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The PPAR γ Agonist Pioglitazone Represses Inflammation In A PPAR α -Dependent Manner In Vitro and In Vivo In Mice

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Abstract

OBJECTIVES—To investigate if the PPAR γ agonist pioglitazone modulates inflammation through PPAR α mechanisms.

BACKGROUND—The thiazolidinediones (TZDs) pioglitazone and rosiglitazone are insulin-sensitizing PPAR γ 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 manifest lower triglycerides (TG) and lacked the increase in soluble VCAM-1 (sVCAM-1) evident in the placebo group. Previously we reported PPAR α but not PPAR γ agonists could repress VCAM-1 expression. Since both TG-lowering and VCAM-1 repression characterize PPAR α activation, we studied pioglitazone's effects via PPAR α .

METHODS—Pioglitazone effects on known PPAR α responses - ligand binding domain (LBD) activation and PPAR α target gene expression - were tested in vitro and in vivo, including in wildtype and PPAR α -deficient cells and mice, and compared to other PPAR γ (rosiglitazone) and PPAR α (WY14643) agonists.

RESULTS—Pioglitazone repressed endothelial TNF α -induced VCAM-1 mRNA expression and promoter activity, and induced hepatic I κ B α in a manner dependent on both pioglitazone exposure and PPAR α expression. Pioglitazone also activated the PPAR α LBD and induced PPAR α target gene expression, with in vitro effects that were most pronounced in endothelial cells. In vivo, pioglitazone administration modulated sVCAM-1 levels and I κ B α expression in wildtype but not PPAR α -deficient mice.

CONCLUSIONS—Pioglitazone regulates inflammatory target genes in hepatic (I κ B α) and endothelial (VCAM-1) settings in a PPAR α -dependent manner. This data offers novel mechanisms that may underlie distinct TZD responses.

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Keywords

Inflammation; VCAM-1; PPARs

INTRODUCTION

The increased risk for atherosclerotic complications evident in individuals with type 2 diabetes mellitus (T2DM) has driven interest in the cardiovascular effects of anti-diabetic 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).

TZDs bind to and activate PPAR γ , a ligand-activated transcription factor that regulates key metabolic pathways, including adipogenesis and insulin sensitivity (6,7). Pioglitazone and rosiglitazone are approved as “highly selective” PPAR γ agonists (8). PPAR γ 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 has raised the possibility of variable responses between these agents and as well as with other PPAR γ agonists. In several studies, including one head-to-head clinical trial, pioglitazone and rosiglitazone have had variable as well as divergent effects on triglycerides (10,11). Meta-analysis data has 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 (13,54). Novel dual PPAR α/γ agonists in development have been abandoned for various adverse effects, including cardiovascular responses (12), raising concerns about dual PPAR α/γ therapeutics (17,18). 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-1 (VCAM-1), an early player in atherogenesis, increased in the placebo group but not those on pioglitazone (19). Previously we reported that PPAR γ activation had no effect on VCAM-1 expression in vitro, although those studies did not include pioglitazone (20). In contrast, PPAR α agonists decrease VCAM-1 in a PPAR α -dependent manner (20,21). Although limited in nature, this clinical data raised the hypothesis that pioglitazone might repress inflammation, including VCAM-1 expression, in part via PPAR α . Although previously raised as a possibility, we studied pioglitazone effects on known PPAR α responses in more definitive models, including PPAR α -deficient cells and mice (22). We provide here the first evidence that pioglitazone represses key endothelial and hepatic inflammatory responses in vitro and in vivo in mice in a PPAR α -dependent manner.

METHODS

Human studies

Subjects—Individuals meeting the American Diabetes Association criteria for T2DM - fasting plasma glucose (FPG) \geq 126 mg/dL with a second confirmatory measurement or a 2 hour plasma glucose \geq 200 mg/dL during oral glucose tolerance testing (2 h-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 non-pharmacological dietary intervention for at least 4 weeks prior to the first study visit; all subjects were either drug-naïve or off any anti-diabetic medication for at least 4 weeks. Exclusion criteria included prior TZD or insulin treatment, hemoglobin A1c (HbA1c) \geq 10%; FPG \geq 260 mg/dL; history of myocardial infarction, unstable angina, cerebral vascular accident, transient ischemic attack, coronary artery bypass graft (CABG) or percutaneous transluminal coronary angioplasty; NYHA class III or IV congestive heart failure; diastolic blood pressure $>$ 100 mmHg and/or systolic blood pressure $>$ 160 mmHg; total cholesterol $>$ 300 mg/dL and/or triglycerides (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 or a matching placebo once a day for the first 4 weeks, with a subsequent increase to pioglitazone 45 mg 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 (23). Plasma sVCAM-1 concentrations in mice and humans were determined in duplicate blinded samples using ELISA (Quantikine, R&D Systems). 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, HbA1c 7%, TG 150 mg/dL, LDL-cholesterol (LDL-C) 100 mg/dL, HDL-C 40 mg/dL (24,25).

Reagents

Pioglitazone hydrochloride (Pioglitazone HCl) was a gift from Takeda Pharmaceuticals North America (Lincolnshire, IL); WY14643 (Biomol, Plymouth Meeting, PA); rosiglitazone (BRL49653, GlaxoSmithkline, Research Triangle Park, NC). All media (BioWhittaker, Walkersville, MD), contained fungizone, penicillin, streptomycin and plasma as indicated. Human and murine TNF α were purchased from R&D Systems (Minneapolis, MN); Escherichia coli O111:B4 lipopolysaccharide (LPS) and 2, 2, 2-tribromoethanol (Sigma-Aldrich, St. Louis, MO).

Cell Culture

Human ECs isolated from saphenous veins (HSVEC) were cultured in M199 medium, endothelial cell growth factor (ECGF), 5% fetal calf serum (20). Bovine Aortic Endothelial Cells (BAEC) were grown in Dulbecco modified Eagle medium (DMEM, 10% fetal bovine serum, glutamine, penicillin, streptomycin and fungizone) (26). TNF α stimulations were done at 10 ng/ml. PPAR $\alpha^{+/+}$ (129S1/SvImJ) mice were obtained from Jackson Laboratories (Bar Harbor, ME). PPAR $\alpha^{-/-}$ mice (129S4/SvJae) were a generous gift from F. Gonzalez (National Institute of Health). Murine ECs from 1-month-old PPAR $\alpha^{+/+}$ and PPAR $\alpha^{-/-}$ mouse hearts were isolated using double selection with intercellular adhesion molecule 2 and platelet endothelial cell adhesion molecule 1 antibodies (BD PharMingen) bound to Dynabeads (DynaL, Lake Success, NY) as before (27).

Plasmids

Human GAL4-PPAR α - or PPAR γ - ligand binding domain (LBD) constructs (pSG5 vector, S. Kliewer, University of Texas Southwestern, Dallas, TX) were used for trans-activation assays. The VCAM-1 promoter construct (755 upstream bp, T. Collins, Children's Hospital, Boston, MA) contains the major regulatory elements (AP-1, NF- κ B, PPAR) (20). cDNA probes for Northern blotting included human VCAM-1 (2.1kb Kpn/SphI fragment, G. Garcia-Cardena, Brigham and Women's Hospital, Boston, MA); mouse full-length PPAR α (2kb cDNA fragment); human acyl-CoA-oxidase (ACO; ATCC, Manassas, VA) 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 2–5, FuGENE 6 (Roche Diagnostics, Indianapolis, IN) (26) in DMEM (1% delipidated fetal calf serum) using human GAL4-PPAR α or γ -LBD, pUASx4-TK-luc, and β -galactosidase (pcDNA- β -Gal) constructs before stimulation (24h later) with the compounds indicated for 16h. For VCAM-1 promoter studies, BAECs were plated in 1% Nutridoma SP (Roche Diagnostics), transfected with the VCAM-1 promoter construct (24h), and then pre-treated with pioglitazone (3 – 30 μ M) or WY14643 (25 – 225 μ M, 3h) before TNF α stimulation (12 h). Responses were normalized to co-transfected β -Galactosidase (pcDNA3) activity using chlorophenol red- β -D-galactopyranoside substrate (Roche Diagnostics) as before (26). For PPAR α reconstitution experiments, murine PPAR α ^{-/-} ECs were plated in 1% delipidated fetal calf serum before transfection with either PPAR α (mouse full-length PPAR α -pSG5) or empty vector (pSG5, 24 h). Cells were pre-treated with pioglitazone (10 μ M) or WY14643 (100 μ M) for 18 h before mouse TNF α (10 h) stimulation as indicated.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated using RNeasy (Qiagen, Valencia, CA) before gel separation and transfer (Hybond-N, Amersham Pharmacia, Piscataway, NJ). Northern and Western blots were quantitated using densitometry (Epson Scan 1200, Image-Pro Plus 5.1).

Western Blotting

Standard Western blot analysis of human EC lysates were performed using rabbit polyclonal antibody against human I κ B α (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibody against GAPDH (1:10,000, Biodesign, Saco, ME). 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% SDS) 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, 1h, 16V). After blocking (5% delipidated milk, 20 mM Tris, 55 mM NaCl, 0.1% Tween 20, 1 h), immunoblotting with the I κ B α and GAPDH antibodies described above was performed using chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

Animal studies

3 month-old age- and sex-matched PPAR α ^{+/+} and PPAR α ^{-/-} mice were divided into two 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 seven 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 One, mice received a survival dose of anesthetic (2, 2, 2 – tribromoethanol, 250 mg/kg body weight) intraperitoneally before retroorbital blood draw for baseline serum measurements. On Day

Eight, retroorbital 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; four hours 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 (2000 × g, 4°C, 20') and serum sVCAM-1 concentrations determined in duplicate as described above.

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 (SPSS, v.16.0), SAS 9.1, and Analyze-it for Microsoft Excel (v1.71). Results are presented as mean ± SD or mean ± SE. Means for baseline clinical characteristics of the human study participants were compared using the independent-Student's *t* test. For within-group analysis (the baseline study vs. the follow-up assessment), two-sided paired Student's *t* test for parametric data was used. For all other among-group comparisons, the Mann-Whitney test was used. Pearson correlation coefficients were calculated to test the association between variables. $p \leq 0.05$ was regarded as significant.

RESULTS

Changes in sVCAM-1 on pioglitazone vs 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). 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 to placebo at baseline versus study end (Table).

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). In contrast, sVCAM-1 levels did not rise among pioglitazone-treated subjects (baseline 470.4 ± 32.3 vs. conclusion 486.7 ± 43.3 ng/mL, ns, within group analysis, Table). Using a mixed design linear regression model to control for baseline levels of multiple parameters, only age had a significantly impact on sVCAM-1 levels (See Supplementary Table A). After controlling for age, sVCAM-1 levels differed significantly between placebo and pioglitazone groups ($p = 0.03$, Supplementary Table B). TNF α 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 hs-CRP 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 intervention arm and subgroups stratified by accepted TG, HDL-C, LDL-C, FPG, and HbA1c cutpoints (see Methods). Only TG subgroups revealed differences in sVCAM-1 levels. Using the National Cholesterol Education Program TG cutpoint of 150 mg/dL (28), 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 TNF α -induced VCAM-1 mRNA expression in endothelial cells

Given the data above, we tested pioglitazone's effects on TNF α -induced VCAM-1 mRNA expression in HSVECs (18 h pre-treatment) using a concentration range commonly used in vascular biology studies and that overlaps pioglitazone levels reported in humans (29). Pioglitazone inhibited VCAM-1 mRNA induction in a dose-dependent manner (Fig. 1A). For comparison, the known repression of VCAM-1 mRNA by the PPAR α agonist WY14643 (100 μ M) 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, 3–30 μ M, $p < 0.05$ for each). As previously reported, rosiglitazone (BRL49653, BRL) had no significant effect on VCAM-1 expression (data not shown) (20,30–32). Pioglitazone-mediated repression of VCAM-1 expression also varied as a function of pioglitazone exposure (3, 6, 18 h; 10 μ M) prior to TNF α 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 PPAR α agonists (20). A human VCAM-1 promoter-luciferase construct was transiently transfected into bovine aortic ECs (BAECs) before testing pioglitazone (3–30 μ M) effects on TNF α -induced VCAM-1 promoter-driven luciferase activity. As expected, TNF α stimulation significantly induced VCAM-1 promoter activity (8.37 ± 0.58 fold, $p < 0.05$, Fig. 1D). Pioglitazone repressed TNF α -induced VCAM-1 promoter activity across a dose range ($p < 0.05$, Fig. 1D); responses to the PPAR α agonist WY14643 are shown for comparison (Fig. 1D).

Pioglitazone regulation of VCAM-1 mRNA expression in the genetic presence or absence of PPAR α

We next considered if pioglitazone's effects on VCAM-1 required the genetic presence of PPAR α . In Northern blots of microvascular ECs isolated from either wildtype (PPAR $\alpha^{+/+}$) or PPAR α -deficient (PPAR $\alpha^{-/-}$) mouse hearts, WY14643 (100 μ M) and pioglitazone (10 μ M) pretreatment decreased VCAM-1 mRNA expression in PPAR $\alpha^{+/+}$ but not in PPAR $\alpha^{-/-}$ ECs while BRL (1 μ M) had no effect in either PPAR $\alpha^{+/+}$ or PPAR $\alpha^{-/-}$ ECs (Fig 2A, B), as seen in Northern blotting. Pioglitazone significantly decreased TNF α -induced VCAM-1 mRNA expression in a dose-dependent manner (3 – 30 μ M, 18 h) in wildtype EC (as compared to TNF α stimulation alone, $p < 0.05$, Fig. 2C) but not in PPAR $\alpha^{-/-}$ 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 PPAR α deficient EC ($p < 0.05$, Fig. 2E).

Reconstitution of PPAR α expression in PPAR α -deficient endothelial cells

To test if reconstituting PPAR α expression in PPAR α -deficient ECs was sufficient to restore pioglitazone-mediated repression of VCAM-1 expression, PPAR $\alpha^{-/-}$ ECs were transiently transfected (24 h) with a full-length mouse PPAR α cDNA (pSG5 expression vector) and compared to cells transfected with the pSG5 vector alone before pretreatment with pioglitazone (10 μ M, 18 h) or WY14643 (100 μ M, 18 h) and TNF α stimulation. Expressing PPAR α in PPAR $\alpha^{-/-}$ ECs restored significant pioglitazone-induced repression of cytokine-induced VCAM-1 expression (Fig. 3A) while transfection of the pSG5 vector alone into PPAR $\alpha^{-/-}$ ECs had no effect on pioglitazone responses (Fig. 3B), as evident on densitometry ($p < 0.05$, Fig. 2C). These results indicate that PPAR α expression is necessary for pioglitazone-mediated repression of TNF α -induced endothelial VCAM-1 mRNA expression.

Pioglitazone effects on expression of canonical PPAR α -regulated target genes

We next asked if pioglitazone also regulated expression in ECs of two other well-established PPAR α -regulated targets: acyl-CoA oxidase (ACO) and I κ B α . ACO contains a defined

PPAR α response element in its upstream promoter region (33). Pioglitazone (3–30 μ M) and the PPAR α agonist WY14643 (100 μ M, 6 h) significantly increased ACO mRNA expression compared to untreated HSVECs (Fig. 4A). Prior reports indicate that PPAR α activation increases expression of I κ B α , a key regulator of inflammation (34). HSVECs were pretreated with pioglitazone (30 μ M) or WY (100 μ M, 16 h) either alone or before TNF α stimulation prior to Western blotting. Both WY14643 and pioglitazone increased I κ B α protein levels in HSVEC (Fig. 4B). TNF α stimulation further increased the I κ B α response to pioglitazone, as previously reported for PPAR α agonists (Fig. 4B) (34).

Pioglitazone's dependency on PPAR α for VCAM-1 repression and its induction of PPAR α -regulated target genes suggests that pioglitazone or one of its metabolites might activate PPAR α . Prior studies considering this issue have varied considerably, with experiments in multiple cell types using PPAR-LBDs from different species (35). Standard Gal4-LBD transfection assays were performed using human PPAR α -LBD transfected into BAECs before pioglitazone (0.01–100 μ M) stimulation. In BAECs, pioglitazone activated the PPAR α -LBD significantly and in a dose-dependent manner (1–100 μ M Fig. 4C). These effects were significant although less than the PPAR γ activation seen with pioglitazone; rosiglitazone had no effects on PPAR α activation (data not shown). Given cell type contributions to variable PPAR-LBD responses previously reported, we compared pioglitazone effects on human PPAR α -LBD assays in NIH/3T3 (fibroblast), HEK293 (epithelial), and Hep-G2 (hepatic) cell lines using either pioglitazone or WY14643 (both 10 μ M), normalizing responses to β -galactosidase (pcDNA- β Gal) activity. PPAR α -LBD activation by either WY14643 or pioglitazone varied significantly according to cell type (Fig. 4D). Interestingly, pioglitazone's PPAR α -LBD effects were 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 PPAR α -dependent effects on hepatic I κ B α protein levels in vivo

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

Pioglitazone's PPAR α -dependent effect on sVCAM-1 levels in vivo

We next tested if pioglitazone repressed sVCAM-1 levels in mice in vivo in a PPAR α -dependent manner. PPAR $\alpha^{+/+}$ and PPAR $\alpha^{-/-}$ 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 PPAR $\alpha^{-/-}$ mice (847.4 ± 75.1 ng/mL, n = 18) versus PPAR $\alpha^{+/+}$ 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 PPAR $\alpha^{+/+}$ mice (1058.11 ± 32.15 ng/mL, n=9, $p < 0.002$). In contrast, LPS-induced sVCAM-1 levels in pioglitazone-treated PPAR $\alpha^{+/+}$ 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 PPAR $\alpha^{-/-}$ mice, pioglitazone had no effect on LPS-induced sVCAM-1 protein levels (pioglitazone, 1034.8 ± 84.8 ng/mL vs. vehicle, 1008.5 ± 62.3 ng/mL, n = 9, Fig. 6).

DISCUSSION

We present pre-clinical data here that the purportedly PPAR γ -specific agonist pioglitazone represses inflammatory responses involving endothelial VCAM-1 and hepatic I κ B α in a PPAR α -dependent manner both in vitro and in vivo in mice. Indeed, reconstituting PPAR α expression in PPAR α -deficient endothelial cells restored pioglitazone-mediated inhibition of TNF α -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 PPAR α . These studies on the role of PPAR α in pioglitazone responses were stimulated by 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 triglyceride-lowering are well-established PPAR α responses in humans (36,37). The pre-clinical evidence presented here that pioglitazone at concentrations overlapping those reported in vivo can activate the PPAR α -LBD and induce expression of PPAR α target genes suggests pioglitazone may directly or indirectly influence PPAR α responses, as previously suggested. Interestingly, PPAR α -LBD responses to pioglitazone varied considerably among cell types and species, with the greatest PPAR α activation evident in EC. This variability may have contributed to pioglitazone's characterization as being PPAR γ -specific (29). Here we have extended prior observations by demonstrating that specific pioglitazone effects in vivo are absent in the PPAR α -deficient mouse. Together these findings have potential implications for TZD mechanisms of action, interpreting TZD studies, especially in preclinical 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 PPAR γ agonists have both shared and distinct gene expression patterns (38–40). Clinically, both pioglitazone and rosiglitazone lack the irreversible liver failure seen with troglitazone, the first clinically-approved PPAR γ agonist (41). In a head-to-head clinical trial, pioglitazone decreased TG significantly while rosiglitazone did not (11), as also suggested by meta-analysis data (42). Although the contribution of differential TZD effects to cardiovascular events remains unclear, our data identifies an additional biologic mechanism that may be involved in pioglitazone responses. Considerable evidence establishes TZDs as limiting inflammation and atherosclerosis in mouse models (44). In clinical studies, both pioglitazone and rosiglitazone lower C-reactive protein (37,45–48), anatomic indicators like carotid intimal medial thickness (48–52) and vessel reactivity (53). In the prospective PROactive study, pioglitazone did not decrease a large, combined primary cardiovascular endpoint although a secondary clinical endpoint of stroke, MI and CV death was improved (54). Various logistical factors have been speculated as contributors to the study's negative primary endpoint (4). Recently, in the PERISCOPE trial, progression of atherosclerosis was seen on coronary intravascular ultrasound in otherwise well-treated patients randomized to glimiperide but not in those receiving pioglitazone (91). Pioglitazone significantly decreased triglycerides and raised HDL in both PROactive and PERISCOPE. No large prospective clinical cardiovascular rosiglitazone trial data is 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. PPAR α activation can increase homocysteine levels and serum creatinine levels (56–59). While the VA-HIT study did show decreased cardiovascular events with the putative PPAR α agonist gemfibrozil, in FIELD, fenofibrate, a more potent PPAR α agonist, did not show a difference in the primary cardiovascular endpoint (60,61). Although the role of pioglitazone-mediated PPAR α activation

in determining clinical responses remains unclear, our data suggests at the very least that PPAR α activation should be considered in interpreting basic science data with this agent.

Both VCAM-1 and I κ B α 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 (62) while VCAM-1 may be elevated in T2DM, perhaps as a result of the hypertriglyceridemia and/or low HDL-C (34,63,64). Reducing TG levels with fibrates or fish oil, both of which can be considered PPAR α activators (67,68), reportedly decrease soluble adhesion molecule levels (32,65,66). VCAM-1 expression is controlled by multiple pathways, including NF- κ B and PPAR α (69). In vitro, VCAM-1 repression has been reported by some PPAR γ agonists but not others, and at drug concentrations that may have PPAR γ -independent effects (30–32). Although we found PPAR α agonists repressed VCAM-1 in wildtype but not PPAR α -deficient EC, rosiglitazone and 15d-PGJ2 had no VCAM-1 effects (20). Differences among reports of PPAR γ agonist VCAM-1 effects may involve differences among agents or the cell types under study.

NF- κ B activation, which is inhibited by I κ B α , increases VCAM-1 expression. PPAR α activation induces I κ B α (34). Here we found that pioglitazone increased I κ B α expression in a PPAR α -dependent manner in HSVECs in vitro and in liver in vivo. 15' deoxy prostaglandin J2 and troglitazone increase I κ B α expression but independent of PPAR γ (70–72). In contrast, the more selective PPAR γ ligand rosiglitazone did not change I κ B α expression in human monocyte/macrophages (72,73). In contrast, pioglitazone reportedly increased I κ B α levels in peripheral mononuclear cells in human subjects (74). These results are potentially consistent with pioglitazone exerting effects through PPAR α as also suggested by studies in which pioglitazone treatment increased expression of PPAR α target genes in subcutaneous fat (43). The possibility that these responses derived from pioglitazone activation of PPAR α was not discussed (43).

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 to other nuclear receptors (75). PPAR activation induces a conformational change in the AF2 domain, which allows co-activator recruitment, co-repressor 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 PPAR γ agonists and the large PPAR LBD (75). Interestingly, other in vitro pharmacologic studies also suggest pioglitazone may activate PPAR α (35). Recently, ligand-independent mechanisms influencing PPAR-mediated anti-inflammatory effects have been reported, for example through SUMOylation (76,77). Differences among PPAR-interacting molecules underlie the concept of selective nuclear receptor modulators, as exist for the estrogen receptor and novel PPAR agents in development (78,79). 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 (79–82).

Dual PPAR α / γ agonists offered the putative clinical benefits of combining HDL-raising/TG-lowering via PPAR α with improved insulin sensitivity through PPAR γ (79). No dual PPAR α / γ agonists have been approved for use (83). Muraglitazar and tesaglitazar reached late stage testing before being abandoned due to adverse effects, including increased cardiovascular events (84,85), raising concerns over dual PPAR α / 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 PPAR α suggests that selective modulators targeting both PPAR α and PPAR γ can be safely developed. Moreover, our findings suggest pioglitazone's description as a PPAR γ -specific agonist may need revisiting. Such studies would need to include analysis of pioglitazone's multiple metabolites, especially ones with biologic activity but unknown PPAR

selectivity (86,87). Pioglitazone metabolite production may differ depending on cell types, tissues, species, or genetic variants. Although pioglitazone demonstrates more potent LBD activation of PPAR γ than PPAR α , the PPAR α -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 PPAR α target genes indirectly, for example altering PPAR α regulatory proteins or inducing the formation of endogenous PPAR α agonists. For example, lipoprotein lipase (LPL), a positively-regulated PPAR γ target gene, can generate PPAR α ligands through VLDL hydrolysis (21). 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 PPAR α agonists (67,88). Prior work identifies increased LPL-mediated lipolysis as a contributor to pioglitazone's triglyceride-lowering effects (89). In clinical studies, pioglitazone induces LPL expression and also decreases the natural LPL inhibitor apoCIII (43,89), further supporting possible indirect PPAR α activation through increased VLDL hydrolysis. Since both LPL and apoCIII are PPAR α -regulated target genes, positive feed-forward mechanisms may amplify these effects (21,90).

Independent of a direct or indirect mechanism, the requirement for PPAR α in order for VCAM-1 repression and I κ B α induction in vitro and in vivo in mice 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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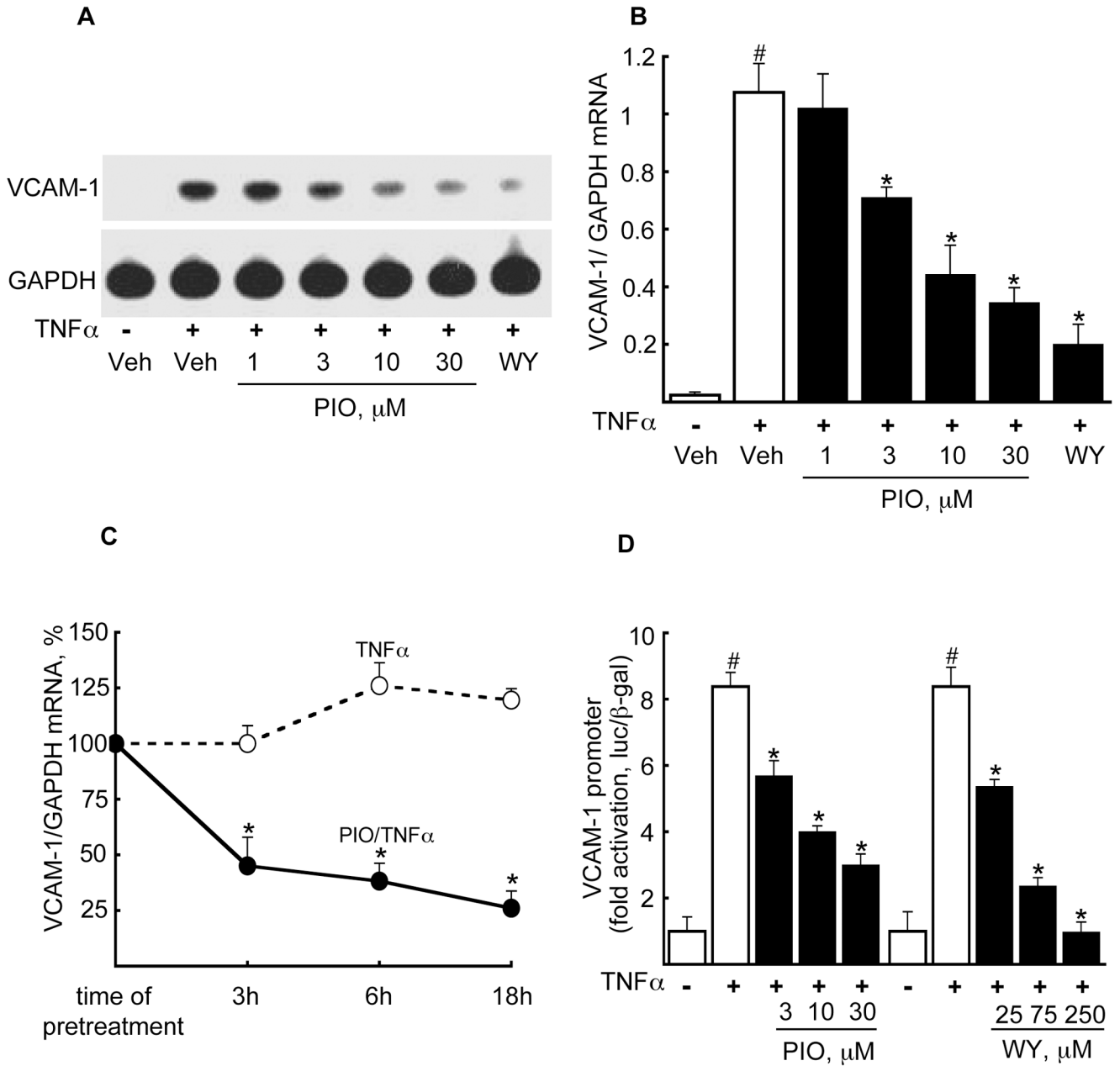


Figure 1. Pioglitazone reduces TNF α -induced VCAM-1 mRNA expression in a dose- and time-dependent manner in human saphenous vein ECs (HSVECs)

(A) Northern blot analysis of TNF α -induced VCAM-1 mRNA expression was performed on HSVECs pretreated in the absence or presence of pioglitazone (18 h) at the concentrations shown prior to TNF α stimulation (10 ng/mL, 10 h). The effects of the PPAR α agonist WY14643 (100 μ M) are provided for comparison. One representative Northern blot (n=3) is shown. (B). The effect of the pioglitazone concentrations on VCAM-1/GAPDH mRNA was quantified from the Northern blots above. (n=3, #p<0.05, TNF α -induced vs. vehicle, *p<0.05, pioglitazone/TNF α vs. TNF α alone, Mann-Whitney test). (C) The time dependent effects of pioglitazone exposure (10 μ M) on TNF α -induced VCAM-1 expression was tested in HSVECs using Northern blotting. Results are shown as a percent of the TNF α 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 BAEC before TNF α stimulation are shown (left). For comparison, the effect of the PPAR α 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 TNF α vs. vehicle; *p<0.05 pioglitazone or WY14643 vs. TNF α alone, Mann-Whitney test).

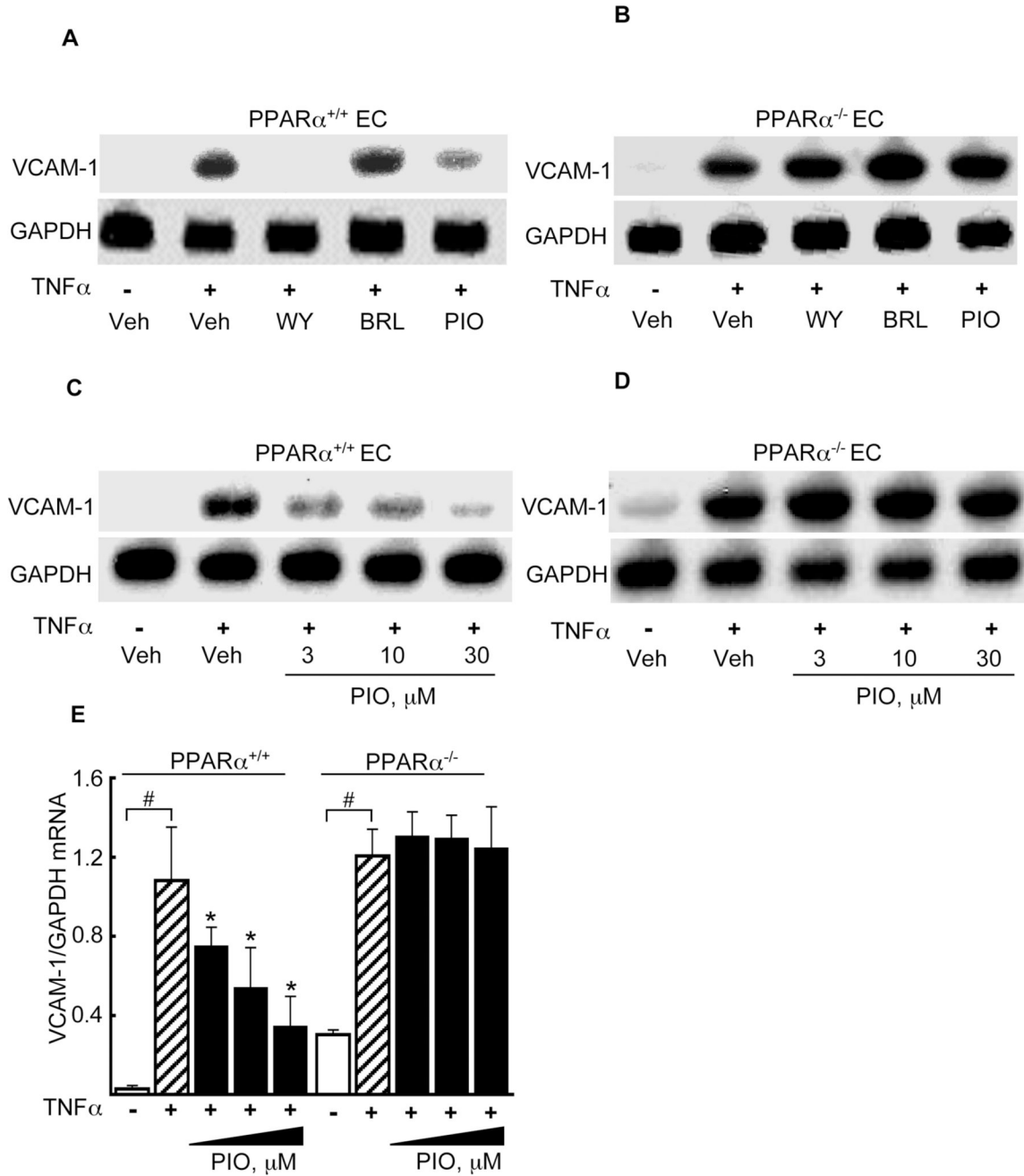


Figure 2. Pioglitazone represses TNF α -induced VCAM-1 expression in a PPAR α -dependent manner

ECs isolated from PPAR $\alpha^{+/+}$ (A) and PPAR $\alpha^{-/-}$ (B) mouse hearts were pretreated with WY14643, rosiglitazone (BRL), or pioglitazone at the concentrations shown (18 h) before mouse TNF α stimulation and subsequent Northern blotting for VCAM-1 mRNA and GAPDH expression. One representative blot of three is shown. Northern blotting for VCAM-1 expression was repeated in the presence of the dose range of pioglitazone shown in EC from PPAR $\alpha^{+/+}$ (C) and PPAR $\alpha^{-/-}$ (D) mice. (E) Quantification of the effects of pioglitazone on VCAM-1 mRNA in PPAR $\alpha^{+/+}$ and PPAR $\alpha^{-/-}$ EC relative to GAPDH mRNA expression (n =

3, # $p < 0.05$ TNF α vs. vehicle; * $p < 0.05$ pioglitazone/TNF α vs. TNF α alone, Mann-Whitney test).

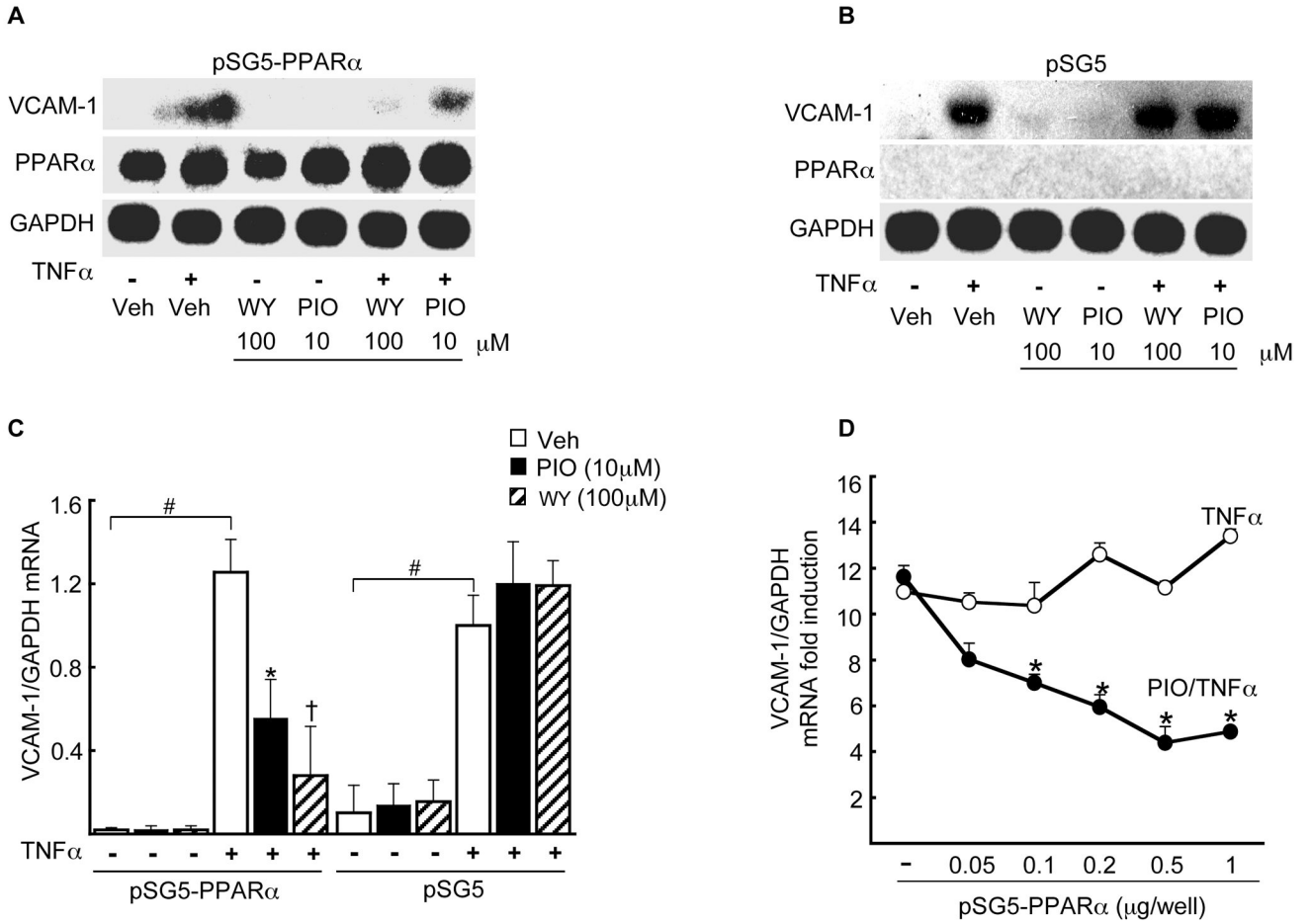


Figure 3. PPAR α is required for pioglitazone-mediated repression of TNF α induced endothelial VCAM-1 mRNA expression

Northern blot analysis was performed on total RNA isolated from PPAR α ^{-/-} ECs transfected either with PPAR α -containing pSG5 overexpression vector (A) or pSG5 alone (B) before stimulation with TNF α in either the absence or presence of WY (100 μ M) or pioglitazone (10 μ M), 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 TNF α vs. vehicle; *p<0.05 pioglitazone/TNF α vs. TNF α alone; †p<0.05 WY14643/TNF α vs. TNF α alone, Mann-Whitney test). (D) Cells were transfected in (A, B) but with a concentration gradient of pSG5-PPAR α as shown before TNF α stimulation in either the absence or presence of pioglitazone 10 μ M (n = 3, *p<0.05 pioglitazone/TNF α vs. TNF α alone).

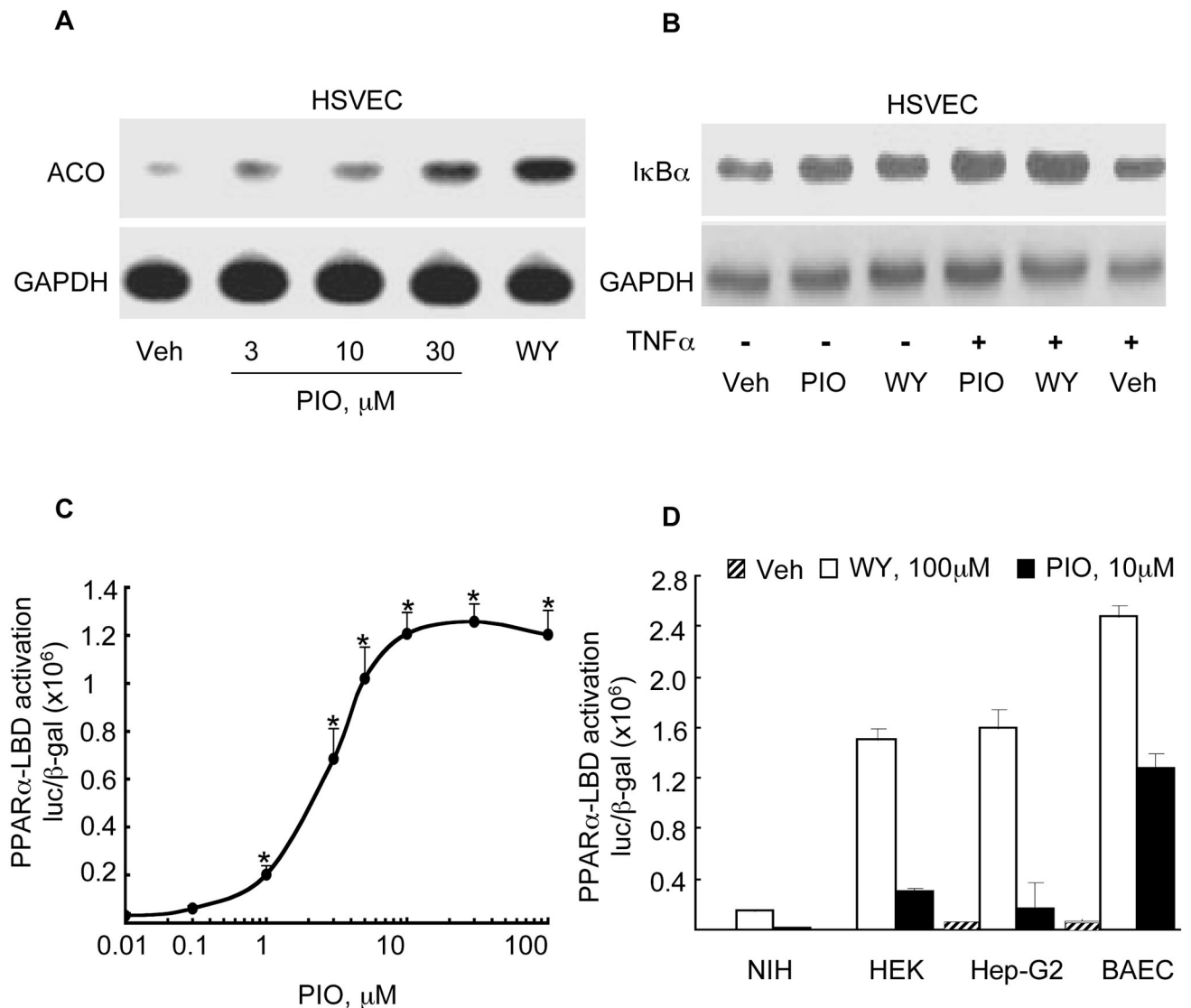


Figure 4. Pioglitazone induces known PPAR α target gene expression and PPAR α -LBD activation in ECs

(A) Northern blot analysis in HSVECs was performed for the PPAR α target gene acyl-CoA-oxidase (ACO) and compared to GAPDH in HSVEC pretreated (16 h) with pioglitazone or WY14643 at the concentrations shown before TNF α stimulation. (B) Western blot analysis for I κ B α 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 TNF α . (C) Standard LBD activation assays were performed in BAECs stimulated with pioglitazone at the concentrations shown (0.01–100 μM). (D) PPAR α -LBD assays were done as before but comparing responses in NIH/3T3 (fibroblasts), HEK293 (human kidney epithelial), Hep-G2 (hepatic) and BAEC cell lines before stimulation with pioglitazone or WY14643 (both 10 μM). Values are expressed as luciferase/ β -Gal activity mean \pm SD (n = 3). *p < 0.05 BAEC vs. NIH/3T3, ** vs. HEK293, *** vs. Hep-G2, both Student's t and Mann-Whitney tests).

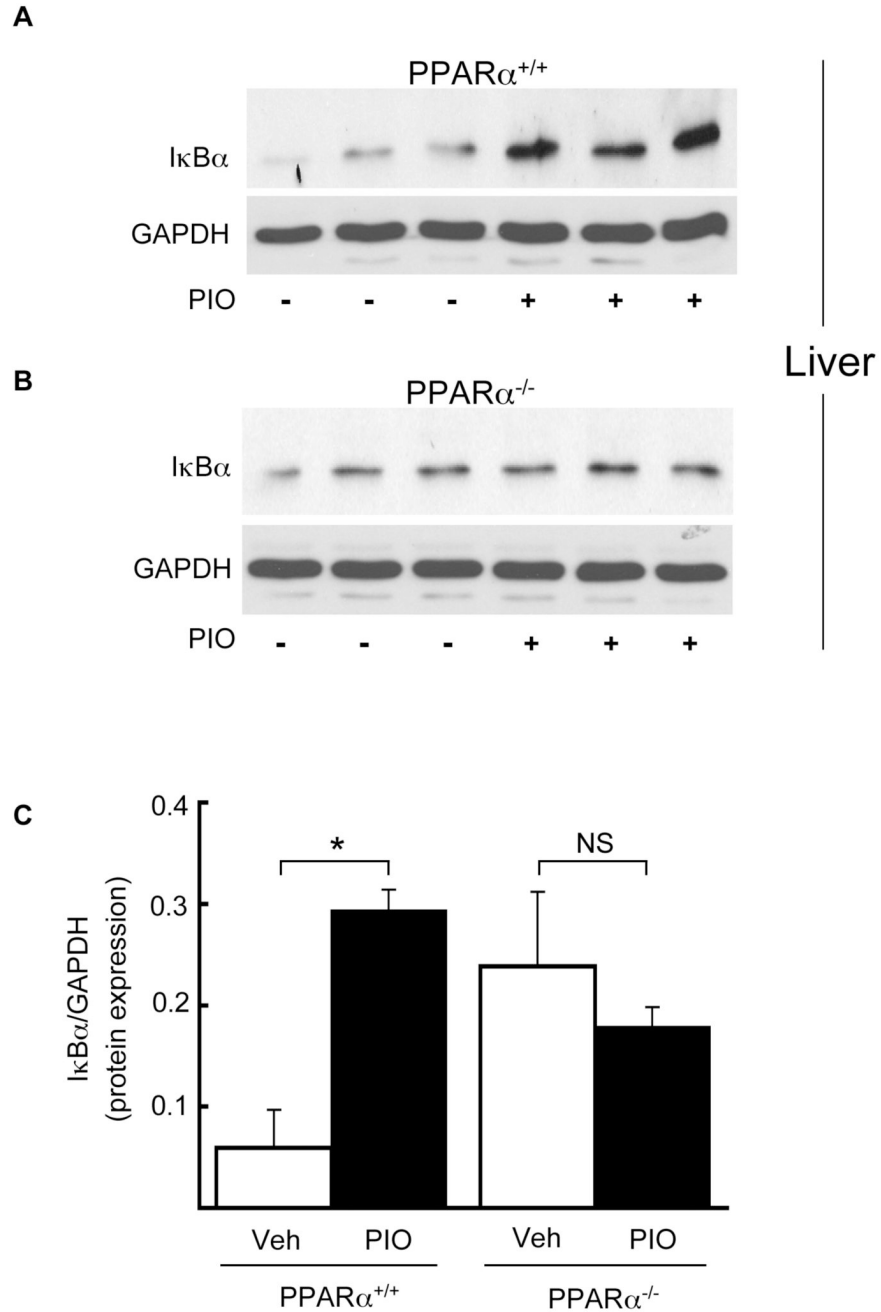


Figure 5. Pioglitazone induces I κ B α protein expression in vivo in a PPAR α dependent manner PPAR $\alpha^{+/+}$ (A) and PPAR $\alpha^{-/-}$ (B) mice were treated with pioglitazone (20mg/kg, 7 days via gavage) before livers were harvested and total protein extracted for Western blot analysis of I κ B α and GAPDH protein levels. Each lane represents a single mouse. (C) The effects of pioglitazone (black bars) and vehicle (white bars) on I κ B α protein expression were quantified and normalized to GAPDH expression. Mean values SD are shown (n = 4 mice/group. *p<0.05 pioglitazone vs. vehicle, Mann-Whitney test).

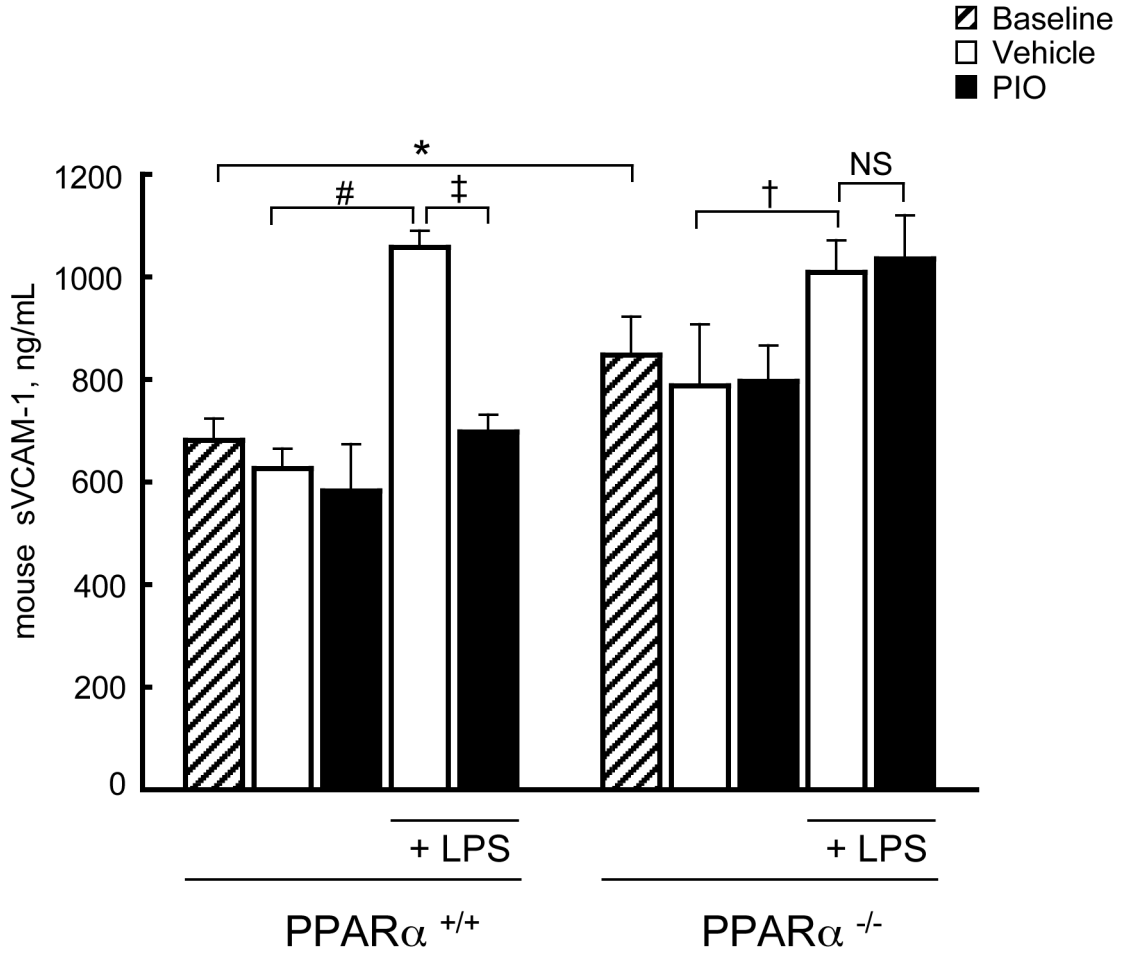


Figure 6. Pioglitazone decreases LPS-induced soluble VCAM-1 in PPAR α ^{+/+} but not PPAR α ^{-/-} mice in vivo

PPAR α ^{+/+} and PPAR α ^{-/-} mice were treated with pioglitazone or vehicle alone before LPS injection (n = 9/genotype as in Methods). sVCAM-1 levels in PPAR α ^{+/+} and PPAR α ^{-/-} mice are shown at baseline (*p<0.007 PPAR α ^{-/-} vs. PPAR α ^{+/+} mice) and after LPS injection in mice treated with either vehicle or pioglitazone (PPAR α ^{+/+}, n=9, #p<0.002 LPS/vehicle vs. vehicle; ‡p<0.01 pioglitazone/LPS vs. vehicle/LPS) and in PPAR α ^{-/-} mice (n=9, †p<0.05 vehicle/LPS vs. vehicle; (NS) non-significant pioglitazone/LPS vs. vehicle/LPS, significance determined using Mann-Whitney test). The mean serum sVCAM-1 concentration of each group \pm SD is shown.

Table 1
 Characteristics of the diabetic patients at baseline and after 16 weeks of intervention with placebo or pioglitazone.

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

Data are showed as mean SE with 95% confidence interval (CI) or median ([†]). No baseline differences in any variables (independent Student's *t* test); p<0.05 shows significant differences within group (paired-Student's *t* test)

Median test (*) between groups pre or post treatment. BMI (Body Mass Index); SBP (Systolic Blood Pressure); DBP (Diastolic Blood Pressure); FPG (Fasting Plasma Glucose); OGTT (Oral Glucose Tolerance Test), ns = non-significant.