARα$^{-/-}$ ECs were plated in 1% delipidated fetal calf serum before transfection with either PPARα (mouse full-length PPARα-pSG5) or empty vector (pSG5, 24 h). Cells were pre-treated with pioglitazone (10 μM) or WY14643 (100 μM) for 18 h before mouse TNFα (10 h) stimulation as indicated.

Ribonucleic acid extraction and Northern blot analysis. Total ribonucleic acid was isolated using RNeasy (Qiagen, Valencia, California) before gel separation and transfer (Hybond-N, Amersham Pharmacia, Piscataway, New Jersey). Northern and Western blots were quantitated using densitometry (Image-Pro Plus 5.1 software).

Western blotting. Standard Western blot analysis of human EC lysates were performed using rabbit polyclonal antibody against human IκBα (1:500, Santa Cruz Biotechnology, Santa Cruz, California) and monoclonal antibody against GAPDH (1:10,000, Biodesign, Saco, Maine). For in-vivo studies, frozen livers were pulverized, added to RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with freshly added protease inhibitors, centrifuged (10', 13,000 rpm, 4°C), and the protein extract boiled in electrophoresis buffer before gel separation (15% polyacrylamide, β-mercaptoethanol-reducing conditions) and transfer (Immobilon-P membranes, semi-dry transfer, 1 h, 16 V). After blocking (5% delipidated milk, 20 mM Tris, 55 mM NaCl, 0.1% Tween 20, 1 h), immunoblotting with the IκBα and GAPDH antibodies described in the previous text was performed using chemiluminescence (PerkinElmer Life Sciences, Boston, Massachusetts).

Animal studies. The 3-month-old age- and gender-matched PPARα$^{+/+}$ and PPARα$^{-/-}$ mice were divided into 2 feeding groups (n = 9/genotype). Group One received pioglitazone (20 mg/kg body weight in 0.5% w/v methylcellulose) by gavage once daily for 7 days. Group Two was treated similarly with vehicle (0.5% w/v methylcellulose). Mice received free access to water, ordinary laboratory diet, and standard animal care (Harvard University guidelines). On Day 1, mice received a survival dose of anesthetic (2, 2, 2—tribromoethanol, 250 mg/kg body weight) intraperitoneally before retro-orbital blood draw for baseline serum measurements. On Day 8, retro-orbital blood draws were repeated on a random subgroup of mice (n = 5). The next day, all mice received LPS (12 mg/kg body weight) intraperitoneally; 4 h later, mice were anesthetized and blood drawn by vena cava puncture and allowed to clot overnight (4°C). Mice were then sacrificed and livers removed immediately, rinsed (0.9% NaCl), and snap-frozen for further analysis. Blood samples were centrifuged (2,000 × *g*, 4°C, 20') and serum sVCAM-1 concentrations determined in duplicate as described in the preceding text.

Statistical analysis. Statistical analysis, performed in conjunction with the Brigham and Women's Hospital Center for Clinical Research Biostatistics Core Laboratory, employed Statistical Package for Social Sciences (version 16.0, SPSS Inc., Chicago, Illinois), SAS 9.1 (SAS Institute, Cary, North Carolina), and Analyze-it for Microsoft Excel (version 1.71). Results are presented as mean ± SD or mean ±

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Table 1 Subject Characteristics at Baseline and After 16 Weeks of Placebo or Pioglitazone

	Placebo (n = 15)			Pioglitazone (n = 19)		
	Baseline	16 Weeks	p Value	Baseline	16 Weeks	p Value
Age, yrs	58.8 ± 11			61.3 ± 5.9		
Men/women	9/6			16/3		
BMI, kg/m^2	31.6 ± 3.1	31.09 ± 3	NS	31.9 ± 3.7	32.1 ± 3.5	NS
Weight, kg	94.1 ± 9.7	93.3 ± 9.1	NS	95.8 ± 13.7	96.6 ± 12	NS
Waist, cm	108.8 ± 7.8			108.8 ± 9.7		
SBP, mm Hg	125.5 ± 15.7			129.3 ± 13.3		
DBP, mm Hg	81.7 ± 9.7			77.4 ± 8.7		
FPG, mg/dl	146.4 ± 10.3	140.9 ± 7.7	NS	162.2 ± 13.6	125.4 ± 7.1	0.002
2h-OGTT, mg/dl	263.3 ± 13.8	265.6 ± 15.2	NS	273.5 ± 19	216.3 ± 12.6	0.001
Total cholesterol, mg/dl	197.4 ± 9.6	177.3 ± 10.9	NS	203.8 ± 9.9	169.2 ± 7.4	NS
LDL-C, mg/dl	119 ± 9.1	88.4 ± 7.5	NS	128.4 ± 8.3	103.1 ± 7	NS
HDL-C, mg/dl	47 ± 1.7	35 ± 1.9	0.001	45.9 ± 2.1	41.4 ± 2.1	NS
TG, mg/dl	169.8 ± 15.3	150.3 ± 16.1	NS	160.7 ± 24.9	129.1 ± 11.4	0.008
TG/HDL ratio	3.5 ± 0.3	4.6 ± 0.6	NS	3.5 ± 0.5	3.1 ± 0.2	0.02
sVCAM-1, ng/ml	512.1 ± 45.7	600.5 ± 41.7	0.008	470.4 ± 33.9	486.7 ± 45.7	NS
hsCRP, mg/l	1.3† (0.44–3.89)	1.3† (0.49–89.3)	NS*	2.6† (0.25–23.8)	0.8† (0.17–8.75)	NS*
TNFα, ng/ml	1.5 ± 0.09	1.8 ± 0.1	NS	1.3 ± 0.08	1.2 ± 0.08	NS

Data are shown as mean ± standard error with 95% confidence interval or medial (†). No baseline differences in any variables (independent Student *t* test); $p < 0.05$ shows significant differences within group (paired Student *t* test); Median test (*) between groups pre- or post-treatment.

BMI = body mass index; DBP = diastolic blood pressure; FPG = fasting plasma glucose; HDL-C = high-density lipoprotein cholesterol; hsCRP = high sensitive C-reactive protein; LDL-C = low-density lipoprotein cholesterol; OGTT = oral glucose tolerance test; SBP = systolic blood pressure; sVCAM = soluble vascular cell adhesion molecule; TG = triglycerides; TNF = tumor necrosis factor.

SE. Means for baseline clinical characteristics of the human study participants were compared using the independent Student *t* test. For within-group analysis (the baseline study vs. the follow-up assessment), 2-sided paired Student *t* test for parametric data was used. For all other among-group comparisons, the Mann-Whitney *U* test was used. Pearson correlation coefficients were calculated to test the association between variables. A value of $p \leq 0.05$ was regarded as significant.

Results

Changes in sVCAM-1 on pioglitazone versus placebo in recently diagnosed T2DM subjects. Pioglitazone (n = 19) and placebo (n = 15) groups were similarly matched on all baseline variables, including sVCAM-1 levels (Table 1). Pioglitazone significantly improved FPG (162.2 ± 13.6 vs. 125.4 ± 7.1 mg/dl, p = 0.002), 2h-OGTT (273.5 ± 19 vs. 216.3 ± 12.6, p = 0.001), TG (160.7 ± 24.9 vs. 129.1 ± 11.4, p = 0.008), and TG/HDL ratio (3.5 ± 0.5 vs. 3.1 ± 0.2, p = 0.02), all as compared with placebo at baseline versus study end (Table 1).

During the study, sVCAM-1 levels rose significantly in patients with recently diagnosed T2DM randomized to placebo alone (baseline 512.1 ± 45.7 ng/ml vs. study conclusion 600.5 ± 41.7 ng/ml, $p < 0.008$, within-group analysis) (Table 1). In contrast, sVCAM-1 levels did not rise among pioglitazone-treated subjects (baseline 470.4 ± 33.9 vs. conclusion 486.7 ± 45.7 ng/ml, p = NS, within-group analysis) (Table 1). Using a mixed design linear regression model to control for baseline levels of multiple parameters, only age had a significant impact on sVCAM-1 levels (Online Table A). After controlling for age, sVCAM-1 levels differed significantly between placebo and pioglitazone groups (p = 0.03) (Online Table B). TNFα levels also increased over time from 1.5 ± 0.09 to 1.8 ± 0.1 ng/ml in the placebo group but decreased from 1.3 ± 0.08 to 1.2 ± 0.08 ng/ml in the pioglitazone group, although not in a statistically significant way. Baseline levels of high-sensitivity C-reactive protein and sVCAM-1 were also significantly correlated (r = 0.45, p = 0.02).

To generate hypotheses as to biologic mechanisms underlying possible pioglitazone effects on repressing the sVCAM-1 increase seen in patients on placebo, responses were analyzed according to the intervention arm and subgroups stratified by accepted TG, HDL cholesterol, LDL cholesterol, FPG, and HbA_{1c} cutpoints (see Methods section). Only TG subgroups revealed differences in sVCAM-1 levels. Using the National Cholesterol Education Program TG cutpoint of 150 mg/dl (29), significant sVCAM-1 increases were restricted to those with higher baseline TG levels (≥150 mg/dl, n = 9; from baseline 506 ± 63.9 ng/ml to 683.1 ± 56.4 ng/ml, $p < 0.03$); sVCAM-1 levels did not differ significantly in placebo-treated subjects with lower baseline TG (<150 mg/dl, n = 6). Among pioglitazone-treated subjects, sVCAM-1 levels did not differ in either higher or lower TG subgroups (data not shown).

Pioglitazone represses TNFα-induced VCAM-1 messenger ribonucleic acid (mRNA) expression in ECs. Given the data above, we tested pioglitazone's effects on TNFα-induced VCAM-1 mRNA expression in HSVECs (18 h pre-treatment) using a concentration range commonly

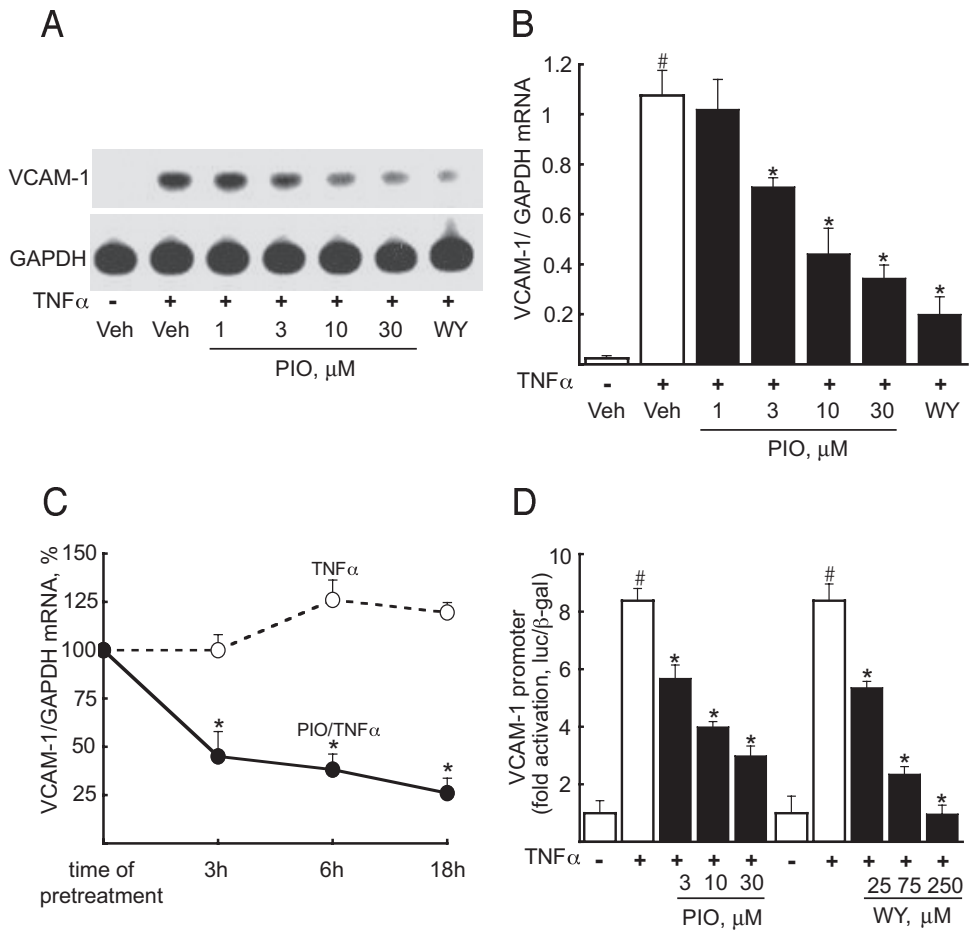

Figure 1 **Pioglitazone Reduces TNFα-Induced VCAM-1 mRNA Expression in a Dose- and Time-Dependent Manner in HSVECs**

(A) Northern blot analysis of tumor necrosis factor (TNF)α-induced vascular cell adhesion molecule (VCAM)-1 messenger ribonucleic acid (mRNA) expression was performed on human saphenous vein endothelial cells (HSVECs) pre-treated in the absence or presence of pioglitazone (PIO) (18 h) at the concentrations shown before TNFα stimulation (10 ng/ml, 10 h). The effects of the PPARα agonist WY14643 (100 μM) are provided for comparison. One representative Northern blot (n = 3) is shown. **(B)** The effect of the pioglitazone concentrations on VCAM-1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was quantified from the Northern blots seen in panel A (n = 3, $^{\#}p < 0.05$, TNFα-induced vs. vehicle, $^{*}p < 0.05$, pioglitazone/TNFα vs. TNFα alone, Mann-Whitney U test). **(C)** The time-dependent effects of pioglitazone exposure (10 μM) on TNFα-induced VCAM-1 expression was tested in HSVECs using Northern blotting. Results are shown as a percent of the TNFα effect alone at 3 h, mean ± SD (n = 3; $^{*}p < 0.05$). **(D)** The effect of pioglitazone versus vehicle on the human VCAM-1 promoter transiently transfected into bovine aortic endothelial cells before TNFα stimulation are shown **(left)**. For comparison, the effect of the PPARα agonist WY14643 on the VCAM-1 promoter is also shown **(right)**. All responses were normalized to β-galactosidase (pCMV-β-Gal) (n = 3 per each treatment, $^{\#}p < 0.05$ TNFα vs. vehicle; $^{*}p < 0.05$ pioglitazone or WY14643 vs. TNFα alone, Mann-Whitney U test).

used in vascular biology studies which overlap pioglitazone levels reported in humans (30). Pioglitazone inhibited VCAM-1 mRNA induction in a dose-dependent manner (Fig. 1A). For comparison, the known repression of VCAM-1 mRNA by the PPARα agonist WY14643 (100 μM) is also shown (Fig. 1A). Quantification of relative changes in VCAM-1/GAPDH mRNA expression using densitometry reveals a significant pioglitazone effect at the concentrations shown (Fig. 1B) (3 to 30 μM, $p < 0.05$ for each). As previously reported, rosiglitazone (BRL49653, BRL) had no significant effect on VCAM-1 expression (data not shown) (21,31–33). Pioglitazone-mediated repression of VCAM-1 expression also varied as a function of pioglitazone exposure (3, 6, 18 h; 10 μM) before TNFα stimulation (maximal 74% reduction at 18 h, $p < 0.05$) (Fig. 1C).

We next considered if pioglitazone could inhibit human VCAM-1 promoter activity, as reported for synthetic PPARα agonists (20). A human VCAM-1 promoter-luciferase construct was transiently transfected into BAECs before testing pioglitazone (3 to 30 μM) effects on TNFα-induced VCAM-1 promoter-driven luciferase activity. As expected, TNFα stimulation significantly induced VCAM-1 promoter activity (8.37 ± 0.58-fold, $p < 0.05$) (Fig. 1D). Pioglitazone repressed TNFα-induced VCAM-1 promoter activity across a dose range ($p < 0.05$) (Fig. 1D); responses to the PPARα agonist WY14643 are shown for comparison (Fig. 1D).

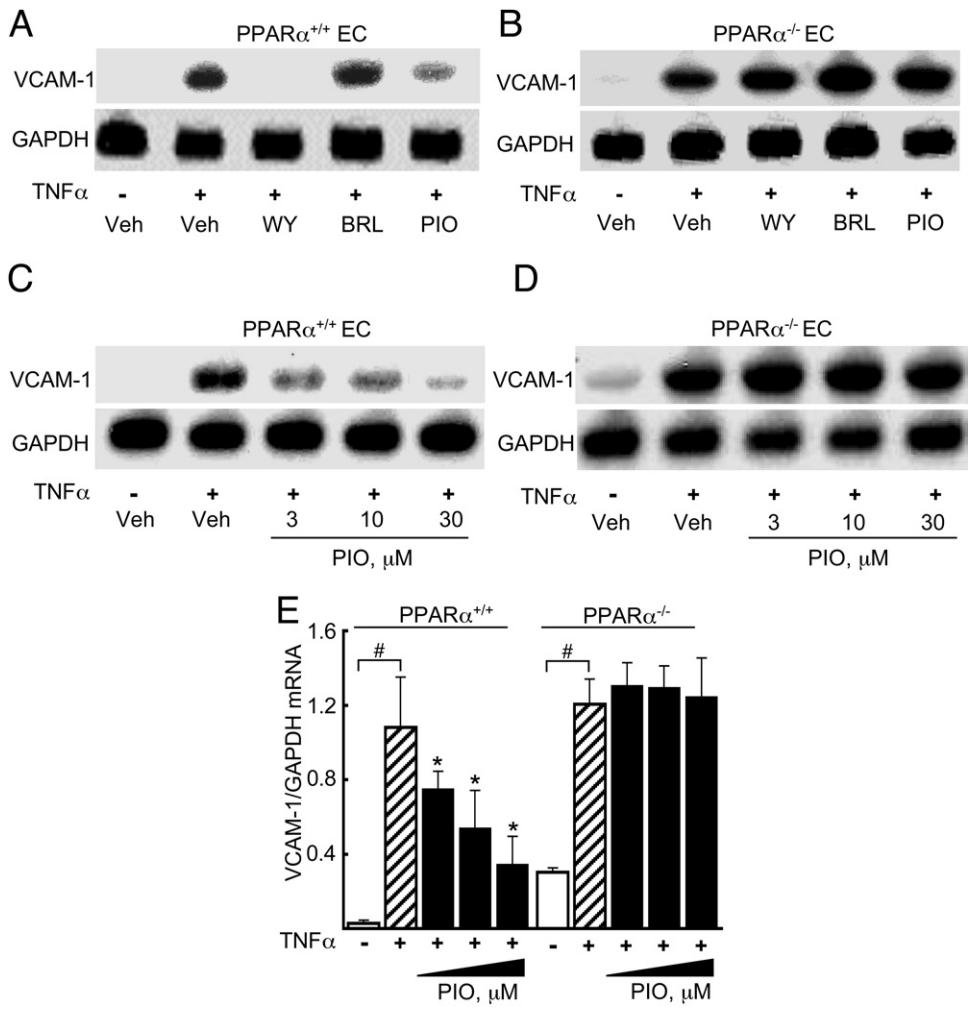

Figure 2 **Pioglitazone Represses TNFα-Induced VCAM-1 Expression in a PPARα-Dependent Manner**

Endothelial cells (ECs) isolated from PPARα$^{+/+}$ **(A)** and PPARα$^{-/-}$ **(B)** mouse hearts were pre-treated with WY14643, rosiglitazone (BRL), or pioglitazone at the concentrations shown (18 h) before mouse TNFα stimulation and subsequent to Northern blotting for VCAM-1 mRNA and GAPDH expression. One representative blot of 3 is shown. Northern blotting for VCAM-1 expression was repeated in the presence of the dose range of pioglitazone shown in EC from PPARα$^{+/+}$ **(C)** and PPARα$^{-/-}$ **(D)** mice. **(E)** Quantification of the effects of pioglitazone on VCAM-1 mRNA in PPARα$^{+/+}$ and PPARα$^{-/-}$ EC relative to GAPDH mRNA expression (n = 3, $^{\#}p < 0.05$ TNFα vs. vehicle; $^{*}p < 0.05$ pioglitazone/TNFα vs. TNFα alone, Mann-Whitney U test). Abbreviations as in Figure 1.

Pioglitazone regulation of VCAM-1 mRNA expression in the genetic presence or absence of PPARα. We next considered if pioglitazone's effects on VCAM-1 required the genetic presence of PPARα. In Northern blots of microvascular ECs isolated from either wild-type (PPARα$^{+/+}$) or PPARα-deficient (PPARα$^{-/-}$) mouse hearts, WY14643 (100 μM) and pioglitazone (10 μM) pre-treatment decreased VCAM-1 mRNA expression in PPARα$^{+/+}$ but not in PPARα$^{-/-}$ ECs while BRL (1 μM) had no effect in either PPARα$^{+/+}$ or PPARα$^{-/-}$ ECs (Figs. 2A and 2B), as seen in Northern blotting. Pioglitazone significantly decreased TNFα-induced VCAM-1 mRNA expression in a dose-dependent manner (3 to 30 μM, 18 h) in wild-type ECs (as compared with TNFα stimulation alone, $p < 0.05$) (Fig. 2C) but not in PPARα$^{-/-}$ ECs (Fig. 2D). Quantification of these responses (n = 3) demonstrated a significant VCAM-1 effect at each pioglitazone dose tested in wild-type but not PPARα-deficient ECs ($p < 0.05$) (Fig. 2E).

Reconstitution of PPARα expression in PPARα-deficient ECs. To test if reconstituting PPARα expression in PPARα-deficient ECs was sufficient to restore pioglitazone-mediated repression of VCAM-1 expression, PPARα$^{-/-}$ ECs were transiently transfected (24 h) with a full-length mouse PPARα cDNA (pSG5 expression vector) and compared with cells transfected with the pSG5 vector alone before pre-treatment with pioglitazone (10 μM, 18 h) or WY14643 (100 μM, 18 h) and TNFα stimulation. Expressing PPARα in PPARα$^{-/-}$ ECs restored significant pioglitazone-induced repression of cytokine-induced VCAM-1 expression (Fig. 3A) while transfection of the

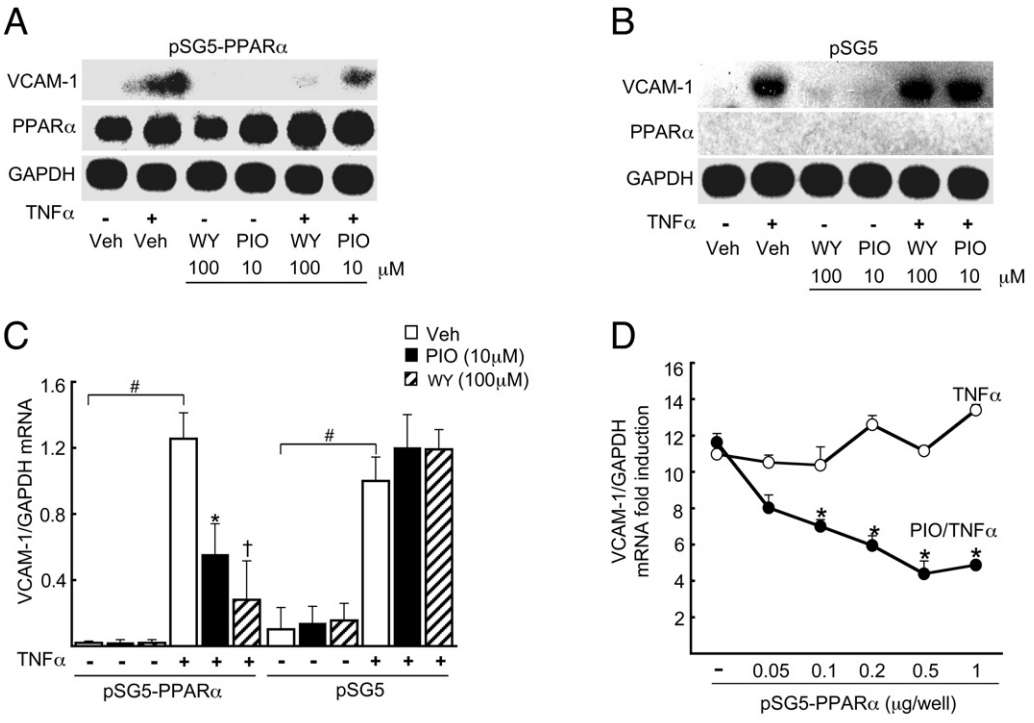

Figure 3 **PPARα Is Required for Pioglitazone-Mediated Repression of TNFα-Induced Endothelial VCAM-1 mRNA Expression**

Northern blot analysis was performed on total RNA isolated from PPARα$^{-/-}$ endothelial cells transfected either with PPARα-containing pSG5 overexpression vector **(A)** or pSG5 alone **(B)** before stimulation with TNFα in either the absence or presence of WY (100 μM) or pioglitazone (10 μM), with subsequent probing for VCAM-1 or GAPDH expression. **(C)** Quantification of the VCAM-1 mRNA response to pioglitazone relative to GAPDH mRNA expression levels (n = 3, #p < 0.05 TNFα vs. vehicle; *p < 0.05 pioglitazone/TNFα vs. TNFα alone; †p < 0.05 WY14643/TNFα vs. TNFα alone, Mann-Whitney U test). **(D)** Cells were transfected in **panels A and B** but with a concentration gradient of pSG5-PPARα as shown before TNFα stimulation in either the absence or presence of pioglitazone 10 μM (n = 3, *p < 0.05 pioglitazone/TNFα vs. TNFα alone). Abbreviations as in Figure 1.

pSG5 vector alone into PPARα$^{-/-}$ ECs had no effect on pioglitazone responses (Fig. 3B), as evident on densitometry ($p < 0.05$) (Fig. 2C). These results indicate that PPARα expression is necessary for pioglitazone-mediated repression of TNFα-induced endothelial VCAM-1 mRNA expression.

Pioglitazone effects on expression of canonical PPARα-regulated target genes. We next asked if pioglitazone also regulated expression in ECs of two other well-established PPARα-regulated targets: acyl-CoA oxidase (ACO) and IκBα. ACO contains a defined PPARα response element in its upstream promoter region (34). Pioglitazone (3 to 30 μM) and the PPARα agonist WY14643 (100 μM, 6 h) significantly increased ACO mRNA expression compared with untreated HSVECs (Fig. 4A). Prior reports indicate that PPARα activation increases expression of IκBα, a key regulator of inflammation (34). HSVECs were pre-treated with pioglitazone (30 μM) or WY (100 μM, 16 h) either alone or before TNFα stimulation before Western blotting. Both WY14643 and pioglitazone increased IκBα protein levels in HSVECs (Fig. 4B). TNFα stimulation further increased the IκBα response to pioglitazone, as previously reported for PPARα agonists (Fig. 4B) (35).

Pioglitazone's dependency on PPARα for VCAM-1 repression and its induction of PPARα-regulated target genes suggests that pioglitazone or one of its metabolites might activate PPARα. Prior studies considering this issue have varied considerably, with experiments in multiple cell types using PPAR-LBDs from different species (36). Standard Gal4-LBD transfection assays were performed using human PPARα-LBD transfected into BAECs before pioglitazone (0.01 to 100 μM) stimulation. In BAECs, pioglitazone activated the PPARα-LBD significantly and in a dose-dependent manner (1 to 100 μM) (Fig. 4C). These effects were significant although less than the PPARγ activation seen with pioglitazone; rosiglitazone had no effects on PPARα activation (data not shown). Given cell type contributions to variable PPAR-LBD responses previously reported, we compared pioglitazone effects on human PPARα-LBD assays in NIH/3T3 (fibroblast), HEK293 (epithelial), and Hep-G2 (hepatic) cell lines using either pioglitazone or WY14643 (both 10 μM), normalizing responses to β-galactosidase (pcDNA-βGal) activity. PPARα-LBD activation by either WY14643 or pioglitazone varied significantly according to cell type (Fig. 4D). Interestingly, pioglitazone's PPARα-LBD effects were

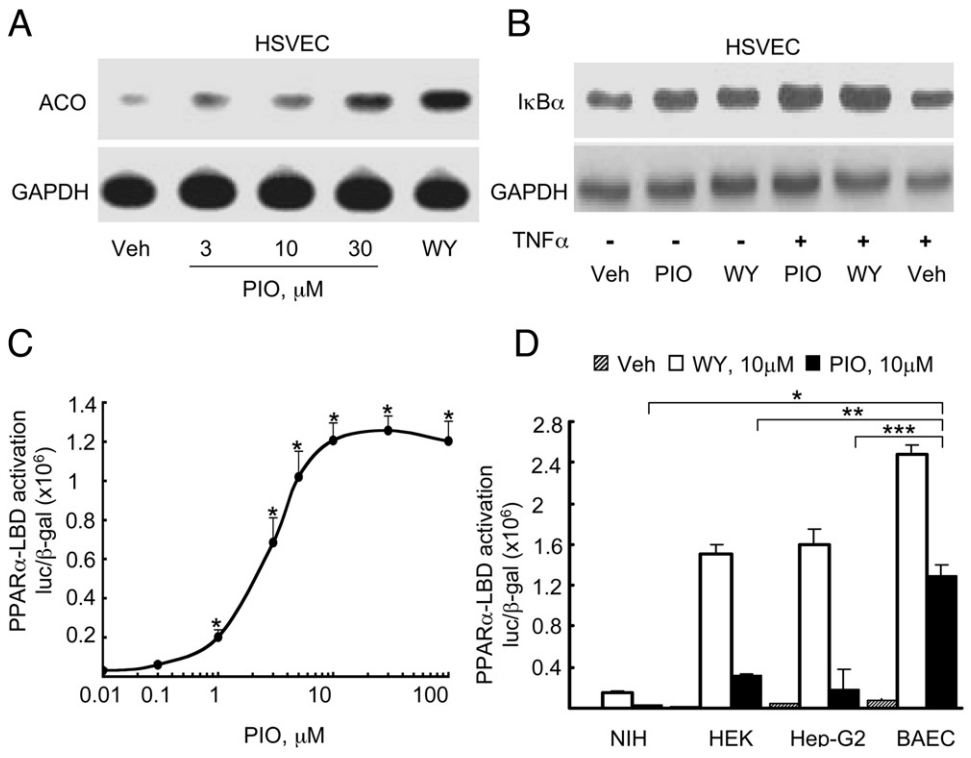

Figure 4 **Pioglitazone Induces Known PPARα Target Gene Expression and PPARα-LBD Activation in ECs**

(A) Northern blot analysis in HSVECs was performed for the PPARα target gene acyl-CoA-oxidase (ACO) and compared with GAPDH in HSVEC pre-treated (16 h) with pioglitazone or WY14643 at the concentrations shown before TNFα stimulation. **(B)** Western blot analysis for IκBα expression was performed on total protein extracts (50 μg) from HSVECs treated with either pioglitazone (10 μM) or WY14643 (250 μM) before stimulation with human TNFα. **(C)** Standard LBD activation assays were performed in bovine aortic endothelial cells stimulated with pioglitazone at the concentrations shown (0.01 to 100 μM). **(D)** PPARα-ligand binding domain (LBD) assays were done as before but responses were compared in NIH/3T3 (fibroblasts), HEK293 (human kidney epithelial), Hep-G2 (hepatic), and bovine aortic endothelial cell lines before stimulation with pioglitazone or WY14643 (both 10 μM). Values are expressed as luciferase/β-Gal activity mean ± SD (n = 3, *p < 0.05 bovine aortic endothelial cells vs. NIH/3T3, **vs. HEK293, ***vs. Hep-G2, both Student *t* and Mann-Whitney U tests). Abbreviations as in Figure 1.

most potent (relative to WY14643) in bovine ECs (52%) compared with all other non-EC cell lines tested: 17% in NIH/3T3 17%, HEK293 21%, and Hep-G2 11% (all $p < 0.05$) (Fig. 4D). Thus, PPAR responses to pioglitazone may vary depending on cell type.

Pioglitazone's PPARα-dependent effects on hepatic IκBα protein levels in vivo. We next considered if pioglitazone regulated PPARα responses in a PPARα-dependent manner in vivo. Given the TG and sVCAM-1 effects shown here (Table 1), we focused on pioglitazone responses in tissues related to lipid metabolism (liver) and inflammation (endothelium), and relevant PPARα-regulated inflammatory target genes in those settings, namely IκBα and VCAM-1. PPARα$^{+/+}$ (n = 4) and PPARα$^{-/-}$ (n = 4) mice were treated with pioglitazone (gavage, 20mg/kg body weight, 7 days) before harvesting livers and performing IκBα Western blotting. Consistent with our in vitro results, pioglitazone significantly increased hepatic IκBα protein expression in PPARα$^{+/+}$ (Fig. 5A) but not PPARα$^{-/-}$ (Fig. 5B) mice, as evident on densitometry ($p < 0.05$) (Fig. 5C)

Pioglitazone's PPARα-dependent effect on sVCAM-1 levels in vivo. We next tested if pioglitazone repressed sVCAM-1 levels in mice in vivo in a PPARα-dependent manner. PPARα$^{+/+}$ and PPARα$^{-/-}$ mice (9 mice/genotype/treatment) were treated (daily gavage, 7 days) with either pioglitazone (20 mg/kg body weight, 0.5% w/v methylcellulose, Group One) or vehicle alone (Group Two) before establishing baseline sVCAM-1 levels followed by LPS intraperitoneal stimulation and blood draws. Basal sVCAM-1 levels were significantly higher in PPARα$^{-/-}$ mice (847.4 ± 75.1 ng/ml, n = 18) versus PPARα$^{+/+}$ mice (680.8 ± 42.4 ng/ml, n = 18), $p < 0.007$ (Fig. 6). As expected, LPS treatment increased sVCAM-1 levels significantly in vehicle-treated PPARα$^{+/+}$ mice (1,058.11 ± 32.15 ng/ml, n = 9, $p < 0.002$). In contrast, LPS-induced sVCAM-1 levels in pioglitazone-treated PPARα$^{+/+}$ mice were unchanged from basal levels (697.55 ± 33.78 ng/ml, n = 9, $p < 0.01$, vs. LPS alone, n = 9) (Fig. 6). In PPARα$^{-/-}$ mice, pioglitazone had no effect on LPS-induced sVCAM-1 protein levels (pioglitazone, 1,034.8 ± 84.8 ng/ml vs. vehicle, 1,008.5 ± 62.3 ng/ml, n = 9) (Fig. 6).

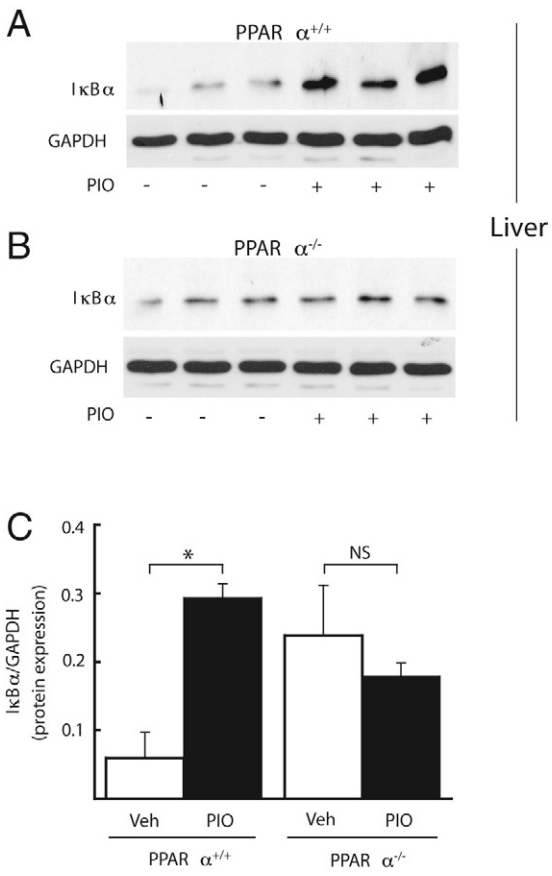

Figure 5 **Pioglitazone Induces IκBα Protein Expression In Vivo in a PPARα-Dependent Manner**

PPARα$^{+/+}$ **(A)** and PPARα$^{-/-}$ **(B)** mice were treated with pioglitazone (20 mg/kg, 7 days via gavage) before livers were harvested and total protein extracted for Western blot analysis of IκBα and glyceraldehyde-3-phosphate dehydrogenase protein levels. Each lane represents a single mouse. **(C)** The effects of pioglitazone **(solid bars)** and vehicle **(open bars)** on IκBα protein expression were quantified and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Mean values ± SD are shown (n = 4 mice/group. *p < 0.05 pioglitazone vs. vehicle, Mann-Whitney U test).

Discussion

We present here pre-clinical data that the reportedly PPARγ-specific agonist pioglitazone represses inflammatory responses involving endothelial VCAM-1 and hepatic IκBα in a PPARα-dependent manner both in vitro and in vivo in mice. Indeed, reconstituting PPARα expression in PPARα-deficient ECs restored pioglitazone-mediated inhibition of TNFα-induced VCAM-1 expression. To the best of our knowledge, this is the first demonstration that defined pioglitazone responses in vivo require the presence of PPARα. Studies on the role of PPARα in pioglitazone responses were prompted by our clinical observations that subjects receiving pioglitazone did not demonstrate the progressive increase in sVCAM-1 levels seen in a small cohort of patients with recently diagnosed T2DM receiving placebo alone. Repression of cytokine-induced VCAM-1 expression and TG-lowering are well-established PPARα responses in humans (37,38). Pre-clinical evidence presented here that pioglitazone at concentrations overlapping those reported in vivo can activate the PPARα-LBD and induce expression of PPARα target genes suggests pioglitazone may directly or indirectly influence PPARα responses, as previously suggested. Interestingly, PPARα-LBD responses to pioglitazone varied considerably among cell types and species, with the greatest PPARα activation evident in EC. This variability may have contributed to pioglitazone's characterization as being PPARγ-specific (36). Here we have extended prior observations by demonstrating that specific pioglitazone effects in vivo are absent in the PPARα-deficient mouse. Together these findings have potential implications for TZD mechanisms of action, interpreting TZD studies, especially in pre-clinical models as well as the development of novel PPAR therapeutic agents.

Agonists for the same PPAR isoform can differ significantly in their biologic and clinical effects. In transcriptional profiling and proteomic assays, different PPARγ agonists have both shared and distinct gene expression patterns (39–41). Clinically, both pioglitazone and rosiglitazone lack the irreversible liver failure seen with troglitazone, the first clinically approved PPARγ agonist (42). In a head-to-head clinical trial, pioglitazone decreased TG significantly while rosiglitazone did not (11), as also suggested by meta-analysis data (43). Although the contribution of dif-

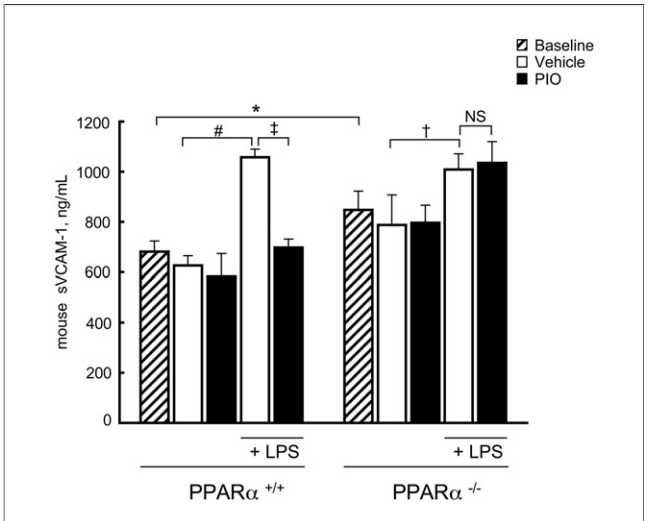

Figure 6 **Pioglitazone Decreases LPS-Induced Soluble VCAM-1 in PPARα$^{+/+}$ But Not PPARα$^{-/-}$ Mice In Vivo**

PPARα$^{+/+}$ and PPARα$^{-/-}$ mice were treated with pioglitazone or vehicle alone before lipopolysaccharide (LPS) injection (n = 9/genotype as in Methods section). Soluble vascular adhesion molecule-1 (sVCAM-1) levels in PPARα$^{+/+}$ and PPARα$^{-/-}$ mice are shown at baseline (*p < 0.007 PPARα$^{-/-}$ vs. PPARα$^{+/+}$ mice) and after LPS injection in mice treated with either vehicle or pioglitazone (PIO) (PPARα$^{+/+}$, n = 9, $^{\#}$p < 0.002 LPS/vehicle vs. vehicle; †p < 0.01 pioglitazone/LPS vs. vehicle/LPS) and in PPARα$^{-/-}$ mice (n = 9, †p < 0.05 vehicle/LPS vs. vehicle; [p = NS] nonsignificant pioglitazone/LPS vs. vehicle/LPS, significance determined using Mann-Whitney U test). The mean serum sVCAM-1 concentration of each group ± SD is shown.

ferential TZD effects to cardiovascular events remains unclear, our data identify an additional biologic mechanism that may be involved in pioglitazone responses. Considerable evidence establishes TZDs as limiting inflammation and atherosclerosis in mouse models (45). In clinical studies, both pioglitazone and rosiglitazone lower C-reactive protein (38,46–49), anatomic indicators like carotid intimal medial thickness (49–53), and vessel reactivity (54). In the prospective PROactive (PROspective pioglitAzone Clinical Trial In macroVascular Events) study, pioglitazone did not decrease a large, combined primary cardiovascular end point although a secondary clinical end point of stroke, myocardial infarction, and cardiovascular death was improved (55). Various logistical factors have been speculated as contributors to the study's negative primary end point (4). Recently, in the PERISCOPE (Pioglitazone Effect on Regression of Intravascular Sonographic Coronary Obstruction Prospective Evaluation) trial, progression of atherosclerosis was seen on coronary intravascular ultrasound in otherwise well-treated patients randomized to glimiperide but not in those receiving pioglitazone (56). Pioglitazone significantly decreased TG and raised HDL in both the PROactive and PERISCOPE trials. No large prospective clinical cardiovascular rosiglitazone trial data is currently available. Recent meta-analyses have raised concern over a possible increase in cardiovascular risk with rosiglitazone (12–14,55), although with limitations in this data as raised by the authors and others (15,55). In similar meta-analyses, no increased risk with pioglitazone was found (13). Given changes induced by TZDs on surrogate markers for cardiovascular disease in basic and clinical studies, it remains possible that offsetting adverse cardiovascular effects could exist with these agents. PPARα activation can increase homocysteine levels and serum creatinine levels (57–60). While the VA-HIT (Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial) study did show decreased cardiovascular events with the putative PPARα agonist gemfibrozil, in the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study, fenofibrate, a more potent PPARα agonist, did not show a difference in the primary cardiovascular end point (61,62). Although the role of pioglitazone-mediated PPARα activation in determining clinical responses remains unclear, our data suggest at the very least that PPARα activation should be considered in interpreting basic science data with this agent.

Both VCAM-1 and IκBα regulate inflammatory responses in atherosclerosis. Endothelial VCAM-1 expression is an important early atherogenic step (2). Circulating levels of VCAM-1 may predict subsequent clinical cardiovascular events (63) while VCAM-1 may be elevated in T2DM, perhaps as a result of the hypertriglyceridemia and/or low HDL cholesterol (35,64,65). Reducing TG levels with fibrates or fish oil, both of which can be considered PPARα activators (66,67), reportedly decreases soluble adhesion molecule levels (35,67,68). VCAM-1 expression is controlled by multiple pathways, including NF-κB and PPARα (69,70). In vitro, VCAM-1 repression has been reported by some PPARγ agonists but not others, and at drug concentrations that may have PPARγ-independent effects (31–33). Although we found PPARα agonists repressed VCAM-1 in wild-type but not PPARα-deficient ECs, rosiglitazone and 15d-PGJ2 had no VCAM-1 effects (21). Differences among reports of PPARγ agonist VCAM-1 effects may involve differences among agents or the cell types under study.

NF-κB activation, which is inhibited by IκBα, increases VCAM-1 expression. PPARα activation induces IκBα (35). Here we found that pioglitazone increased IκBα expression in a PPARα-dependent manner in HSVECs in vitro and in liver in vivo; 15 deoxyprostaglandin J2 and troglitazone increase IκBα expression but independent of PPARγ (71–73). The more selective PPARγ ligand rosiglitazone did not change IκBα expression in human monocyte/macrophages (73,74). In contrast, pioglitazone reportedly increased IκBα levels in peripheral mononuclear cells in human subjects (75). These results are potentially consistent with pioglitazone exerting effects through PPARα as also suggested by studies in which pioglitazone treatment increased expression of PPARα target genes in subcutaneous fat (44). The possibility that these responses derived from pioglitazone activation of PPARα was not discussed (44).

PPAR biology suggests several mechanisms for how agonists for the same PPAR isotype might exert distinct effects. PPARs have a particularly large LBD, even as compared with other nuclear receptors (76). PPAR activation induces a conformational change in the AF2 domain, which allows coactivator recruitment, corepressor release, and formation of the heterodimeric PPAR-RXR complex. These critical determinants of transcriptional responses can vary as a function of different interactions between structurally distinct PPARγ agonists and the large PPAR-LBD (76). Interestingly, other in vitro pharmacologic studies also suggest pioglitazone may activate PPARα (36). Recently, ligand-independent mechanisms influencing PPAR-mediated anti-inflammatory effects have been reported, for example through SUMOylation (Small Ubiquitin-related Modifier) (77,78). Differences among PPAR-interacting molecules underlie the concept of selective nuclear receptor modulators, as has been raised for the estrogen receptor and novel PPAR agents in development (79,80). The potential variability among specific PPAR-interacting molecules is apparent in the reports of full agonists, selective partial agonists, inverse agonists, antagonists, as well as pan-PPAR and dual PPAR agonists (80–83).

Dual PPARα/γ agonists offered the putative clinical benefits of combining HDL-raising/TG-lowering via PPARα with improved insulin sensitivity through PPARγ (80). No dual PPARα/γ agonists have yet been approved for use (84). Muraglitazar and tesaglitazar reached late stage testing before being abandoned due to adverse effects, including increased cardiovascular events (85,86), raising concerns over dual PPARα/γ agonists as a drug class. Given the evidence that pioglitazone can be used safely (13), our

data that pioglitazone may act, at least in part, through PPARα suggest that selective modulators targeting both PPARα and -γ may be able to be safely developed. Moreover, our findings suggest pioglitazone's description as a PPARγ-specific agonist may need revisiting. Further studies would need to include analysis of pioglitazone's metabolites with biologic activity but unknown PPAR selectivity (87,88). Pioglitazone metabolite production may differ depending on cell types, tissues, species, or genetic variants. Although pioglitazone demonstrates more potent LBD activation of PPARγ than PPARα, the PPARα-LBD activation seen is within a range that could influence biologic responses, especially in EC. LBD activation may also underestimate functional PPAR effects in vivo as a result of mechanisms such as preferential generation, stabilization, or transport of a specific drug metabolite.

Pioglitazone could also regulate PPARα target genes indirectly, for example altering PPARα regulatory proteins or inducing the formation of endogenous PPARα agonists. For example, lipoprotein lipase (LPL), a positively regulated PPARγ target gene, can generate PPARα ligands through VLDL hydrolysis (22). Increased LPL expression and activity would be associated with lower TG and higher HDL, as occurs with gain of function LPL polymorphisms and after treatment with synthetic PPARα agonists (66,89). Prior work identifies increased LPL-mediated lipolysis as a contributor to pioglitazone's TG-lowering effects (90). In clinical studies, pioglitazone induces LPL expression and also decreases the natural LPL inhibitor apoCIII (44,90), further supporting possible indirect PPARα activation through increased VLDL hydrolysis. Since both LPL and apoCIII are PPARα-regulated target genes, positive feed-forward mechanisms may amplify these effects (22,91).

Independent of a direct or indirect mechanism, PPARα is required in order for VCAM-1 repression and IκBα induction to occur in vitro and in vivo in mice, which expands potential mechanisms of action for this agent, at least in vitro and in mouse models. This data also underscores the need to fully understand the effects of both existing and emerging PPAR agonists and their biologically active metabolites. Indeed, the complexity of PPAR biology, the number of variables dictating transcriptional and, hence, clinical responses, and the fact that agonist structure can determine biologic response argues that the notion of general PPAR-activating drug classes may be limited.

Acknowledgment

The authors thank Ruzena Tupy for excellent editorial assistance.

Reprint requests and correspondence: Dr. Jorge Plutzky, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, NRB 742, Boston, Massachusetts 02115. E-mail: jplutzky@rics.bwh.harvard.edu.

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Key Words: inflammation ■ VCAM-1 ■ PPAR.

APPENDIX

For a supplementary table on the impact of various baseline parameters on sVCAM-1 changes and the sVCAM-1 levels in pioglitazone versus placebo groups after controlling for age, as well as a supplementary figure on Northern blot analysis of PPARα mRNA expression and the quantification of PPARα expression levels by quantitative RT-PCR after concentration-dependent PPARα transfection, please see the online version of this article.