Original Article

PPARα Agonists Suppress Osteopontin Expression in Macrophages and Decrease Plasma Levels in Patients With Type 2 Diabetes

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Osteopontin (OPN) is a proinflammatory cytokine implicated in the chemoattraction of monocytes and the development of atherosclerosis. Peroxisome proliferator–activated receptor (PPAR)α, a ligand-activated transcription factor with pleiotropic anti-inflammatory effects in macrophages, is the molecular target for fibrates, which are frequently used to treat dyslipidemia in patients with type 2 diabetes at high risk for cardiovascular disease. In the present study, we examined the regulation of OPN by PPARα agonists in macrophages and determined the effect of fibrate treatment on OPN plasma levels in patients with type 2 diabetes. Treatment of human macrophages with the PPARα ligands bezafibrate or WY14643 inhibited OPN expression. PPARα ligands suppressed OPN promoter activity, and an activator protein (AP)-1 consensus site conferred this repression. Overexpression of c-Fos and c-Jun reversed the inhibitory effect of PPARα ligands on OPN transcription, and, in chromatin immunoprecipitation assays, PPARα ligands inhibited c-Fos and phospho–c-Jun binding to the OPN promoter. Moreover, c-Fos and phospho–c-Jun protein expression was inhibited by PPARα agonists, indicating that PPARα ligands suppress OPN expression through negative cross talk with AP-1–dependent transactivation of the OPN promoter. This inhibitory effect of PPARα ligands on OPN expression was absent in PPARα-deficient macrophages, suggesting a receptor-mediated mechanism of OPN suppression. Finally, treatment of type 2 diabetic patients with bezafibrate significantly decreased OPN plasma levels. These results demonstrate a novel mechanism whereby PPARα ligands may impact macrophage inflammatory responses and decrease early proinflammatory markers for cardiovascular disease. *Diabetes* 56:1662–1670, 2007

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Received for publication 21 August 2006 and accepted in revised form 27 February 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 14 March 2007. DOI: 10.2337/db06-1177.

AP, activator protein; ChIP, Chromatin immunoprecipitation; FBS, fetal bovine serum; IL, interleukin; OPN, osteopontin; PMA, phorbol myristic acid; PPAR, peroxisome proliferator–activated receptor; TNF, tumor necrosis factor.

Osteopontin (OPN) is a secreted extracellular matrix protein and a proinflammatory cytokine that has recently been identified as a key component of cell-mediated immunity (1). OPN is abundantly secreted by macrophages and mediates their recruitment and activation at sites of inflammation by regulating monocyte adhesion, migration, differentiation, and inflammatory gene expression (2,3). OPN is expressed in atherosclerotic lesions, where it is abundantly synthesized by macrophages and, to a lesser extent, by smooth muscle cells and endothelial cells (4). Using murine models, we have recently demonstrated that OPN deficiency in macrophages prevents the development of atherosclerosis (5). Similarly, Matsui et al. (6) have demonstrated decreased atherosclerosis and macrophage accumulation in OPN-deficient mice. In humans, OPN plasma levels significantly correlate with the extent of coronary atherosclerosis (7,8). Moreover, OPN levels are particularly elevated in patients with type 2 diabetes (9,10), and a variety of factors associated with increased cardiovascular risk induce OPN expression in vascular cells including hyperglycemia (11), hyperlipidemia (12), and hypertension (13). This evidence supports the concept that OPN may be causally involved in the premature development of pathogenesis in patients at increased risk for cardiovascular disease.

Peroxisome proliferator–activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that have emerged as key regulators of inflammation, lipid homeostasis, and proliferation of vascular cells (14). PPARα is expressed in macrophages and in atherosclerotic lesions (15). PPARα is the molecular target for fibrates, such as bezafibrate and fenofibrate, which are clinically used to treat dyslipidemia in patients at increased cardiovascular risk (16). In addition to these beneficial lipid-modifying effects, PPARα ligands exert pleiotropic effects to inhibit inflammatory gene expression (17). These pleiotropic effects are thought to contribute to the prevention of cardiovascular diseases observed in large clinical trials such as VA-HIT (Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial) (18) or BECAIT (Bezafibrate Coronary Atherosclerosis Intervention Trial) (19). Considering the widespread clinical use of PPARα ligands, understanding of molecular mechanisms responsible for their beneficial efficacy in cardiovascular

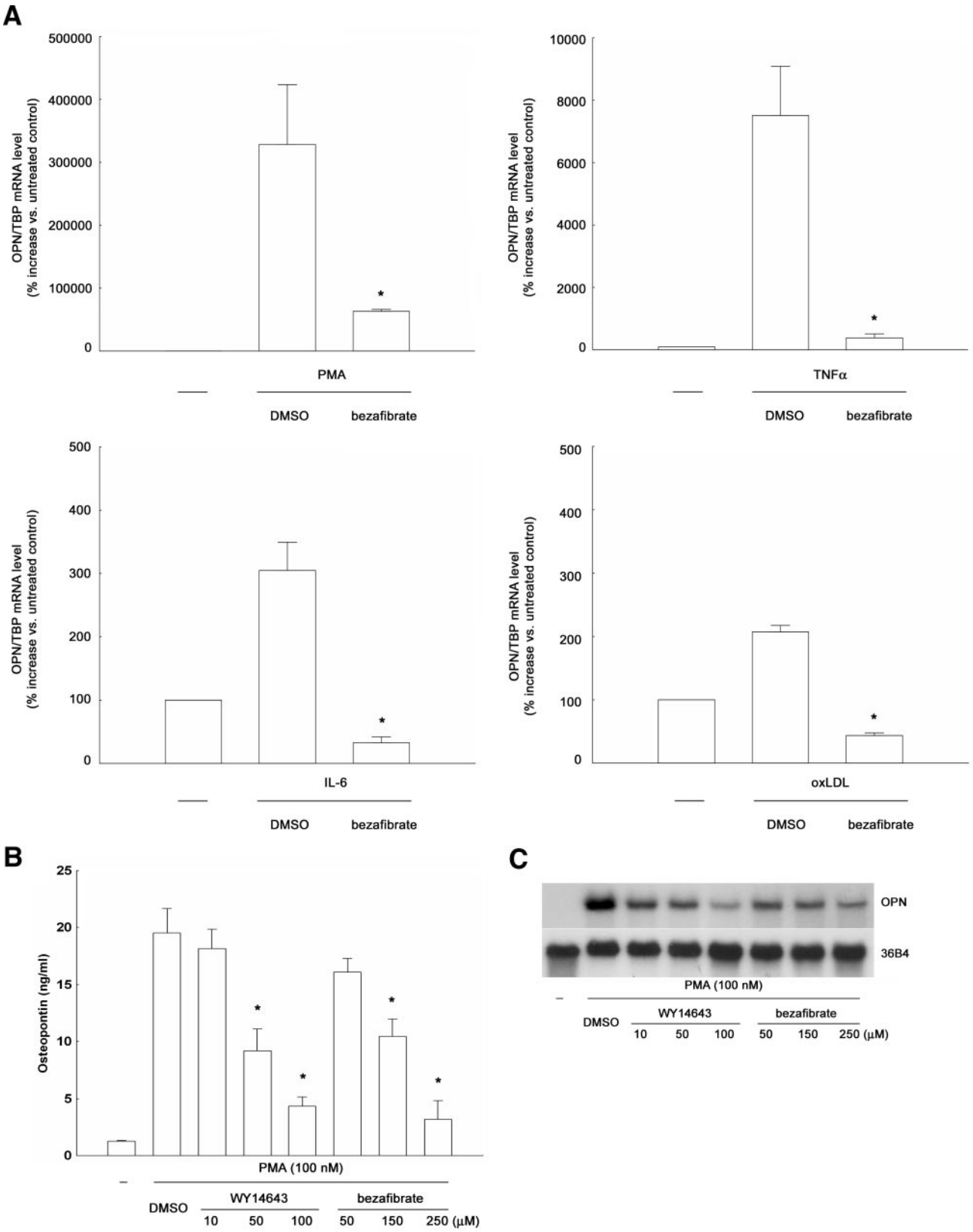

FIG. 1. PPARα ligands inhibit OPN expression in human macrophages. *A*: Human THP-1 monocytes were left untreated (—) or incubated with either vehicle (DMSO) or the PPARα ligand bezafibrate (250 μmol/l) for 24 h before stimulation with 100 nmol/l PMA, 50 ng/ml TNF-α, 100 ng/ml IL-6, or 50 μg/ml oxidized LDL in the presence of the ligand. Cells were harvested after 48 h and analyzed for OPN mRNA by real-time RT-PCR. OPN mRNA expression levels were normalized to the housekeeping gene TATA-binding protein and expressed as percentage of increase over untreated cells from three independently performed experiments (mean ± SEM; *$P < 0.05$ vs. the vehicle DMSO). *B*: Cells were pretreated with the indicated PPARα ligand and stimulated with 100 nmol/l PMA as described in *A*. At 72 h after stimulation, the supernatant was analyzed for OPN secretion by enzyme-linked immunosorbent assay. Results are presented as means ± SEM from three independently performed experiments (*$P < 0.05$ vs. vehicle). *C*: Cells were harvested after 48 h and analyzed for OPN and 36B4 mRNA expression by Northern blotting. The blot depicted is representative of three independently performed experiments.

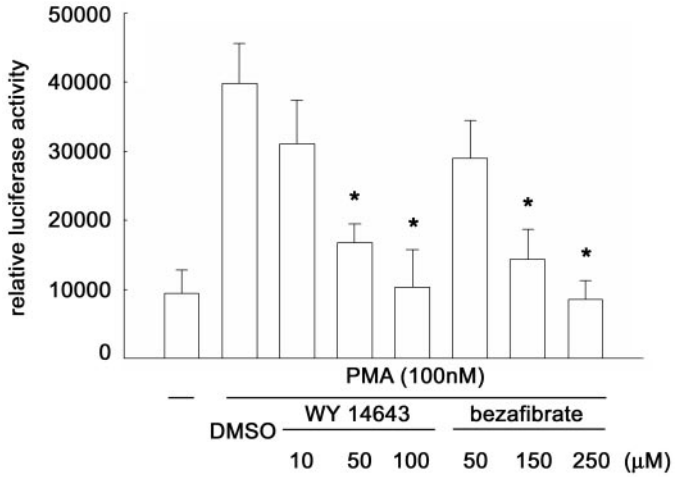

FIG. 2. PPARα agonists inhibit OPN promoter activity. RAW 264.7 macrophages were transiently transfected with a full-length OPN promoter construct. Transfected macrophages were pretreated for 12 h with vehicle (DMSO) or the indicated PPARα agonist and stimulated with PMA (100 nmol/l) in the presence of the agonist. After 24 h, luciferase activities were analyzed as described in RESEARCH DESIGN AND METHODS. Data are expressed as normalized luciferase activity and presented as means ± SEM from three independently performed experiments (*$P < 0.05$ vs. vehicle).

disease provides an important basis for the future development of these agents.

In the present study, we demonstrate that PPARα ligands suppress OPN expression in macrophages in vitro and that bezafibrate reduces OPN plasma levels in patients with type 2 diabetes. These results outline a previously unrecognized mechanism by which PPARα ligands may impact macrophage inflammatory responses and reduce plasma levels of inflammatory markers in patients at high risk for the development of cardiovascular diseases.

RESEARCH DESIGN AND METHODS

Cell culture. Human THP-1 monocytes and RAW 264.7 macrophages (American *Type Culture* Collection) were cultured in RPMI-1640 medium and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), respectively. For ligand treatment, THP-1 monocytes and RAW 264.7 macrophages were serum deprived by culture in 0.5% FBS and pretreated with the PPARα ligand WY14643 or bezafibrate for 24 h before stimulation with 100 nmol/l phorbol 12-myristate 13-acetate (PMA), 50 ng/ml tumor necrosis factor (TNF)-α, 100 ng/ml interleukin (IL)-6, or 50 mg/ml oxidized LDL for the indicated time points. Thioglycollate-elicited peritoneal macrophages isolated from C57BL6 wild-type and PPARα-deficient mice were cultured as described (20) and treated with ligands and PMA using the same protocol. WY14643 was purchased from Cayman Chemical. Bezafibrate was commercially obtained from Sigma-Aldrich. For all data shown, individual experiments were repeated at least three times with different lots or preparation of cells.

Analysis of cellular OPN protein secretion by enzyme-linked immunosorbent assay. THP-1 monocytes were cultured in 0.5% FBS and pretreated with the indicated PPARα ligand for 24 h. Following this pretreatment, cells were stimulated with 100 nmol/l PMA in the presence of the ligand for 72 h, and OPN protein secretion into the media was analyzed using a commercially available Osteopontin (human) enzyme immunoassay kit (Assay Designs).

Northern blotting and quantitative real-time RT-PCR. Northern blotting for OPN RNA levels, densitometry, and normalization to the constitutively expressed housekeeping gene 36B4 was performed as previously described (5). Quantitative real-time RT-PCR for c-Fos, c-Jun, and OPN was performed as described (21) using an iCycler (Bio-Rad), SYBR Green I system (Bio-Rad), and the following primers: murine OPN (forward 5′-TCCCTCGATGTCATC CCTGT-3′ and reverse 5′-CCCTTTCCGTTGTTGTCCTG-3′), human OPN (forward 5′-AGGCTGATT CTGGAAGTTCTGAGG-3′ and reverse 5′-ACTCC TCGCTTTCCATGTGTGAGG-3′), murine c-Fos (forward 5′-AGAGCGGGb AATGGTGAAG-3′ and reverse 5′-GGATTCTCCGTTTCTCTTCC-3′), or murine

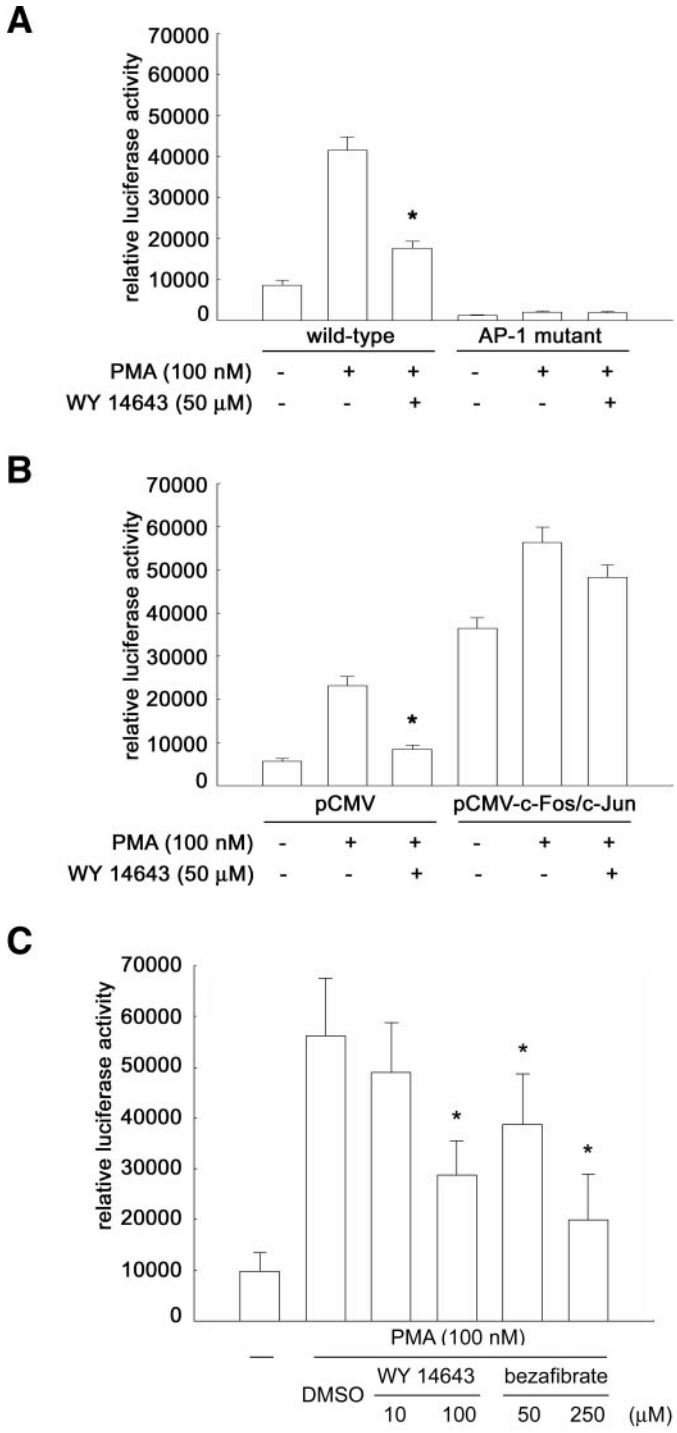

FIG. 3. PPARα agonists suppress OPN promoter activity by negative interference with AP-1 signaling pathways. *A*: RAW 264.7 macrophages were transiently transfected with the full-length wild-type or AP-1–mutated OPN promoter. Following transfection, macrophages were pretreated for 12 h with 50 μmol/l WY14643 and stimulated for 24 h with 100 nmol/l PMA as indicated. *B:* RAW 264.7 macrophages were transfected with the OPN promoter construct alone or cotransfected with the empty pCMV vector (400 ng) or pCMV-c-Fos (200 ng) and pCMV-c-Jun (200 ng) expression vectors. After transfection, cells were treated as described in *A*. *C*: RAW 264.7 macrophages were transfected with an AP-1–driven heterologous promoter. Transfected macrophages were pretreated for 12 h with vehicle (DMSO) or the indicated PPARα agonist and stimulated for 24 h with 100 nmol/l PMA in the presence of the agonist. Following stimulation, luciferase activities were analyzed. Data are expressed as normalized luciferase activity and presented as means ± SEM from three independently performed experiments (*$P < 0.05$ vs. vehicle).

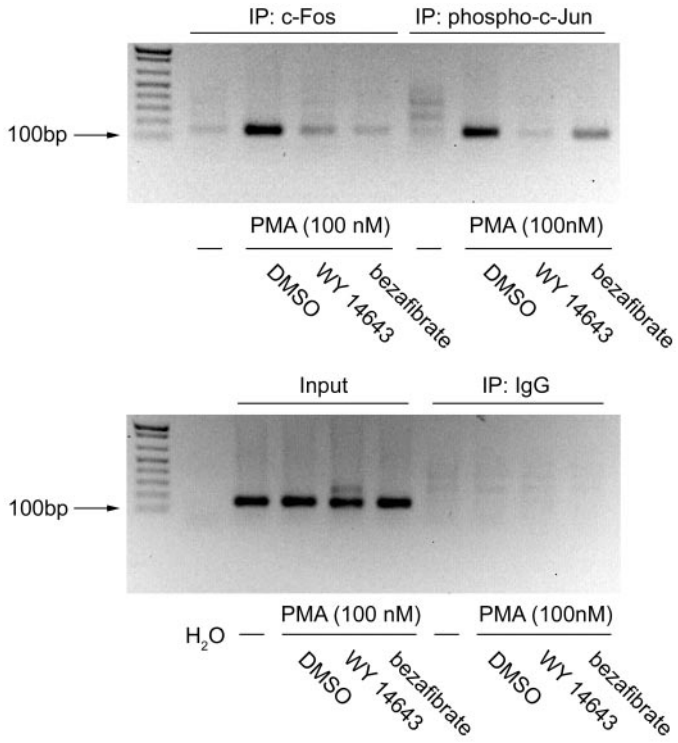

FIG. 4. PPARα agonists inhibit AP-1 binding to the proximal OPN promoter. RAW 264.7 macrophages were pretreated for 24 h with vehicle (DMSO), 50 μmol/l WY14643, or 250 μmol/l bezafibrate and stimulated with 100 nmol/l PMA for 24 h. ChIP assays were performed using c-Fos and phospho–c-Jun antibodies. Total extract (input) and rabbit IgG were used as controls. Ethidium bromide–stained agarose gels shown are representative of three independently performed experiments.

c-Jun (forward 5′-CGCACAGCCCAGGCTAAC-3′ and reverse 5′-TGAGGG CATCGTCGTAGAA-3′). mRNA expression levels were normalized to the housekeeping genes TATA-binding protein or cyclophilin.

Western blot analysis. Cells were harvested at the indicated time points, and nuclear extracts were isolated in the presence of a protease inhibitor cocktail using the NE-PER (Nuclear and Cytoplasmic Extraction Reagents) kit according to the manufacturer's instruction (Pierce Biotechnology). Western blotting was performed as recently described (5). Primary antibodies were purchased from Santa Cruz Biotechnology (c-Fos: sc-8047 or sc-253; phospho–c-Jun: sc-822) or Abcam (Histone H3: ab32151).

Plasmids and transient transfections. The OPN promoter constructs, AP-1 luciferase reporter construct, and c-Fos and c-Jun expression vectors were used as previously described (22). RAW 264.7 macrophages were transfected with 1 mg DNA using LipofectAMINE 2000 (Invitrogen). At 8 h after transfection, cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.5% FBS in the presence of the indicated PPARα ligand for 12 h before PMA stimulation. Luciferase activity was assayed 48 h after stimulation as described (22). All experiments were repeated at least three times and in triplicates with different cell preparations.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate) according to the manufacture's instructions. Soluble chromatin was prepared from RAW 264.7 macrophages treated with 100 mmol/l WY14643 or 250 mmol/l bezafibrate for 24 h, followed by stimulation with PMA (100 nmol/l) for 24 h. Chromatin was immunoprecipitated with antibodies (2 mg) directed against c-Fos (sc-8047) and phospho–c-Jun (sc-822). Final DNA extractions were PCR amplified using primer pairs that cover AP-1 consensus sequence at −76 in the OPN promoter to yield a 151-bp PCR product (forward 5′-ACCACAAAACCA GAGGAGGA-3′ and reverse 5′-TTCAGTGTGAGCTGCTGGTG-3′).

Study population and study design. Type 2 diabetic patients (diagnosed by World Health Organization criteria) were referred to the outpatient clinic at the Dokkyo University Hospital for treating dyslipidemia. Ambulatory male or female patients aged 40–75 years with triglyceride levels between <400 and ≥150 mg/dl, duration of type 2 diabetes >2 years, A1C <10%, and stable glycemic control (defined as a variation in A1C of no more than 2% in the last 2 years) were eligible. Furthermore, patients on concomitant cardiovascular risk–modifying medications must have been stable on these drugs for at least 6 months before entering the study. Exclusion criteria included BMI >30 kg/m^2; evidence of thyroid, liver, or gallbladder disease or renal dysfunction (creatinine >2 mg/dl); or any known intolerance for fibrate treatment. Ten patients were enrolled into this study and treated with 200 mg bezafibrate twice daily for 28 days as add-on therapy to any preexisting medication. Blood samples after an overnight fast were taken at baseline and 28 days after treatment for measurements of metabolic parameters and serum OPN levels. Serum OPN levels at baseline and 28 days after bezafibrate treatment were analyzed using a new commercially available human OPN Osteopontin enzyme immunoassay kit (Assay Designs). It has previously been demonstrated that OPN levels determined with this new assay are lower than measured with older assay systems (23). Metabolic parameters were assessed as previously described (24). All patients gave informed consent, and the study was approved by the Dokkyo University institutional review board.

Statistical analysis. ANOVAs using one-way ANOVA with Bonferroni's *t* test for post hoc analysis and paired or unpaired *t* test were performed for statistical analysis as appropriate. Differences in OPN plasma levels and metabolic parameters before and after bezafibrate treatment were calculated using the paired *t* test. Skewed data were reported as median (interquartile range); all other data were reported as means ± SEM or SD as indicated. *P* values <0.05 were considered statistically significant.

RESULTS

PPARα agonists inhibit OPN expression in human macrophages. Our recent studies have demonstrated that OPN mediates monocyte infiltration into the arterial wall during atherosclerosis development and that deficiency of OPN secretion from macrophages reduces atherosclerosis in apoE (apolipoprotein E)-deficient mice (5). Based on this evidence, we analyzed whether antiinflammatory PPARα ligands modulate OPN expression in human monocytes. Treatment of human THP-1 monocytes with inflammatory mediators known to be elevated in patients with type 2 diabetes and cardiovascular disease such as TNF-α, IL-6, and oxidized LDL (25) or PMA resulted in a profound induction of OPN mRNA expression, which was inhibited by the PPARα agonist bezafibrate (Fig. 1*A*). Since PMA has been previously reported to be a potent stimulus for OPN secretion (26) and resulted in the highest induction of OPN expression in monocytes (Fig. 1*A*), subsequent experiments were performed using PMA as stimulus for OPN expression. Pretreatment of monocytes with either the PPARα ligand WY14643 or bezafibrate resulted in a dose-dependent inhibition of OPN protein secretion into the supernatant as assessed by enzyme-linked immunosorbent assay (Fig. 1*B*). Similarly, Northern blotting experiments demonstrated that PMA-induced OPN mRNA expression was dose-dependently suppressed by both PPARα agonists used, indicating that the suppression of OPN in macrophages by PPARα ligands occurs at the gene expression level (Fig. 1*C*).

PPARα ligands suppress OPN transcription. To further analyze the effect of PPARα ligands on OPN transcription, we next transiently transfected RAW 264.7 macrophages with a 2-kb OPN promoter fragment. PMA treatment resulted in a significant induction of OPN promoter activity, which was suppressed by both PPARα ligands used (Fig. 2). The inhibition of OPN promoter activity by the PPARα ligands was dose dependent and paralleled the inhibition of OPN mRNA and protein expression. These results suggest that PPARα ligands suppress PMA-induced OPN expression by inhibiting transcription of the OPN gene.

PPARα agonists suppress OPN promoter activity by negatively interfering with AP-1–dependent transactivation of the OPN promoter. Based on our previous studies demonstrating an important role of the AP-1 site located at −76 from the transcription initiation site for the

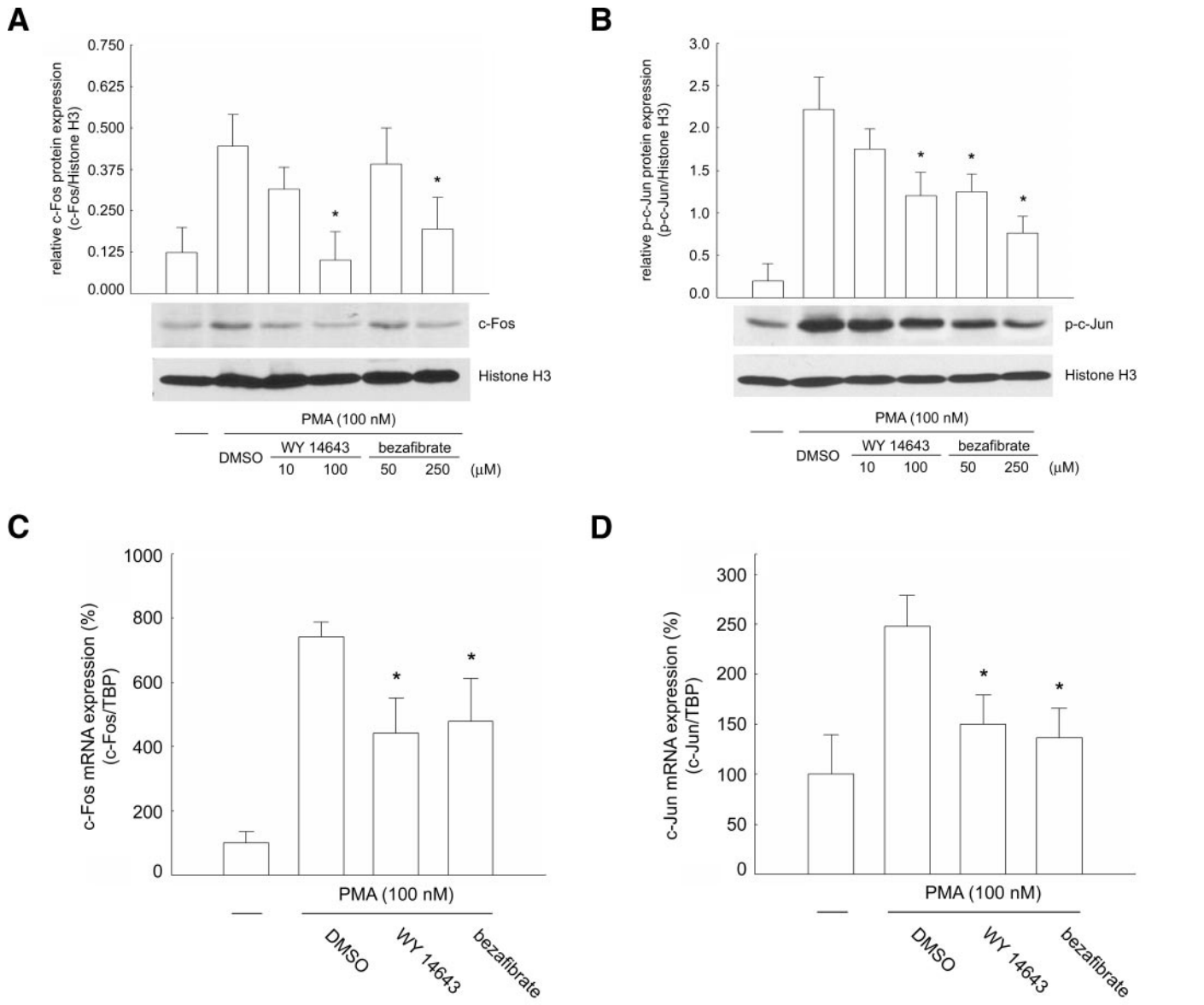

FIG. 5. PPARα agonists inhibit c-Fos and phospho–c-Jun expression. RAW 264.7 macrophages were left untreated (—) or incubated with either vehicle (DMSO) or the indicated PPARα agonist for 24 h before stimulation with 100 nmol/l PMA in the presence of the ligand. Following stimulation for 3 h, nuclear extracts were isolated and analyzed for c-Fos (*A*) and phospho–c-Jun (*B*) protein expression. Quantification was performed by densitometry and normalization to Histone H3 of three independently performed experiments. Results are presented as means ± SEM (P* < 0.05 vs. vehicle). c-Fos (*C*) and c-Jun (*D*) mRNA expression levels were analyzed by real-time RT-PCR following stimulation with PMA for 30 min. Quantification was performed from three independently performed experiments and normalization to the housekeeping gene TATA-binding protein. Data are expressed as percentage of increase over unstimulated cells and presented as means ± SEM (**P* < 0.05 vs. vehicle).**

regulation of the OPN promoter in macrophages (22), we generated a site-directed mutation in this AP-1 consensus element. Transient transfection of macrophages with the OPN wild-type promoter construct and treatment with the PPARα ligand WY14643 resulted in an almost complete inhibition of OPN transcription (Fig. 3*A*). In marked contrast, the OPN promoter construct bearing a mutation of the AP-1 site exhibited low basal activity and was not induced by PMA. To further confirm an important role of this AP-1 site for the inhibition of OPN promoter activity by PPARα ligands, we next cotransfected macrophages with the wild-type OPN reporter construct and eukaryotic expression vectors for c-Fos and c-Jun (Fig. 3*B*). Overexpression of c-Fos and c-Jun resulted in a complete loss of the effect of the PPARα ligand to inhibit PMA-induced OPN promoter activity. To further corroborate the observation that PPARα ligands suppress AP-1–dependent transactivation, we performed transfection experiments using a heterologous promoter driven by multiple AP-1 response elements. PMA-induced transcriptional activity of this AP-1–driven reporter construct was dose-dependently inhibited by both PPARα ligands (Fig. 3*C*). In concert, these findings suggest that the suppression of PMA-induced OPN promoter activity by PPARa ligands is mediated through negative interference with c-Fos/c-Jun acting on the proximal OPN promoter.

PPARα ligands inhibit AP-1 binding to the proximal OPN promoter. ChIP assays using primer pairs that cover the AP-1 site at −76 in the OPN promoter were next performed to confirm that PPARα ligands interfere with c-Fos and phospho–c-Jun binding to the endogenous OPN promoter. As depicted in Fig. 4, stimulation of RAW 264.7

macrophages with PMA resulted in binding of c-Fos and phospho–c-Jun to the AP-1 site at −76 of the endogenous OPN promoter, an effect which was inhibited by both PPARα ligands WY14643 and bezafibrate. To further determine whether the inhibition of c-Fos and phospho–c-Jun binding to the OPN promoter by PPARα ligands reflects changes in c-Fos or phospho–c-Jun expression levels, protein and mRNA levels of both transcription factors were next analyzed. Western blotting and real-time RT-PCR experiments demonstrated that PMA-induced c-Fos and phospho–c-Jun proteins (Fig. 5*A* and *B*) and mRNA (Fig. 5*C* and *D*) expression were significantly inhibited by treatment with PPARα ligands. Thus, suppression of PMA-induced binding to the AP-1 site at −76 by PPARα ligands reflects, at least in part, an inhibition of c-Fos and c-Jun expression.

The inhibition of OPN expression by PPARα ligands is receptor dependent. Since ligands for PPARα have previously been reported to also have nongenomic receptor-independent effects (27), we investigated the suppression of OPN by PPARα ligands in macrophages isolated from wild-type and PPARα-deficient mice. Treatment of wild-type macrophages with PMA resulted in an induction of OPN mRNA expression, and three different PPARα agonists significantly suppressed PMA-induced OPN mRNA expression (Fig. 6*A*). Interestingly, PMA-induced OPN mRNA expression in PPARα-deficient macrophages was substantially higher compared with wild-type macrophages (27.81 ± 3.76 – and 2.42 ± 0.31–fold increase vs. control, respectively). In contrast to wild-type macrophages, PPARα ligands were without effect on PMA-induced OPN mRNA expression in macrophages isolated from PPARα-deficient mice. These data indicate that the suppression of OPN by PPARα ligands in macrophages is PPARα dependent and further suggest that PPARα represses basal OPN mRNA levels.

Bezafibrate treatment decreases OPN plasma levels in patients with type 2 diabetes. OPN plasma levels are associated with the presence and extent of coronary artery disease and have recently been recognized as an independent predictor of future adverse cardiac events in patients with chronic stable angina (7,8). In addition, OPN levels have been reported to be significantly elevated in patients with type 2 diabetes (9,10). Therefore, we next performed a clinical proof-of-concept study to determine whether treatment with bezafibrate modulates OPN plasma levels in patients with type 2 diabetes. A total of 10 patients (6 female and 4 male) with type 2 diabetes without previous treatment for dyslipidemia were recruited from an outpatient clinic. Baseline characteristics of these patients are shown in Table 1. No significant correlations between OPN plasma levels and age or sex were observed at baseline. Treatment with bezafibrate at a dose of 400 mg/day for 28 days resulted in a significant decrease of plasma triglyceride levels and an increase in plasma HDL cholesterol levels, demonstrating that bezafibrate exhibited its expected metabolic effects. In addition, bezafibrate treatment resulted in a slight but significant decrease of total cholesterol levels. No significant changes were observed in BMI, LDL cholesterol, glucose, A1C, or blood pressure after treatment. As depicted in Fig. 7*A* and *B*, treatment with bezafibrate significantly decreased OPN plasma levels by 22.8% (median 229.22–177.07 ng/ml after treatment, $P = 0.0059$). In concert, these results indicate that short-term treatment of type 2 diabetic patients with the PPARα ligand bezafibrate decreases OPN plasma levels.

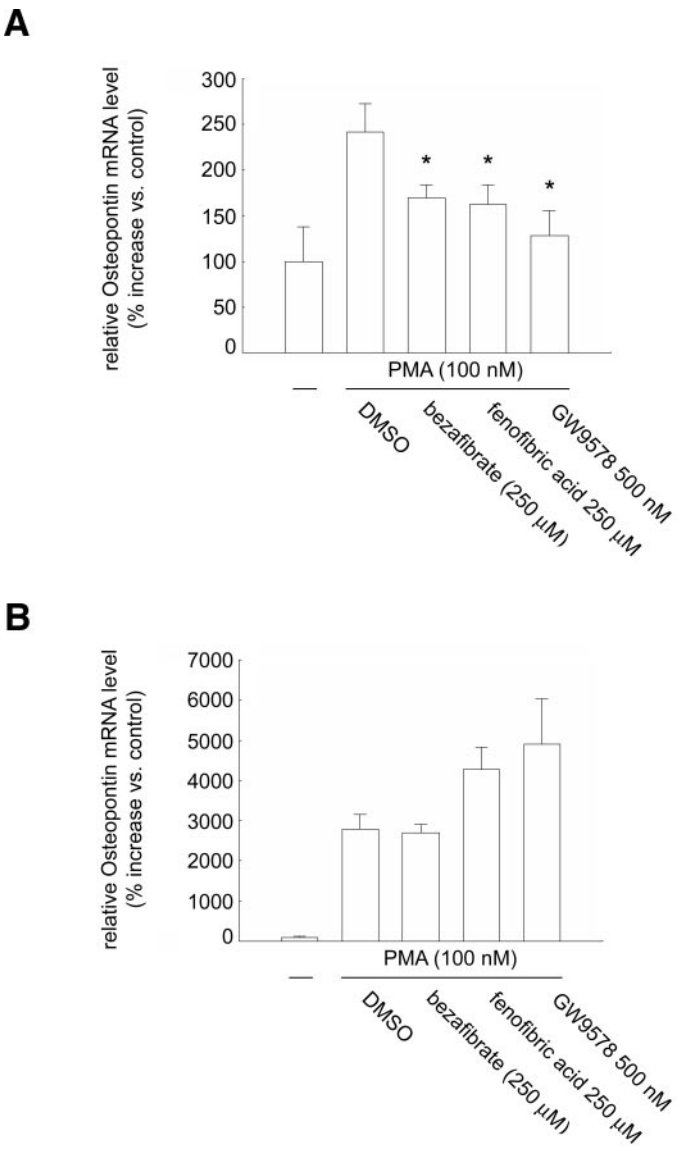

FIG. 6. The inhibition of OPN by PPARα ligands is mediated through a receptor-dependent mechanism. Peritoneal macrophages from wild-type (*A*) and PPARα-deficient (*B*) mice were pretreated with the PPARα ligands bezafibrate (250 μmol/l), fenofibric acid (250 μmol/l), or GW9578 (500 nmol/l) for 24 h before stimulation with 100 nmol/l PMA. mRNA was isolated after 24 h and analyzed for OPN expression by real-time RT-PCR. Quantification was performed from three independently performed experiments and normalization to the housekeeping gene cyclophilin. Data are expressed as percentage of increase over unstimulated cells and presented as means ± SEM (*$P < 0.05$ vs. vehicle).

DISCUSSION

Type 2 diabetes and cardiovascular disease share a common metabolic milieu characterized by insulin resistance, dyslipidemia, and chronic inflammation (25,28). A key component of cell-mediated inflammation constitutes OPN, which is elevated in both type 2 diabetes and cardiovascular disease (7,10). PPARα agonists are frequently used to treat dyslipidemia in these patients, and accumulating evidence supports pleiotropic anti-inflammatory effects of PPARα agonists in vitro as well as in preclinical and clinical studies (14,17). In the present

TABLE 1
Characteristics of the study subjects before and after bezafibrate treatment

Characteristics	Baseline	28 days after treatment	*P*
Sex (female/male)	(6/4)	—	—
Age (years)	61.6 ± 10.7	—	—
BMI (kg/m^2)	25.3 ± 4.7	25.1 ± 5.9	0.19
Cholesterol (mg/dl)			
Triglycerides	195.0 (164–230)	93.0 (86–142)	0.005
Total	227.5 (226–232)	212.5 (169–230)	0.03
LDL	129.5 (118–164)	121.0 (104–147)	0.31
HDL	47.5 (44–54)	55.0 (49–59)	0.0007
Glucose (mg/dl)	145.8 (123–163)	144.5 (126–163)	0.28
A1C (%)	8.2 (7.1–9.4)	8.2 (6.2–9.1)	0.21
Blood pressure (mmHg)			
Systolic	132 (122–136)	129 (112–132)	0.21
Diastolic	70 (70–80)	70 (64–72)	0.14

Data are means ± SD or median (interquartile range).

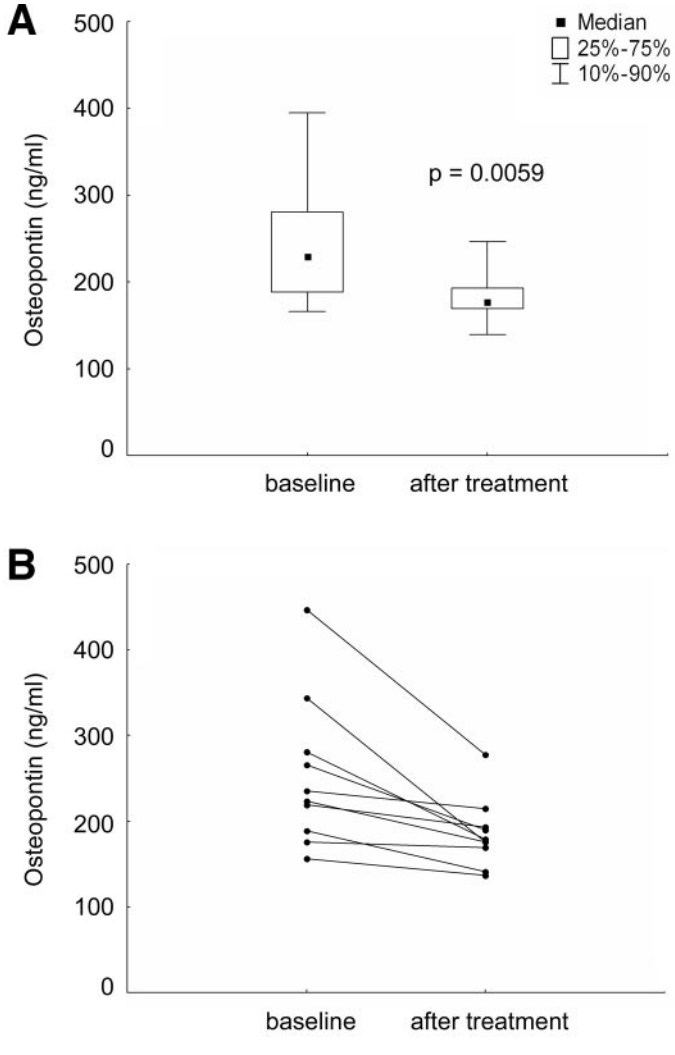

FIG. 7. Bezafibrate decreases OPN plasma levels in patients with type 2 diabetes. Ten patients with type 2 diabetes and dyslipidemia were treated for 28 days with 400 mg bezafibrate/day. Plasma OPN levels were measured by enzyme-linked immunosorbent assay before and after treatment. *A*: Median, interquartile range, and percentiles 10.0 and 90.0 before and after treatment ($P = 0.0059$). *B*: The individual changes in OPN plasma levels before and after treatment with bezafibrate.

study, we demonstrate that OPN expression in macrophages is induced by proinflammatory mediators known to be elevated in type 2 diabetes and cardiovascular disease, including TNF-α, IL-6, and oxidized LDL (25). This induction of OPN expression in macrophages is suppressed by PPARα ligands, which further supports pleiotropic effects of PPARα agonists and identifies OPN as a previously unrecognized PPARα target gene.

PPARα ligands suppress OPN expression at the transcriptional level, an effect that was absent in PPARα-deficient macrophages demonstrating a receptor-dependent suppression of OPN. Therefore, we further sought to identify the *cis*-regulatory elements in the OPN promoter involved in this suppression. Since PPARα ligands suppress OPN and analysis of the OPN promoter did not reveal the presence of any putative PPAR response elements, inhibition of OPN transcription by PPARα ligands likely involves an indirect mechanism through regulation of other transcription factors supporting OPN transcription. In our previous studies, we have identified an AP-1 consensus site in the OPN promoter located between −80 and −71 that supports basal and induced transcriptional activity of the OPN promoter in macrophages (22). Using ChIP assays, we demonstrate in this study that PPARα agonists inhibit c-Fos and phospho–c-Jun binding to this AP-1 site in the proximal OPN promoter. The effect of PPARα ligands to suppress OPN promoter activity is lost in cells overexpressing c-Fos and c-Jun. These results, combined with our observation that PPARα ligands inhibit transactivation of a heterologous AP-1–driven promoter, support the notion that PPARα ligands suppress OPN expression by interfering with AP-1–dependent transactivation of the proximal OPN promoter.

Inhibition of AP-1 binding to the OPN promoter by PPARα ligands could be the result of either decreased expression of c-Fos and phospho–c-Jun or of cross talk through physical interaction between PPARα and c-Fos or phospho–c-Jun. Consistent with recent studies in cardiomyocytes (29), we observed that PPARα ligands inhibit protein expression of c-Fos and phospho–c-Jun in macrophages. This inhibition was at least in part mediated through a transcriptional suppression of c-Fos and c-Jun mRNA levels by PPARα ligands. However, we have also previously demonstrated that PPARα physically interacts with c-Jun and thereby decreases AP-1 binding to target genes (30). Therefore, posttranslational mechanisms and

physical interaction between PPARα and c-Fos or c-Jun could potentially provide an additional mechanism by which PPARα ligands inhibit AP-1–dependent activation of the OPN promoter.

Although a number of preclinical studies demonstrate the suppression of proinflammatory genes by PPARα ligands in vitro (14,17), there is a lack of studies using translational clinical approaches to confirm that the expression of putative candidate genes is modified by fibrate treatment in patients. Based on our in vitro observations, we performed a study designed as proof of concept to demonstrate that a clinically used PPARα agonist decreases OPN plasma levels in patients at high risk for cardiovascular diseases. Although the number of patients treated is relatively small and a limitation of the study, we provide evidence that OPN plasma levels in patients with type 2 diabetes are decreased by 22% in response to treatment with bezafibrate. Bezafibrate exerted its expected metabolic efficacy to improve dyslipidemia (i.e., decrease triglyceride and increase HDL cholesterol plasma levels), and, since it has previously been demonstrated that high-fat diet feeding of mice increases vascular OPN expression (12), the observed decrease of OPN plasma levels may also result from improved dyslipidemia. However, in these studies, dietary cholesterol supplementation in particular increased vascular OPN expression, and total cholesterol was only modestly affected by bezafibrate, with no changes in LDL cholesterol. Therefore (as further supported by extensive evidence from in vitro and in vivo preclinical models [17]), pleiotropic effects of the fibrate may likely contribute to the observed decreases of OPN plasma levels following treatment with bezafibrate.

Accumulating evidence has demonstrated that a chronic low-grade state of inflammation links type 2 diabetes and cardiovascular disease (31,32). OPN levels have been reported to be significantly elevated in patients with type 2 diabetes (9,10). OPN transcription is induced in response to high glucose (11) and OPN expression in the diabetic artery elevated (10), raising the possibility that increased OPN secretion in diabetes may play a direct causal role for the development of diabetic vascular complications. Although we and other investigators have demonstrated a causal role for OPN in the development of atherosclerosis using murine models (5,6), several recent studies link elevated OPN plasma levels in patients to cardiovascular disease. The pioneering study by Panda et al. (33) first demonstrated that OPN plasma levels are increased in patients with atherosclerosis. A second study by Ohmori et al. (7) provided evidence that OPN plasma levels correlate with the extent of coronary atherosclerosis. Finally, an important recent study by Minoretti et al. (8) has shown that OPN plasma levels predict the development of nonfatal myocardial infarction and death from cardiovascular causes in patients with chronic stable angina. These clinical studies may provide initial evidence that OPN may also play a causal role for the development of cardiovascular disease in humans and that elevated OPN plasma levels could provide a novel marker for a chronic inflammatory state in patients at high risk for cardiovascular complications.

In summary, data presented in this study demonstrate that OPN expression in macrophages is suppressed by PPARα ligands through a mechanism involving an inhibition of AP-1–dependent transactivation of the proximal OPN promoter. Using a translational approach, we further provide evidence that in a patient cohort with type 2 diabetes, treatment with the PPARα ligand bezafibrate decreases OPN plasma levels. Since OPN is a key component of macrophage-derived inflammatory processes (34) and promotes the development of atherosclerosis in preclinical studies (5,6), inhibition of OPN expression by PPARα ligands points to a novel mechanism by which these agents reduce cardiovascular disease and its complications.

ACKNOWLEDGMENTS

These studies were in part supported by grants from the National Institutes of Health (HL084611), the American Diabetes Association (Research Award 1-06-RA-17), and the American Heart Association (Scientist Development Grant 0435239N).

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