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Activation of Peroxisome Proliferator-Activated Receptor γ Does Not Inhibit IL-6 or TNF-α Responses of Macrophages to Lipopolysaccharide In Vitro or In Vivo

Rolf Thieringer,[1]* Judy E. Fenyk-Melody,[†] Cheryl B. Le Grand,* Beverly A. Shelton,[†] Patricia A. Detmers,* Elizabeth P. Somers,* Linda Carbin,* David E. Moller,[‡] Samuel D. Wright,* and Joel Berger[‡]

We have investigated the potential use of peroxisome proliferator-activated receptor γ (PPARγ) agonists as anti-inflammatory agents in cell-based assays and in a mouse model of endotoxemia. Human peripheral blood monocytes were treated with LPS or PMA and a variety of PPARγ agonists. Although 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) at micromolar concentrations significantly inhibited the production of TNF-α and IL-6, four other high affinity PPARγ ligands failed to affect cytokine production. Similar results were obtained when the monocytes were allowed to differentiate in culture into macrophages that expressed significantly higher levels of PPARγ or when the murine macrophage cell line RAW 264.7 was used. Furthermore, saturating concentrations of a potent PPARγ ligand not only failed to block cytokine production, but also were unable to block the inhibitory activity of 15d-PGJ_2. Thus, activation of PPARγ does not appear to inhibit the production of cytokines by either monocytes or macrophages, and the inhibitory effect observed with 15d-PGJ_2 is most likely mediated by a PPARγ-independent mechanism. To examine the anti-inflammatory activity of PPARγ agonists in vivo, *db/db* mice were treated with a potent thiazolidinedione that lowered their elevated blood glucose and triglyceride levels as expected. When thiazolidinedione-treated mice were challenged with LPS, they displayed no suppression of cytokine production. Rather, their blood levels of TNF-α and IL-6 were elevated beyond the levels observed in control *db/db* mice challenged with LPS. Comparable results were obtained with the corresponding lean mice. Our data suggest that compounds capable of activating PPARγ in leukocytes will not be useful for the treatment of acute inflammation. *The Journal of Immunology,* **2000, 164: 1046–1054.**

The peroxisome proliferator-activated receptors (PPARs)[2] are members of the nuclear receptor supergene family that function in ligand-activated transcription (1–3). PPARs consist of three isoforms, encoded by separate genes. The PPARγ is highly expressed in adipose tissue, colon, spleen, adrenal gland, and macrophages. Due to alternative promoter use and RNA splicing this receptor is present as two isoforms: PPARγ1 and PPARγ2 (4–6). The latter has additional amino acids at the amino terminus and is the isoform primarily expressed in adipocytes; the former appears to be the major isoform in all other tissues. PPARγ has been shown to play a major regulatory role in adipogenesis and the expression of adipocyte genes involved in lipid metabolism. Its forced overexpression in fibroblasts and myocytes causes these cells to differentiate into adipocytes (7, 8). Recently, it has been demonstrated that the naturally occurring arachidonic acid metabolite, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) as well as thiazolidinedione (TZD) and certain novel non-TZD insulin-sensitizing agents are ligands and agonists of this receptor (9–13). PPARα is expressed at high levels in macrophages (14) and tissues that demonstrate high levels of lipid catabolism, especially liver (15). Activation of hepatic PPARα results in increased expression of enzymes involved in fatty acid β-oxidation and, in rodents, peroxisome proliferation and hepatocarcinogenesis (as reviewed in Refs. 1, 16). Drugs that serve as hypolipidemic agents in humans, including numerous fibrates and WY-14653, are PPARα ligands and agonists (17–20). PPARδ is widely expressed in a variety of tissues, including the brain (21, 22). While it has been shown that it may play a role in regulating cholesterol metabolism in an animal model of insulin resistance (M. D. Leibowitz, C. Fievet, N. Hennuyer, J. Peinado-Onsurbe, J. Duez, J. Berger, C. A. Cullinan, C. P. Sparrow, J. Baffic, G. D. Berger, C. Santini, R. W. Marquis, R. Tolman, C. Fruchart, R. G. Smith, D. E. Moller, and J. Auwerx, manuscript in preparation), the physiological role of PPARδ is yet to be fully delineated.

Recently, several laboratories have examined the effects of PPARγ activation on the inflammatory responses of monocytes and macrophages (23, 24). One group presented data demonstrating that PPARγ agonists could abrogate IFN-γ activation of nitric oxide synthase (iNOS) and gelatinase b expression in murine macrophages (24). In addition, induction of promoters for proinflammatory genes that are regulated by the AP-1, STAT and NF-κB transcription factors was antagonized by activation of PPARγ in transfected cell lines (24). A second group of researchers showed that PPARγ agonists could inhibit production of inflammatory cytokines by pharmacologically activated human monocytes (23). It

Departments of *Endocrinology and Chemical Biology, [†]Laboratory Animal Resources, and [‡]Molecular Endocrinology, Merck Research Laboratories, Rahway, NJ 07065

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[1] Address correspondence and reprint requests to Dr. Rolf Thieringer, Department of Endocrinology and Chemical Biology, Merck Research Laboratories, 126 East Lincoln Avenue, Rahway, NJ 07065. E-mail address: rolf_thieringer@merck.com

[2] Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; D-PBS, Dulbecco's PBS; AD-5075, 5-[4-[2-(5-methyl-2-phenyl-4-oxazoly)-2-hydroxyethoxy-]benzyl]-2,4-thiazolidinedione; L-165,041, 4-[3-[2-propyl-3-hydroxy-4-acetyl]phenoxy]propyloxyphenoxy acetic acid; L-796,449, 3-chloro-4-(3-(3-phenyl-7-propylbenzofuran-6-yloxy)propylthio)phenylacetic acid; L-165,461, 3-chloro-4-(3-(3-ethyl-7-propylbenzisoxazol-6-yloxy)propylthio)phenylacetic acid.

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was suggested that these inhibitory effects were occurring at the transcriptional level, because PPARγ agonists blocked induction of the TNF-α and IL-2 promoters in a transfected, macrophage-like cell line. Taken together, these results suggest that compounds that activate PPARγ may be able to serve as anti-inflammatory agents.

We have recently identified and characterized a number of non-TZDs that serve as ligands and agonists of PPARs (12). These compounds have been shown to alter the conformation of the receptors that they activate and promote their interaction with nuclear receptor coactivators. In addition, these compounds have been used to demonstrate that activation of PPARγ results in an insulin-sensitizing effect in vivo. In the present study we employed a subset of these compounds as well as 15d-PGJ_2 and TZDs to determine whether PPARγ activation affects the production of cytokines by monocytes or macrophages in vitro or in vivo. We found that, with the exception of 15d-PGJ_2, PPARγ agonists were unable to significantly inhibit cytokine production by primary human monocytes, differentiated human macrophages, or murine RAW 264.7 macrophage-like cells stimulated by LPS or PMA. Additionally, AD-5075, a potent TZD, was unable to attenuate LPS-induced TNF-α and IL-6 production in obese diabetic or lean mice after a dosing protocol that provided significant antidiabetic relief in the former. These results raise doubts as to whether PPARγ can modulate acute macrophage-dependent inflammatory events.

Materials and Methods

Reagents

LPS from *Salmonella minnesota* R595 and *Escherichia coli* K235 was obtained from List Biologicals (Campbell, MA). A protein-free preparation of LPS from *E. coli* K235 was prepared as previously described (25). The final preparation was devoid of protein as detected by the colloidal gold total protein staining method (Bio-Rad, Hercules, CA). All LPS preparations were prepared as 1 mg/ml stocks in Dulbecco's PBS without calcium and magnesium (D-PBS) and were sonicated briefly in a water bath sonicator before dilution and addition to the cells. Recombinant human TNF-α and recombinant human IL-6 were purchased from R&D Systems (Minneapolis, MN). FCS was obtained from HyClone (Logan, UT), and human serum was obtained from BioWhittaker (Walkersville, MD) or Gemini (Calabasas, CA). PMA, penicillin G, streptomycin sulfate, DMSO, protease inhibitors, and BSA (fraction V) were purchased from Sigma (St. Louis, MO). D-PBS, RPMI 1640, and Ham's F-12 were obtained from Mediatech (Herndon, VA). 15d-PGJ_2 was obtained from Biomol (Plymouth Meeting, PA). AD-5075 (5-[4-[2-(5-methyl-2-phenyl-4-oxazoly)-2-hydroxyethoxy] benzyl]-2,4-thiazolidinedione), L-165,041 (4-[3-[2-propyl-3-hydroxy-4-acetyl] phenoxy]propyloxyphenoxyaceticacid), L-796,449 (3-chloro-4-(3-(3-phenyl-7-propylbenzofuran-6-yloxy)propylthio)phenylacetic acid), and L-165,461 (3-chloro-4-(3-(3-ethyl-7-propylbenzisoxazol-6-yloxy)propylthio)phenylacetic acid) were provided by Gerard Kieczykowski, Philip Eskola, Joseph F. Leone, Mark S. Levorse, Peter A. Cicala, Gregory D. Berger, Robert Marquis, Conrad Santini, Soumya P. Sahoo, and Richard L. Tolman (Merck Research Laboratories, Rahway, NJ).

Purification of monocytes

Human PBMC were obtained by plasmapheresis (University of Pennsylvania, Philadelphia, PA). The cells were washed in RPMI 1640 medium that was supplemented with L-glutamine, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate and purified further using Lymphocyte Separation Medium (ICN, Aurora, OH). After centrifugation at 1500 × *g* for 30 min at room temperature, the interface containing the mononuclear cells was harvested and washed twice in complete RPMI medium. T lymphocytes were then removed using the SRBC rosetting method (26). The monocyte preparation was further washed three times with ice-cold D-PBS before the cells were used in cell-based assays.

Monocyte culture using Teflon beakers

Culture of mononuclear phagocytes in suspension by incubation on a Teflon surface to which cells do not adhere has been described previously (27, 28). Briefly, 1×10^7 monocytes were resuspended in 10 ml of RPMI 1640 medium with L-glutamine and 14% normal human serum/60-ml Teflon beaker. The loosely capped beakers were incubated at 37°C in a 5% CO_2 atmosphere. Cell recovery from each beaker was ~90%.

Culture of RAW 264.7 cells

RAW 264.7 cells (TIB-71) were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Ham's F-12 medium with L-glutamine supplemented with 10% FCS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate in an atmosphere containing 5% CO_2.

Induction of cytokine expression in monocytes and macrophages

Freshly isolated or differentiated monocytes were seeded at a density of 1×10^5 cells/well in RPMI 1640 medium with L-glutamine and 10% normal human serum into Costar 96-well tissue culture plates (Corning, Corning, NY). RAW 264.7 cells were seeded at a density of 3×10^4 cells/well in Ham's F-12 medium containing 10% FCS 15–24 h before the experiment into 96-well plates. The cells were treated with compound for 1 h before the addition of either 0.1 ng/ml ReLPS from *S. minnesota* R595 or 30 ng/ml PMA. After 4-h or overnight (18- to 24-h) incubation at 37°C in 5% CO_2 and 95% air, the conditioned cell medium was harvested. The IL-6 and TNF-α concentrations were determined by ELISA as described below.

Cytokine ELISAs

Cytokines (IL-6 or TNF-α) were quantitated using a sandwich ELISA with commercially available Abs. Briefly, 100 μl/well of 4 μg/ml solutions of mAbs to human IL-6 (MAB206, R&D Systems), mouse IL-6 (MAB406, R&D Systems), human TNF-α (MAB610, R&D Systems), or mouse TNF-α (1221-00, Genzyme, Cambridge, MA) in D-PBS were immobilized on Dynatech Immulon-4 96-well plates (Dynex Technologies, Chantilly, VA) by overnight incubation. The plates were blocked for 1 h with a blocking buffer containing 1% BSA, 5% sucrose, and 0.05% NaN_3 in D-PBS. The blocked plates were washed five times with wash solution (Kirkegaard & Perry, Gaithersburg, MD). The cell medium or plasma samples were appropriately diluted in ELISA diluent containing 1% BSA and 0.05% NaN_3 in D-PBS. The diluted supernatants were added to the wells and incubated for 2 h. The plates were then washed as described above. Biotinylated Ab (anti-human IL-6 BAF206, R&D Systems, 25 ng/ml; anti-mouse IL-6, BAF406, R&D Systems, 200 ng/ml; anti-human TNF-α, BAF210, R&D Systems, 200 ng/ml; anti-mouse TNF-α, 80-4895-01, Genzyme, 5 μg/ml) were added to the wells in ELISA diluent, and the plates were incubated for an additional 2 h. After washing, a 1/20,000 dilution of HRP-conjugated streptavidin (Zymed, San Francisco, CA) was added to the wells. The plates were incubated for 30 min. After washing, 100 μl of tetramethylbenzidine peroxidase substrate solution (Kirkegaard & Perry) was added to each well, and the color reaction was stopped by adding 50 μl of 1 M phosphoric acid. The absorbance at 450 nm was determined using a SpectraMAX 250 plate reader (Molecular Devices, Sunnyvale, CA). Cytokines were quantitated relative to a standard curve representing a range of dilutions of recombinant IL-6 or TNF-α (R&D Systems). All steps were conducted at room temperature.

RT-PCR

Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD). To remove potential contamination by genomic DNA for downstream procedures, total RNA was first treated with 1 U of DNase I (Life Technologies). Reverse transcription was then performed using the RT-for-PCR kit from Clontech Laboratories (Palo Alto, CA). Briefly, 1 μg of total RNA was incubated with 20 pmol of oligo$(dT)_{18}$, 20 U of RNase inhibitor, and 200 U of Moloney murine leukemia virus reverse transcriptase in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ in a total volume of 20 μl at 42°C for 1 h, followed by an incubation at 94°C for 5 min. Kit-provided human placental RNA (1 μg) was used as a control. Aliquots (1/25th of the RT reaction) were subjected to PCR amplification using 2 U of Taq polymerase (Fisher Biotech, Pittsburgh, PA) and a total of 35 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C). The following primers were used: human G3PDH amplimer set 5406 (Clontech), mouse G3PDH amplimer set 5409 (Clontech), and human PPARγ forward primer, 5′-GGAAAGACAACAGACAAATCAC; human PPARγ reverse primer, 5′-TGCATTGAACTTCACAGCAAAC; mouse PPARγ forward primer, 5′-TCATACATAAAGTCCTTCCC; and mouse PPARγ reverse primer, 5′-TGTCTGTCTCTGTCTTCTTG. Plasmids pSG5/hPPARγ1 encoding human PPARγ1 (5) and pSG5/mPPARγ2 containing mouse PPARγ2 cDNA (provided by Dr. Bruce Spiegelman, Dana-Farber Institute, Boston, MA) were used as positive controls in the

PCR reactions. These plasmids were constructed by subcloning the full-length cDNAs into the mammalian expression vector pSG5 (Stratagene, La Jolla, CA).

Immunoblot analysis

Purified human monocytes were cultured in Teflon beakers as described above. On days 0 and 5 in culture, cells were harvested from two beakers, and cell lysates were prepared. Cells were washed once with 10 ml of PBS (Mediatech) with protease inhibitors (0.3 U/ml aprotinin, 2 mM PMSF, 50 μg/ml benzamidine, 3 mM di-isopropyl fluorophosphate, and 5 μg/ml each of antipain, leupeptin, chymostatin, and pepstatin A). Recovered cells were directly lysed in 125 μl of SDS sample buffer for 15 min on ice. The SDS sample buffer consisted of 10% glycerol, 2% SDS, 0.03% bromophenol blue, 1 mM EDTA (pH 7.0), and 0.06 M Tris (pH 6.8) with protease inhibitors as described above. The lysates were briefly sonicated and centrifuged for 5 min at 12,000 × *g*, and supernatants were collected. The detergent-compatible protein assay (Bio-Rad) was performed to determine the protein concentration in each sample. The SDS-PAGE samples were made under reducing conditions using 250 μg of protein/well. The SDS-PAGE was run on 10% Tris-glycine gels using standard buffers (NOVEX, San Diego, CA). Following separation, protein samples were transferred to nitrocellulose filters (NOVEX) for 1.5 h at 300 mA. Filters were blocked with Superblock (Pierce, Rockford, IL) overnight at 4°C, washed twice with Tris-buffered saline/0.1% Tween-20, and incubated with mAb E-8 directed against PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml for 2 h at room temperature. The filters then were washed twice as described above and incubated with HRP-conjugated goat anti-mouse IgG diluted 1/3,000 for 2 h at room temperature. The filters were again washed as described above, and bound Ab was detected using chemiluminescence (ECL, Amersham, Arlington Heights, IL).

In vivo studies

Specific pathogen-free, 8- to 9-wk-old, male *db/db* (C57BL6/J$^{+/+}$*Lepr*^{db}) or lean control heterozygous mice (The Jackson Laboratory, Bar Harbor, ME) were housed five per cage in static microisolators and allowed ad libitum access to pelleted chow (Purina 5001, Ralston Purina, Richmond, IN) and water. The animal room was maintained on a 12-h light, 12-h dark cycle. The institutional animal care and use committee of Merck Research Laboratories reviewed and approved all animal use, and all animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources), National Research Council, Washington, DC, 1996).

The animals were treated daily for 5 days by oral gavage (0.2 ml/mouse) with vehicle (0.5% carboxymethyl cellulose) with or without AD-5075 (10 mg/kg). On day 5 of the treatment, vehicle (saline) with or without a protein-free preparation of LPS (50 μg/mouse) from *E. coli* K235 (26) was injected i.p. (0.1 ml/mouse) 1 h after the final dose of vehicle or AD-5075. Blood samples were collected into lithium heparin Microtainer tubes (Becton-Dickinson, Franklin Lakes, NJ), 90 min (tail nick) and 5 h (terminal, CO_2 overdose, cardiocentesis) after LPS or vehicle challenge. Cytokines were quantitated by ELISA as described above. Glucose and triglyceride levels were determined by hexokinase and glycerophosphate oxidase methods, respectively (Hitachi 911, Roche, Indianapolis, IN).

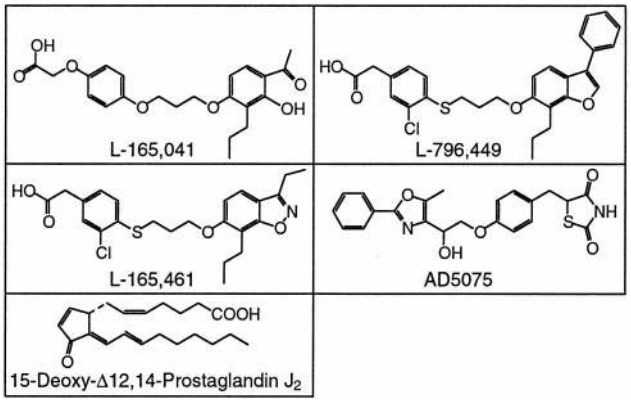


FIGURE 1. Chemical structures of PPAR agonists used in this study.

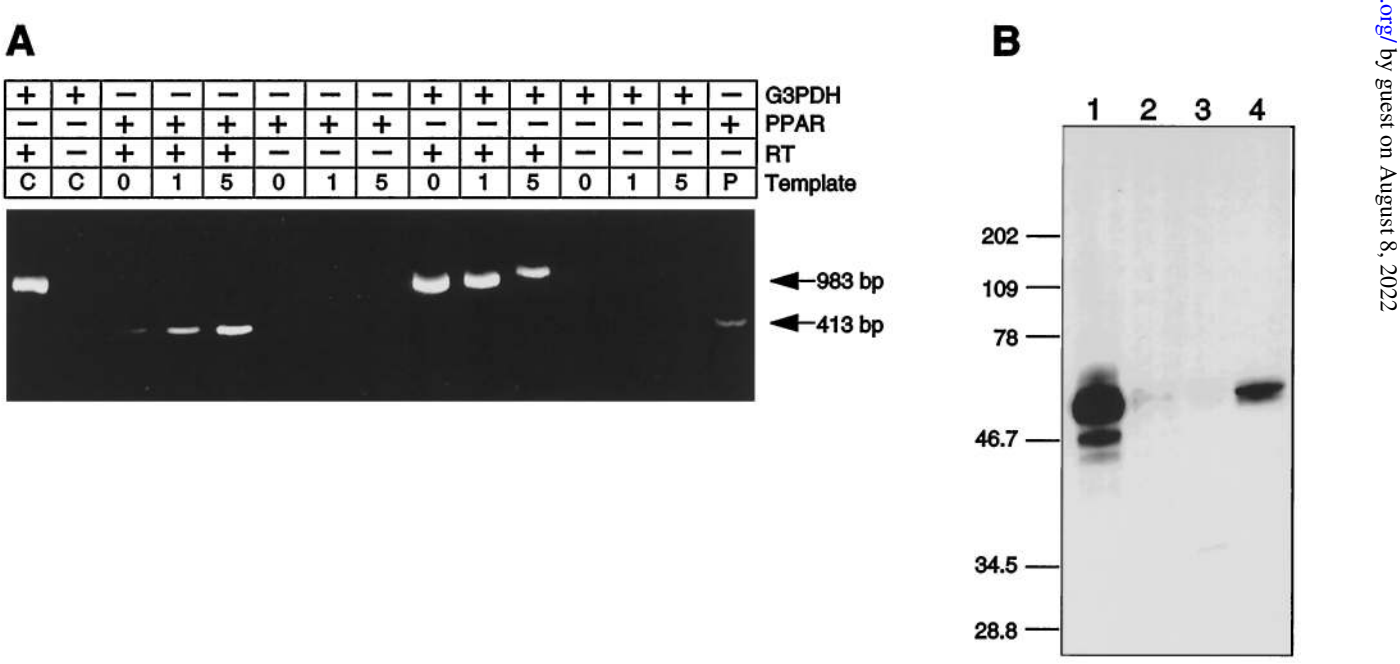


FIGURE 2. PPARγ is expressed in human peripheral blood monocytes. *A*, The RT-PCR was performed on mRNA isolated from freshly isolated human peripheral blood monocytes or after 1 or 5 days culture in Teflon beakers (designated 0, 1, and 5 in the template panel, respectively). The RT-PCR was performed with primers specific for PPARγ or G3PDH in the presence or the absence of reverse transcriptase (RT), as indicated. A plasmid containing human PPARγ cDNA (P) was used as a positive control for the PCR reaction. Kit-supplied human placental mRNA (C) was used as a positive control for the RT-PCR reaction. The positions of the expected PCR products after agarose gel electrophoresis are indicated. *B*, Immunoblot analysis of cell extracts was performed as outlined in *Materials and Methods* with a monoclonal anti-PPARγ Ab. Cell lysates were prepared from freshly isolated human peripheral blood monocytes (*lane 3*) or after 5 days of culture in Teflon beakers (*lane 4*). As controls, lysates were prepared from COS-1 cells transfected either with a mammalian expression plasmid encoding human PPARγ1, pSG5/hPPARγ1 (*lane 1*), or with the noncoding control plasmid, pSG5 (*lane 2*). The positions of the molecular mass markers (in kilodaltons) are indicated.

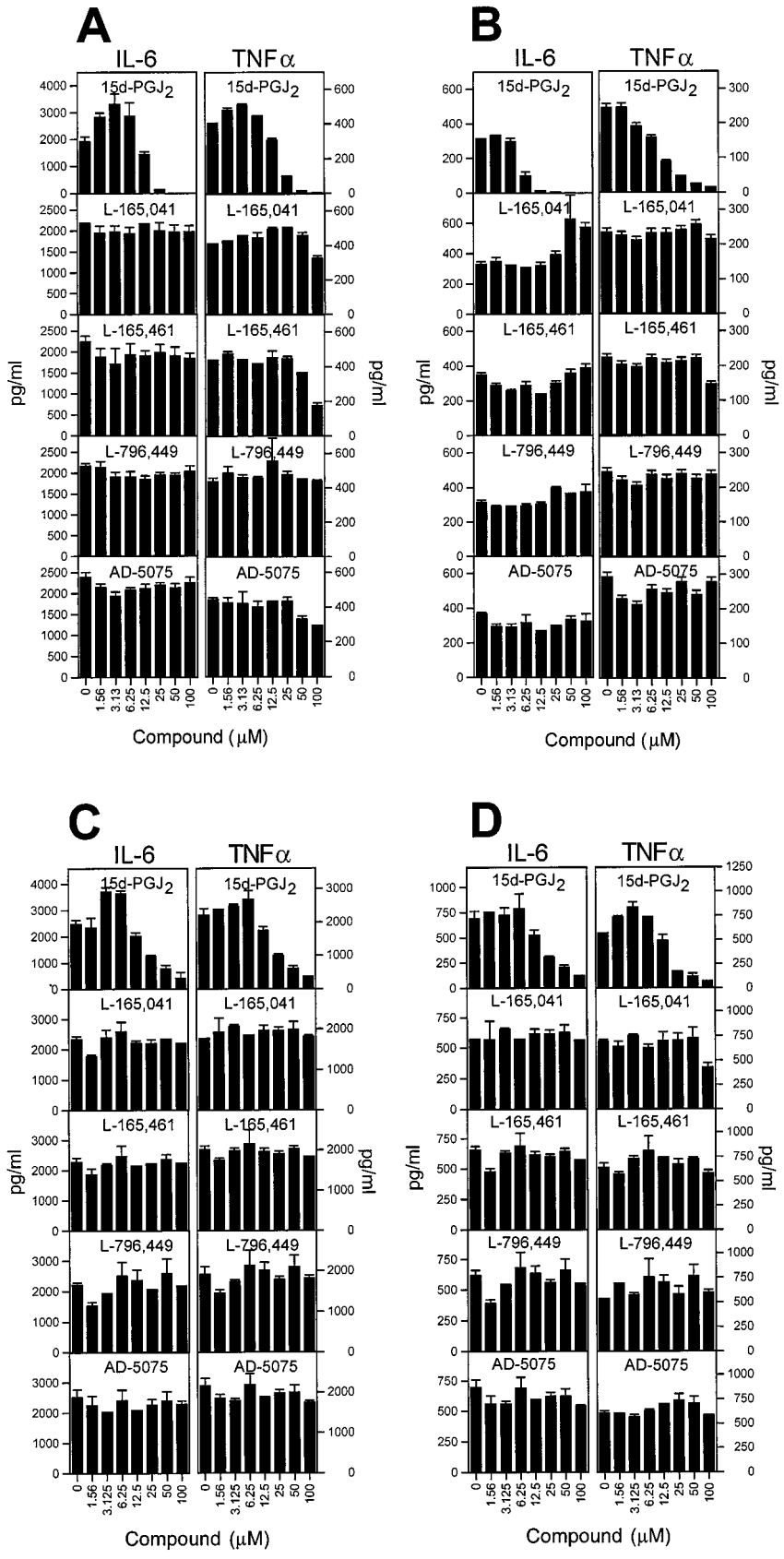
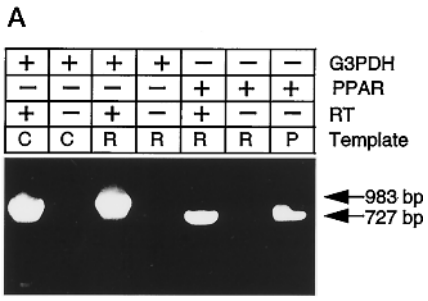


FIGURE 3. Effects of PPAR agonists on LPS- or PMA-induced cytokine expression from human peripheral blood monocytes. Peripheral blood monocytes (1×10^6 cells/well) were plated in a 96-well plate cell culture dish either immediately after isolation (*A* and *B*) or after 5-day culture in Teflon beakers (*C* and *D*) to allow for differentiation of the cells to a macrophage phenotype. Compounds were added to the wells, and after 60-min incubation, 0.1 ng/ml LPS (*A* and *C*) or 30 ng/ml PMA (*B* and *D*) were added. Cell media were harvested after 4-h (LPS) or overnight (PMA) incubation at 37°C. Cytokine levels were determined by ELISA. The results are shown as the mean of each condition performed in duplicate with SDs.



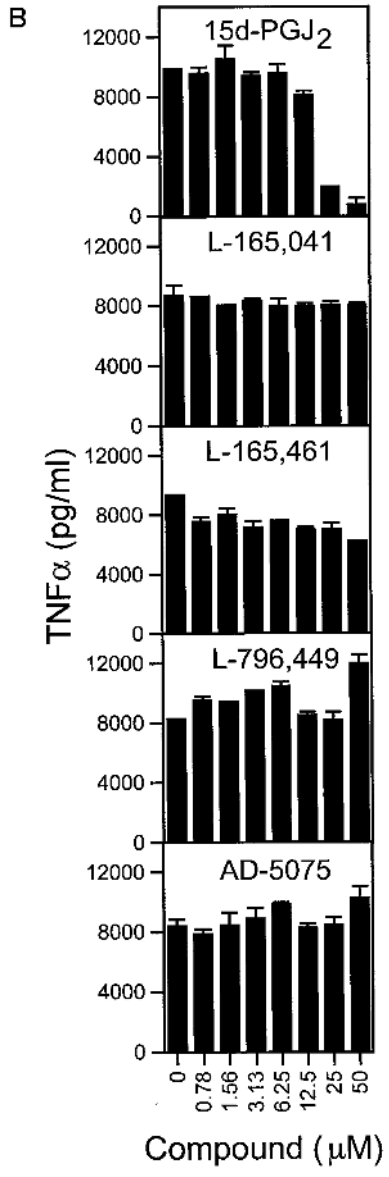


FIGURE 4. Effects of PPARγ agonists on LPS-mediated TNF-α secretion from RAW 264.7 cells. *A*, RT-PCR was performed with mRNA isolated from RAW 264.7 cells (R) using primers specific for PPARγ or G3PDH in the presence or the absence of reverse transcriptase (RT), as indicated. A plasmid, pSG5/mPPARγ2, containing mouse PPARγ2 cDNA (P) was used as a control for the PCR reaction. Kit-supplied human placental mRNA (C) was used as a positive control for the RT-PCR reaction. The positions of the expected amplification products are indicated. *B*, RAW 264.7 cells (3×10^4 cells/well) were preincubated for 1 h with the indicated compound. Then, LPS (0.1 ng/ml) was added to the cells, the medium was harvested after further incubation for 4 h, and TNF-α levels were determined by ELISA. The results are shown as the mean of each condition performed in duplicate with SDs.

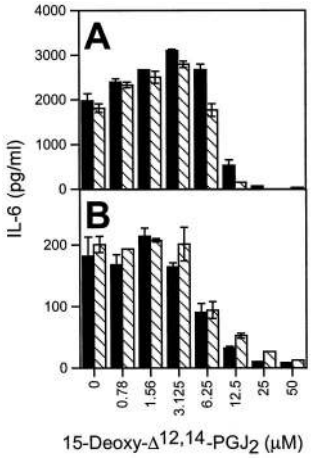


FIGURE 5. Effect of 15d-PGJ_2 on LPS- or PMA-mediated cytokine expression in the presence of a PPARγ agonist. Freshly isolated human peripheral blood monocytes (1×10^6 cells/well) were plated in 96-well plate cell culture dishes. Each well received 15d-PGJ_2 at the indicated concentration in the absence (■) and the presence (▨) of 50 μM AD-5075. After a 60-min preincubation period, 0.1 ng/ml LPS (*A*) or 30 ng/ml PMA (*B*) was added. Cell medium was harvested after 4-h (LPS) or overnight (PMA) incubation at 37°C, and cytokine levels were determined by ELISA. The results are shown are the mean of each condition performed in duplicate with SDs.

Results

PPARγ agonists do not inhibit cytokine production by monocytes or macrophages

A structurally diverse array of PPARγ agonists was selected for this study to test the hypothesis that inflammatory cytokine production can be inhibited in monocytic cells by a mechanism involving PPARγ (Fig. 1). The TZD antidiabetic agent, AD-5075, is a potent PPARγ agonist (13). The non-TZD insulin-sensitizing agents L-796,449, L-165,461, and L-165,041 serve as potent, moderate, and weak PPARγ agonists, respectively (12). Finally, the prostanoid 15d-PGJ_2 has demonstrated PPARγ agonist activity at micromolar concentrations (11) .

Before treating human monocytes with the above pharmacological agents, we examined their level of PPARγ expression. Freshly isolated human peripheral blood monocytes contained only relatively small amounts of PPARγ mRNA when assessed by RT-PCR, consistent with previous results (14, 29). In contrast, G3PDH mRNA was readily detectable (Fig. 2*A*). Cultivation of monocyte preparations in Teflon beakers leads to differentiation into cells possessing a mature monocyte phenotype (27, 28). During the maturation process, increased expression of PPARγ was observed (Fig. 2*A*). The increase in PPARγ mRNA was apparent after only 1 day of culture and was even greater following culture of the cells for 5 days. Immunoblot analysis of cell lysates using a commercially available anti-PPARγ mAb failed to demonstrate immunodetectable protein in fresh monocytes (Fig. 2*B*, *lane 3*), but resulted in strong staining after 5 days of differentiation in the Teflon dishes (Fig. 2*B*, *lane 4*). Similar results were obtained with two different anti-PPARγ peptide Abs (data not shown).

We next used the human monocyte preparations to test the effect of the previously described PPARγ agonists on LPS- or PMA-mediated cytokine induction. Freshly prepared monocytes or those previously differentiated for 5 days were incubated with each of the inflammatory agents alone or in the presence of increasing concentrations of PPARγ activators. Following these incubations (4 h for LPS; overnight for PMA), TNF-α and IL-6 concentrations

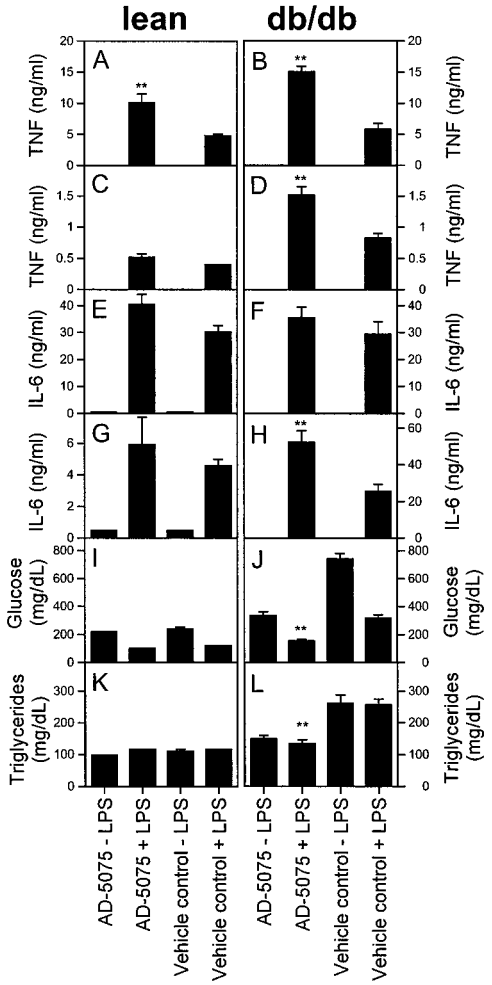


FIGURE 6. LPS challenge of *db/db* and lean mice after chronic treatment with AD-5075. Mice (10 animals/group) were treated orally with daily doses of 10 mg/kg AD-5075 or vehicle for 5 days. One hour after the last administration, 50 μg/mouse of LPS (*E. coli* K235) or vehicle was injected i.p. into the animals. Serum samples were taken 90 min (*A, B, E,* and *F*) or 5 h (*C, D,* and *G–L*) after LPS administration for cytokine, glucose, and triglyceride measurements. Results are shown as the mean ± SEM. Variations between values from the LPS-challenged groups treated with or without AD-5075 that were found to be statistically significant by Student's *t* test are indicated by asterisks (**, $p < 0.005$).

were measured in the cell medium as described in *Materials and Methods*. Unstimulated cells did not produce measurable cytokine (data not shown), while addition of LPS or PMA caused strong cytokine expression (Fig. 3). Treatment of freshly isolated monocytes (Fig. 3, *A* and *B*) or 5-day cultured monocytes (Fig. 3, *C* and *D*) with L-165,041, L-165,461, L-796,449, or AD-5075 at concentrations up to 50 μM did not inhibit the TNF-α or IL-6 synthesis and secretion caused by LPS (Fig. 3, *A* and *C*) or PMA (Fig. 3, *B* and *D*). Of all the compounds tested, only 15d-PGJ_2 significantly abrogated cellular cytokine production, and it did so in freshly isolated monocytes lacking immunodetectable PPARγ as well as in the cultured monocyte preparations (Fig. 3, *A–D*). The prostanoid effectively blocked cytokine secretion in a concentration-dependent manner, with IC_{50} values generally between 12.5 and 25 μM. Similar results were obtained when LPS incubations were allowed to proceed overnight (not shown). The above results were reliably repeated when using cell preparations from different healthy donors.

To examine the effects of PPARγ agonists on cytokine induction in murine cells, RAW 264.7 cells, a well-established mouse tumor cell line with a mature macrophage phenotype (30), were used. It has previously been demonstrated by researchers examining the anti-inflammatory effects of PPARγ agonists that these cells express PPARγ, albeit at low levels (24). We confirmed the expression of PPARγ in our RAW 264.7 culture by RT-PCR (Fig. 4*A*). Similar to the results we observed with human monocytes, all the PPARγ agonists, with the exception of 15d-PGJ_2, were ineffective in inhibiting production of TNF-α in responses to LPS in RAW 264.7 cells (Fig. 4*B*). In contrast, the prostaglandin once again blocked the induction of cytokine production in a dose-responsive manner.

We further investigated whether the effectiveness of 15d-PGJ_2 for cytokine suppression could be ablated by simultaneous addition of a saturating concentration of a TZD PPARγ agonist that had proven ineffective (as described above) as an anti-inflammatory agent. Treatment of fresh human monocytes with 15d-PGJ_2 resulted in almost identical inhibition curves for IL-6 production regardless of whether 50 μM AD-5075 was absent or present during the incubation. The results presented were obtained after stimulation of the cells with LPS for 4 h (Fig. 5*A*) or after overnight incubation with PMA (Fig. 5*B*), and nearly identical results were observed when TNF secretion was determined or when overnight incubations with LPS were used as the stimulus (data not shown). Similar observations were made when AD-5075 was replaced by the other PPARγ agonist used in this study (not shown).

In aggregate, our results do not support the conclusion that activation of PPARγ serves to inhibit inflammatory responses of monocytic cells in vitro. In contrast, 15d-PGJ_2 appears to possess anti-inflammatory activity that is most likely mediated by a PPARγ-independent mechanism.

Chronic treatment with a potent PPARγ agonist fails to reduce LPS-induced cytokine production in mice

Animal studies were conducted to determine whether macrophages in vivo respond to the PPARγ agonist, AD-5075, and to allow prolonged treatment of the cells (5 days). Importantly, these studies allowed a positive measure of AD5075 efficacy. Obese, diabetic *db/db* mice were used in these experiments because their responsiveness to AD-5075 treatment, previously demonstrated to be mediated through activation of PPARγ (13), can be easily monitored by measuring decreases in the animals' elevated blood glucose and triglyceride levels. The effects of this TZD were also examined in metabolically normal, lean mice. The animals were treated daily with 10 mg/kg of AD-5075 orally for 5 days. Subsequently, they were injected i.p. with 50 μg/mouse of LPS to induce acute inflammation, and plasma levels of TNF-α and IL-6 were measured at 90 min and 5 h postinjection as described in *Materials and Methods*.

Treatment of *db/db* mice with the PPARγ agonist significantly lowered blood glucose and triglyceride levels (all $p \leq 0.005$; Fig. 6, *J* and *L*) to approximately those observed in lean mice (Fig. 6, *I* and *K*). As previously described (13), AD-5075 did not lower glucose or triglyceride levels in the lean mice. Glucose levels were lowered in normal and diabetic animals by the acute administration of LPS (Fig. 6, *I* and *J*). Such hypoglycemic effects of LPS in *db/db* and lean mice are well documented (31) and were, therefore, expected.

The synthesis and secretion of proinflammatory cytokines such as TNF-α and IL-6 by activated monocytes and macrophages are two of the hallmarks of the host response to endotoxin (32–34).

Following injection of *db/db* and lean mice with LPS, plasma levels of these cytokines increased dramatically at both 90 min and 5 h compared with those in mice that did not receive LPS (Fig. 6, *A–H*). AD-5075 pretreatment did not diminish plasma cytokine levels in mice subsequently administered LPS. Rather, chronic treatment with the potent PPARγ agonist resulted in increases in TNF-α and IL-6 plasma levels at both time points after LPS administration. These increases reached high statistical significance ($p < 0.005$) for TNF-α at 90 min in both the lean (Fig. 6*A*) and*db/db* (Fig. 6*B*) mice as well as for TNF-α (Fig. 6*D*) and IL-6 (Fig. 6*F*) at 5 h in *db/db* mice. Additional studies examined LPS-induced plasma nitrate and nitrite production resulting from the in-duction of inducible nitric oxide synthase. The LPS-induced nitrite/nitrate production was not significantly altered in *db/db* mice using the TZD treatment protocol (data not shown).

These results support the conclusion that activation of PPARγ does not blunt the production of TNF-α, IL-6, or iNOS in vivo when the challenge is LPS.

Discussion

Macrophages play a key role in local inflammatory responses in the vascular wall, contributing, for example, to the formation and progress of atherosclerotic lesions (35, 36). It was previously demonstrated that lesional macrophages from human atheroma (the so-called foam cells) and cultured monocyte-derived macrophages, differentiated by adherence, express PPARγ mRNA and protein (14, 29, 37). Recent in vitro data suggested that activation of macrophage PPARγ by 15d-PGJ_2 as well as other synthetic PPARγ ligands inhibited the expression of proinflammatory agents, macrophage scavenger receptor A, and inducible nitric oxide synthase; matrix metalloproteinase-9 and gelatinase activities were also diminished (23, 24, 29). On the basis of transfection studies performed in macrophage-like cell lines, antagonism of the transcription factors AP-1, NF-κB, and STAT was implicated as the mechanism of the observed anti-inflammatory effects (24). These findings suggested the possibility of using PPARγ agonists in novel treatment protocols for acute and chronic inflammatory diseases that involve activated macrophages, such as atherosclerosis and rheumatoid arthritis.

Our experiments were aimed at further exploring the anti-inflammatory potential of PPARγ agonists. We initially chose in vitro experiments that model a key function of monocytes and macrophages, the synthesis and secretion of cytokines in response to an inflammatory stimulus such as bacterial LPS. These cellular responses strongly depend upon the maximal activation of cytokine expression by transcription factors such as NF-κB and AP-1. We tested the effects of TZD and non-TZD PPARγ agonists of varying potencies in freshly cultured and differentiated human monocytic cells after stimulation with either LPS or PMA. Our results demonstrated that regardless of the state of monocyte differentiation and PPARγ expression, both synthetic TZD and non-TZD PPARγ agonists were without effect. Similar results were obtained with the murine macrophage cell line RAW 264.7. In addition to the compounds presented here, other TZD PPARγ agonists were also ineffective in suppressing cytokine release (data not shown). Our results make it unlikely that PPARγ agonists would demonstrate anti-inflammatory effects in vivo through a mechanism involving the repression of NF-κB and/or AP-1.

The observations that PPARγ is expressed in monocytic cells and that the naturally occurring prostaglandin D_2 metabolite 15d-PGJ_2 is a PPARγ agonist have suggested a potential role for this receptor not only in lipid metabolism but also in control of inflammation (10, 11, 38). When tested in our monocyte assays, 15d-PGJ_2 was, indeed, the only PPARγ agonist that effectively blocked LPS- or PMA-induced cytokine expression. Based on the full weight of the data, it appears unlikely that 15d-PGJ_2 effects are directly mediated through PPARγ activation. The anti-inflammatory potency of 15d-PGJ_2 in our experiments and in those previously published (23, 24) substantially exceeded that of other more potent TZD and non-TZD PPARγ agonists. Importantly, we demonstrate here that synthetic PPARγ ligands were unable to block the inhibitory activity of 15d-PGJ_2 when used at concentrations that should displace the prostaglandin all but completely from the receptor. These findings, we believe, support the contention that 15d-PGJ_2 may act through mechanisms not involving PPARγ. Vaidya et al. have rendered a similar conclusion from studies showing that 15d-PGJ_2, but not AD-5075, inhibited responses of neutrophils to TNF-α and formyl peptides (39). It was hypothesized that the prostanoid may exert its effects through interaction with an as yet unknown prostaglandin receptor. In neutrophils, this receptor appears to be unrelated to the prostaglandin D_2 receptor, because a potent specific agonist of the receptor did not affect peroxide production. Given the similarity of these observations, it is conceivable that monocytes and neutrophils share a common signaling pathway initiated by 15d-PGJ_2. Collectively, these findings emphasize the need to exercise caution when interpreting results obtained with 15d-PGJ_2 and the importance of studying the actions of a broad spectrum of PPARγ agonists before invoking this receptor as a mediator of critical biological responses.

The above studies suggest that PPARγ does not affect the acute response of macrophages to a stimulant such as LPS. Macrophages exhibit a distinct phenomenon, macrophage activation, which entails differentiation to a state characterized by unique patterns of gene expression and responsiveness to stimuli. PPARγ was first described as a nuclear receptor that plays a critical role in adipocyte differentiation (1, 7, 8), and it is thus possible that PPARγ may critically regulate monocyte activation or differentiation. Because monocyte differentiation is controlled by poorly defined factors acting locally in tissues, we tested the role of PPARγ agonists in animals by measuring responses of animals to challenges with LPS, a widely used model of acute inflammation. The use of obese diabetic mice (*db/db*) in the study in addition to normal lean animals allowed us to determine the effectiveness of the TZD treatment on PPARγ activity by measuring the decline in blood glucose and triglyceride levels at the time of endotoxin administration (12). In both lean and *db/db* mice the chronically administered PPARγ agonist did not show effects on IL-6 and TNF-α production and, therefore, confirmed the results we had previously obtained in vitro with cultured monocytes and macrophages. Instead, the blood levels of TNF-α and IL-6 in the mice actually increased, often significantly, compared with those in vehicle-treated animals. Using the same treatment protocol, similar results were obtained with C57BL/6J mice maintained on either a low fat diet or a high fat, obesity-inducing diet (data not shown). The rise in LPS-induced cytokine production is consistent with PPARγ playing a role in macrophage activation or differentiation, but with effects opposite those proposed by prior authors.

Recent studies of a homologous transcription factor, PPARα, are consistent with our studies of PPARγ. Hill et al. reported that chronic treatment of animals with PPARα agonists (fenofibrate and Wy-14,643) results in a marked increase in TNF-α levels and significantly lowers 50% lethal doses of LPS in a mouse model of endotoxemia (40). Although these agents modestly down-regulated TNF expression in primary macrophages in vitro (40), important differences between PPARα and PPARγ must be noted. The PPARα agents both increase liver weight and decrease serum

lipoprotein levels. Because hepatic macrophages may contribute importantly to plasma TNF levels, and because lipoproteins may strongly neutralize LPS, these two effects may explain the larger increase in cytokine levels observed by Hill et al. (40).

Our data cannot rule out the possibility that the activation of PPARγ may prove effective in antagonizing macrophage function in other settings. Certain stimuli, for example treatment with live or killed bacteria, are known to raise serum IFN-γ levels in animals and produce hypersensitivity to the effects of LPS (41, 42). It is conceivable that under these experimental conditions PPARγ agonists would prove effective in alleviating inflammation. Interestingly, mice with a targeted deletion of the IFN-γ receptor demonstrate a significant decrease in their disposition to develop atherosclerotic lesions, suggesting that macrophage activation driven by IFN-γ promotes atherosclerosis (43). As mentioned above, PPARγ is expressed in macrophages from atherosclerotic lesions (24, 29), and recently published data provided evidence that a PPARγ agonist, troglitazone, can effectively reduce atherosclerosis in animals (44). It is therefore likely that PPARγ agonists may not act globally as regulators of all inflammatory mediators but, rather, may control only a specific subset of proinflammatory genes. In keeping with this idea, recent studies have observed that both PPARα and PPARγ agonists failed to block production of the cytokine IL-8, but at the same time strongly inhibited MMP-9 secretion from a monocytic cell line, THP-1 (H. Shu, B. Wong, G. Zhou, Y. Li, J. P. Berger, J. W. Woods, S. D. Wright, and T.-Q. Cai, manuscript in preparation).

In summary, we have shown that PPARγ agonists other than 15d-PGJ_2 do not inhibit cytokine production in in vitro and in vivo models of acute inflammation. The prostanoid appears to exert its actions via a PPARγ-independent mechanism. The results from our study raise significant doubts about the potential global utility of PPARγ agonists as anti-inflammatory agents. Rather, PPARγ agonists may function selectively by regulating proinflammatory genes involved in the development of inflammatory diseases such as atherosclerosis. This may at least in part explain the protective role of activators of PPARγ in atherosclerosis (44).

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