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Oxidized Low Density Lipoprotein Inhibits Interleukin-12 Production in Lipopolysaccharide-activated Mouse Macrophages via Direct Interactions between Peroxisome Proliferator-activated Receptor-γ and Nuclear Factor-κB*

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Lipopolysaccharide (LPS) increases the production of interleukin-12 (IL-12) from mouse macrophages via a κB site within the IL-12 p40 promoter. In this study, we found that oxidized low density lipoprotein (oxLDL) inhibited this LPS-stimulated production of IL-12 in a dose-dependent manner while native LDL did not. OxLDL inhibited p40 promoter activation in monocytic RAW264.7 cells transiently transfected with p40 promoter/reporter constructs, and the repressive effect mapped to a region in the p40 promoter containing a binding site for nuclear factor-κB (NF-κB) (p40-κB). Activation of macrophages by LPS in the presence of oxLDL resulted in markedly reduced binding to the κB site, as demonstrated by the electrophoretic mobility shift assays. In contrast, native LDL did not inhibit the IL-12 p40 promoter activation and NF-κB binding to the κB sites, suggesting that oxidative modification of LDL was crucial for the inhibition of NF-κB-mediated IL-12 production. 9-Hydroxyoctadecadienoic acid, a major oxidized lipid component of oxLDL, significantly inhibited IL-12 production in LPS-stimulated mouse macrophages and also suppressed NF-κB-mediated activation in IL-12 p40 promoter. The NF-κB components p50 and p65 directly bound peroxisome proliferator-activated receptor-γ (PPAR-γ) *in vitro*. In cotransfections of CV-1 and HeLa cells, PPAR-γ inhibited the NF-κB transactivation in an oxLDL-dependent manner. From these results, we propose that oxLDL-mediated suppression of the IL-12 production from LPS-activated mouse macrophages may, at least in part, involve both inhibition of the NF-κB-DNA interactions and physical interactions between NF-κB and PPAR-γ.

Interleukin (IL)[1] 12, a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes, was originally identified in the supernatant fluid of Epstein-Barr virus-transformed human B-cell lines (1, 2). IL-12 is produced by phagocytic cells and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products (3, 4). IL-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of interferon-γ (IFN-γ), which in turn augments their cytotoxicity, and by enhancing their proliferation potential. IL-12 production is critical for the development of T helper type 1 (Th1) cells and the initiation of cell-mediated immune responses (reviewed in Ref. 5). The key role of IL-12 in inflammation as well as the cell-mediated immune responses (6, 7) has raised considerable interests in the mechanisms of IL-12 gene transcription. Inducible expression of IL-12 has been documented in macrophages and dendritic cells after stimulation by microbial antigens or via CD40-CD40L interaction (8, 9). In lipopolysaccharide (LPS)- and IFN-γ-treated monocytes, the expression of IL-12 p40 has been shown to be primarily regulated at the transcriptional level, which involved at least two transcription factors that belong to the NF-κB and Ets families (10–12). Expression of IL-12 p35 is also known to be subject to similar transcriptional regulation, although characterized to a much lesser extent than p40 (13, 14).

The low density lipoprotein (LDL) particle acquires a number of important biological activities as a result of oxidative modification. Oxidized LDL (oxLDL) is both a potent chemoattractant for circulating monocytes and a potent inhibitor of resident macrophage motility (15). OxLDL has also been shown to be a powerful regulator of macrophage gene expression. A number of genes involved in the inflammatory response, including those encoding tumor necrosis factor-α, IL-1α, IL-1β, IL-6, and platelet-derived growth factor, are known to be modulated by exposure to oxLDL (16–18).

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of nuclear receptor superfamily of ligand-dependent transcription factors that is predominantly expressed in adipose tissue, adrenal gland, and spleen (19, 20). The PPAR-γ is related to the T_3 and vitamin D_3 receptors and bind to a hexameric direct repeat as a heterodimeric complex with retinoid receptor Xα. PPAR-γ serves as a transcription regulator of genes involved adipocyte lipid metabolism (21). Naturally occurring compounds such as fatty acids and the prostaglandin

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[1] The abbreviations used are: IL, interleukin; IFN-γ, interferon-γ; Th1, T helper type 1; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; oxLDL, oxidized low density lipoprotein; PPAR-γ, peroxisome proliferator-activated receptor-γ; 9-HODE, 9-hydroxyoctadecadienoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LDL, low density lipoprotein; TBARS, thiobarbituric acid reactive substance.

D_2 metabolite 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 bind to PPAR-γ and stimulate transcription of target genes (22, 23). OxLDL is also known to stimulate PPAR-γ expression in macrophages and monocytic cell lines (24). Recently, two major oxidized lipid components of oxLDL, 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), were identified as endogenous activators and ligands of PPAR-γ (25), suggest that the oxLDL particle itself may be a source of endogenous PPAR-γ ligand. In addition, PPAR-γ agonists such as thiazolidinediones (oral anti-diabetic agents) and a variety of nonsteroidal anti-inflammatory drugs suppress monocyte elaboration of inflammatory cytokines (26). Inhibition of cytokine production may help to explain the incremental therapeutic benefit of nonsteroidal anti-inflammatory drugs observed in the treatment of rheumatoid arthritis.

The transcription factor NF-κB is important for the inducible expression of a wide variety of cellular and viral genes (reviewed in Ref. 27). NF-κB is composed of homo- and heterodimeric complexes of members of the Rel (NF-κB) family of polypeptides. In vertebrates, this family comprises p50, p65 (RelA), c-Rel, p52, and RelB. These proteins share a 300-amino acid region, known as the Rel homology domain, which binds to DNA and mediates homo- and heterodimerization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105, and p100 (28). In the majority of cells, NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins. Treatment of cells with various inducers results in the degradation of IκB proteins. The bound NF-κB is released and translocates to the nucleus, where it activates appropriate target genes. Interestingly, members of steroid receptors including glucocorticoid receptor (29, 30), estrogen receptor (31, 32), progesterone receptor (33), and androgen receptor (34), have been shown to inhibit NF-κB activity and can physically interact with NF-κB *in vitro*. Since RelA represses ligand-dependent activation of steroid receptor-regulated promoters, a mutually inactive complex formed by a direct protein-protein interaction of steroid receptors and RelA has been proposed.

In this report, we have demonstrated that oxLDL inhibited IL-12 production in LPS-stimulated mouse macrophages while native LDL did not. The experimental results indicate that oxLDL-mediated suppression of the IL-12 production from LPS-activated macrophages may involve, at least in part, both inhibition of the NF-κB-DNA interactions and direct interactions between PPAR-γ and NF-κB.

EXPERIMENTAL PROCEDURES

Mice, Cell Lines, Culture Medium, and Transient Transfection—Female DBA/2 mice were obtained from the Japan SLC, Inc. (Tokyo, Japan) and used at 6–10 weeks of age. RAW264.7 cells, CV-1 cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics (Life Technologies, Inc.). Spleen cell populations and macrophages from mice were maintained in RPMI 1640 supplemented with 10% FBS. For transfections, cells were grown in 24-well plates with medium supplemented with 10% FBS for 24 h and transfected with indicated plasmid in the presence of Superfectam according to the manufacturer's protocol (Qiagen, Germany). After 20 h, cells were washed and refed with DMEM containing 10% FBS. Cells were harvested 20 h later, luciferase activity was assayed as described previously (35), and the results were normalized to the *LacZ* expression. Similar results were obtained in more than two separate experiments.

Monoclonal Antibodies, Cytokines, and Reagents—Anti-IL-12 p40 mAbs C17.8 and C15.6 (36) were purified from ascitic fluid by ammonium sulfate precipitation followed by DEAE-Sephagel chromatography (Sigma). Anti-IL-12 p35 mAb Red-T/G297–289 was obtained from PharMingen (San Diego, CA). Recombinant murine IL-12 was generously provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). LPS (from *Escherichia coli* 0111:B4) was purchased from Sigma. 9-HODE and 13-HODE were obtained from BIOMOL Research Laboratory, Inc. (Plymouth Meeting, PA).

Preparation and Modification of Lipoproteins—Human LDL (d = 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation of plasma from healthy donors, and LDL and oxidized LDL (oxLDL) were made as described previously (37). In brief, oxidation of LDL was performed by incubating 0.1 mg of LDL protein/ml in phosphate-buffered saline containing 5 μM $CuSO_4$ for 24 h at 37 °C and stopped by adding butylated hydroxytoluene (2, 6-di-*t*-butyl-*p*-cresol) (Sigma) to a final concentration of 0.1 mM. Oxidized LDL was separated from $CuSO_4$ and equilibrated into the cell culture medium over a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden). All reagents were endotoxin-free. LPS levels of LDL preparations were confirmed with a chromogenic *Limulus* assay (38) and contained <0.3 pg of LPS/μg of LDL protein. The extent of oxidation of the lipoprotein preparations was determined by the thiobarbituric acid reactive substance (TBARS) assay (39). The native LDL had <3 nM TBARS/mg of cholesterol, whereas the oxidized LDL had 20–25 nM TBARS/mg of cholesterol.

Plasmids—The −689/+98 fragment of mIL-12 p40 promoter from pXP2 (11) was subcloned into *Kpn*I/*Xho*I sites of pGL3-basic luciferase vector (Promega Co., Madison, WI). All the deletion mutants were generated by polymerase chain reaction using an upstream primer containing *Bam*HI site. A linker-scanning mutant was generated by a two-step polymerase chain reaction procedure with overlapping internal primers that contain mutated sequences for the NF-κB site. The PPAR-γ gene fragment (Dr. Spiegelman, Dana-Farber Cancer Institute, Boston, MA) was subcloned into *Kpn*I-*Eco*RI/*Dra* restriction site of the CMV/T7 expression vector (Invitrogen, San Diego, CA). The reporter constructs κB-Luc, Gal4/p65, GST/p65. GST/p50 fusion protein, mammalian expression vectors for p65, and the transfection indicator pRSV-β-gal were constructed as described previously (40).

Preparation of Splenic Macrophages Stimulated with LPS—Spleen cells were cultured at 10^6 cells/ml for approximately 3 h at 37 °C. The nonadherent cells were removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The adherent cells were removed from plates by incubating for 15 min with ice-cold phosphate-buffered saline and rinsing repeatedly. The isolated adherent cell population was stimulated with 5 μg/ml LPS in the absence or presence of LDL or oxLDL at 1, 5, 10, 20, and 50 μg/ml at 1×10^5 cells/well in 96-well culture plates for 48 h. In some experiments, the cells were stimulated with LPS in the absence or presence of ciglitazone, a selective PPAR-γ agonist (BIOMOL).

Cytokine Assays—The quantities of IL-12 p40 and IL-12 p70 in culture supernatants were determined by sandwich ELISAs using mAbs specific for each cytokine, as described previously (41). The mAbs for coating the plates and the biotinylated second mAbs were as follows: for IL-12 p40, C17.8 and C15.6; for IL-12 p70, Red-T/G297–289 and C17.8. Standard curves were generated using recombinant cytokine. The lower limit of detection was 30 pg/ml for IL-12 p40 and 50 pg/ml for IL-12 p70.

Electrophoretic Mobility Shift Assay—The nuclear extracts were prepared from the cells, as described previously (42). An oligonucleotide containing an NF-κB-binding site within the Ig κ-chain (5′-CCG GTT AAC AGA GGG GGC TTT CCG AG-3′) was used as a probe. Labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 μg of nuclear extracts, in 20 μl of binding buffer (10 mM Tris·HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI-dC), and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris borate buffer. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

GST Pull-down Assay—The GST fusions or GST alone was expressed in *E. coli*, bound to glutathione-Sepharose-4B beads (Amersham Pharmacia Biotech), and incubated with labeled proteins expressed by *in vitro* translation by using the TNT-coupled transcription-translation system, which conditions as described by the manufacturer (Promega, Madison, WI). Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described (43).

Statistical Analysis—Student's t test was used to determine the statistical differences between various experimental and control groups. A p value of <0.01 was considered as significant.

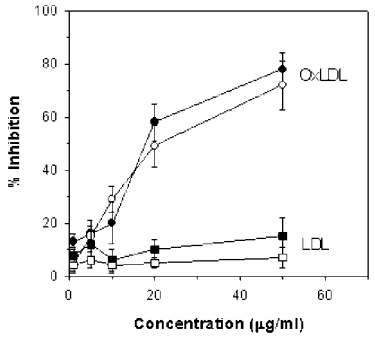

FIG. 1. **Inhibition of IL-12 production in primary macrophages by oxLDL.** Macrophages were stimulated with LPS (5 μg/ml) in the absence or presence of varying concentrations of LDL or oxLDL. Cytokine levels were evaluated by ELISA, and results are presented as mean ± standard deviations of the percentage response of cytokine production of the treated macrophages compared with untreated control macrophages stimulated with LPS. Mean cytokine levels in the absence of LDL or oxLDL were as follows: IL-12 p70, 1.7 ng/ml; IL-12 p40, 3.5 ng/ml. *Closed* and *open symbols* indicate the IL-12 p70 heterodimer and the IL-12 p40, respectively.

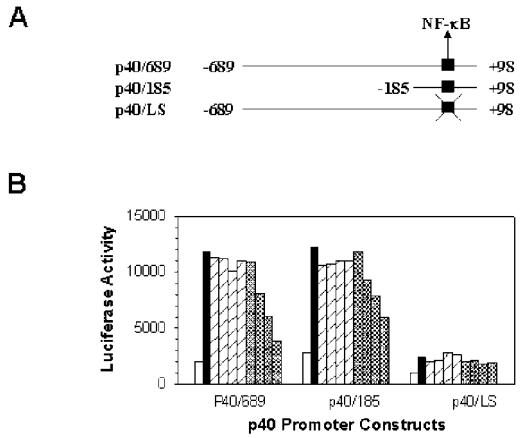

FIG. 2. **Analysis of oxLDL-mediated transcriptional repression of p40 promoter constructs activated by LPS.** *A*, schematic representation of the mouse p40 promoter constructs as well as a linker-scanning mutant for NF-κB site are as shown, along NF-κB binding site. The nucleotide sequence numbers for each construct are shown. *B*, transient transfection of RAW264.7 cells with the p40 promoter constructs, followed by stimulation with LPS in the absence or presence of LDL or oxLDL. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions, and the standard deviations are less than 5%. *Open*, *closed*, *striped*, and *checked* boxes indicate no LPS added, 5 μg/ml LPS, 5 μg/ml LPS plus LDL (1, 10, 50, and 100 μg/ml each), and 5 μg/ml LPS plus oxLDL (1, 10, 50, and 100 μg/ml each), respectively. The data are representative of three similar experiments.

RESULTS

OxLDL Inhibits IL-12 Production from LPS-activated Macrophages—We examined the effect of oxLDL on the production of IL-12 by primary macrophages stimulated with LPS. LPS readily induced the production of IL-12 heterodimer as well as the p40 subunit, as expected. However, oxLDL inhibited this LPS-induced IL-12 production in a dose-dependent manner while native LDL did not (Fig. 1). By trypan blue exclusion assay, we found that native LDL and oxLDL at concentrations of $<$100 μg/ml were not toxic to the cells (viability = 100%).

OxLDL Inhibits NF-κB-mediated Activation of IL-12 p40 Promoter by LPS—An IL-12 p40 subunit was known as the highly inducible and tightly regulated component of IL-12 (5). To identify the region involved in these oxLDL actions, we generated a series of luciferase reporter constructs containing the p40 promoter sequences from positions −689 and −185 to +98 relative to the transcription initiation site (Fig. 2*A*). Mouse RAW264.7 monocytic cells were transfected with each of these constructs and stimulated with LPS either in the absence or presence of LDL (or oxLDL), and the luciferase activity was determined. All of these constructs showed strong stimulation with LPS in the absence of oxLDL but impaired stimulation with oxLDL (Fig. 2*B*). In particular, deleting sequences to −185 (p40/185) did not diminish the LPS-dependent promoter activities and the inhibitory effect of oxLDL was still observed, suggesting that the target site for oxLDL should reside within this region. To directly test the role of a κB site found between −121 and −131 of the p40 promoter in the oxLDL-mediated inhibitory actions, we introduced a linker scanning mutation into the κB site within the context of the −689/+98 construct (p40/LS). The LPS-dependent promoter activation was still observed with p40/LS although significantly reduced (Fig. 2*B*), consistent with the previous findings in which the κB site was shown to be important for the LPS induction of p40 promoter (10). However, addition of oxLDL to LPS-stimulated cells did not have any repressive effects with p40/LS, clearly indicating that the inhibitory effect of oxLDL on IL-12 production was mediated through the κB site. In contrast, in consistent with the experiment of IL-12 production, native LDL could not significantly inhibit the LPS induction of p40 promoter activity (Fig. 2*B*).

Next, to determine whether oxidized components in the oxLDL were crucial for the inhibition of IL-12 production in mouse macrophages, the cells were stimulated with LPS in the absence or presence of 9-HODE or 13-HODE, two major oxidized lipid components of oxLDL (25). Afterward, the IL-12 levels in culture supernatants were determined. Like oxLDL, 9-HODE and 13-HODE significantly inhibited IL-12 production in LPS-activated mouse macrophages (Fig. 3*A*). In addition, 9-HODE significantly suppressed NF-κB-mediated IL-12 p40 promoter activation (Fig. 3*B*).

Physical Interaction of PPAR-γ with NF-κB—With the precedents of direct physical interactions of NF-κB with steroid receptors (29–34), we hypothesized that associations of NF-κB with PPAR-γ may have led to the NF-κB-inhibitory action of oxLDL. Indeed, *in vitro* translated, labeled PPAR-γ interacted with GST fusions to the NF-κB components p50 and p65 but not with GST alone, in a ligand-independent manner (Fig. 4). The band intensity is not significantly different between groups in the absence or presence of the ligand, as demonstrated by densitometric analysis.

NF-κB Binding to the κB Site Inhibited by OxLDL—Steroid receptors have been shown to inhibit NF-κB binding to κB sites in a ligand-dependent manner (29–34). To examine whether oxLDL-mediated inhibition of the NF-κB transactivation also exploits similar mechanisms, we analyzed the κB binding activity present in nuclear extract of unstimulated or LPS-stimulated macrophages, either in the absence or presence of oxLDL or LDL. As expected, nuclear extracts from LPS-stimulated macrophages exhibited strong κB binding activity in the electrophoretic mobility shift assays using a labeled oligonucleotide containing a consensus Ig-κB site (44) (Fig. 5). The binding was specific since it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, nonspecific oligonucleotide, and was absent with nuclear extracts from unstimulated cells. Similar to steroid receptors, nuclear extracts from macrophages stimulated by LPS in the presence of oxLDL showed much diminished κB binding activities (Fig. 5). In contrast, native LDL had no effect on the LPS-induced DNA-binding of NF-κB to the κB site.

An Inhibitory Complex of NFκB-PPARγ—To test if this oxLDL-mediated inhibition of NF-κB activities in macrophages are generally observed in other cell types, we employed a re-

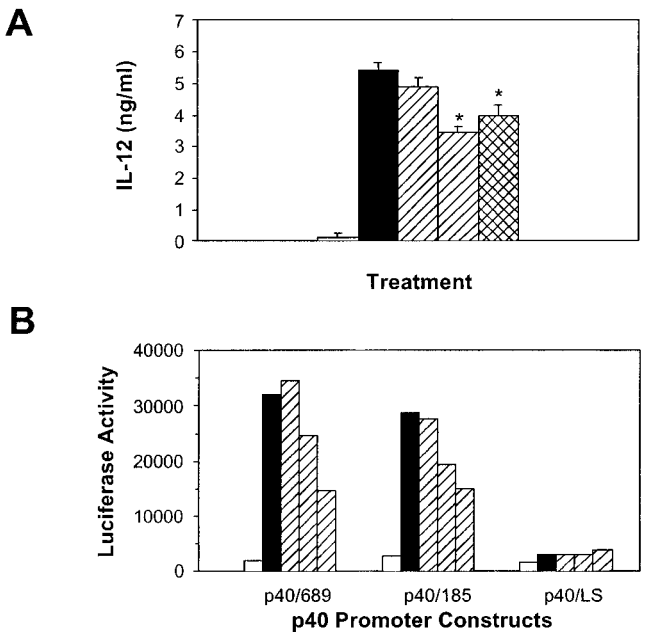

FIG. 3. **Inhibition of IL-12 production in LPS-activated macrophages by HODE.** *A*, macrophages were stimulated with LPS (5 μg/ml) in the absence or presence of 9-HODE or 13-HODE. IL-12 levels were evaluated by IL-12 p40 ELISA, and results are presented as mean ± standard deviations of triplicate determinations. *Open*, *closed*, *striped*, and *checked boxes* indicate no LPS added, 5 μg/ml LPS, 5 μg/ml LPS plus 9-HODE (1.25, 2.5 μg/ml), and 5 μg/ml LPS plus 13-HODE (1.25 μg/ml), respectively. *, $p < 0.01$, relative to LPS-stimulated group in the absence of 9-HODE or 13-HODE. *B*, transient transfection of RAW264.7 cells with the p40 promoter constructs, followed by stimulation with LPS in the absence or presence of 9-HODE. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions, and the standard deviations are less than 5%. *Open*, *closed*, and *striped boxes* indicate no LPS added, 5 μg/ml LPS, and 5 μg/ml LPS plus 9-HODE (0.1, 1.0, and 10 μg/ml each), respectively.

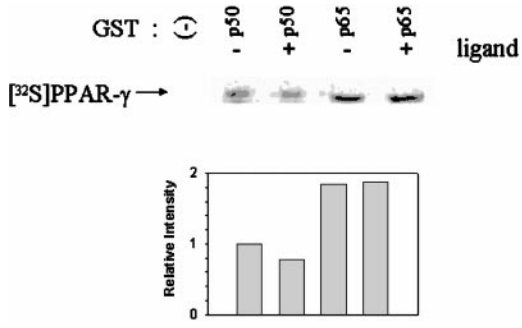

FIG. 4. **Interactions of PPAR-γ with p50 and p65 *in vitro*.** The wild type PPAR-γ was labeled with [^{35}S]methionine by *in vitro* translation and incubated with glutathione beads containing GST alone or GST fusions to p50 and p65, either in the absence or presence of 10 μg/ml oxLDL, as indicated. Beads were washed, and specifically bound material was eluted with reduced glutathione and resolved by SDS-polyacrylamide gel electrophoresis. Approximately 10–20% of total input was typically retained. Intensities of specific bands were quantitated and were plotted as relative intensity.

porter construct κB-LUC, previously characterized to efficiently mediate the NF-κB-dependent transactivations in various cell types, that consists of a minimal promoter from the IL-2 gene and four upstream κB sites from the IL-6 gene (45). Cotransfection of HeLa-1 cells with PPAR-γ had minimal effect on the p65-induced reporter gene expression in the presence of LDL. In the presence of oxLDL or 9-HODE, however, increasing amount of cotransfected PPAR-γ inhibited the reporter gene expression in a PPAR-γ dose-dependent manner (Fig. 6). Similarly, cotransfection of increasing amounts of p50 or p65

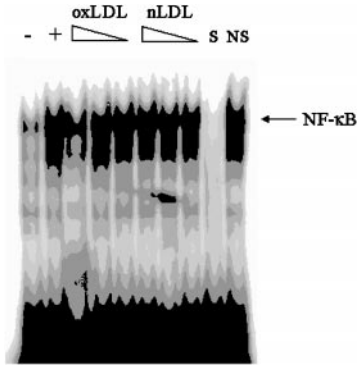

FIG. 5. **OxLDL-mediated inhibition of κB binding by NF-κB.** Nuclear extracts prepared from macrophage cells stimulated by LPS in the absence or presence of LDL or oxLDL (1, 10, and 50 μg/ml each) were examined for κB binding activity in the electrophoretic mobility shift assays using a labeled oligonucleotide containing a consensus Ig-κB site, as indicated. *S* and *NS* indicate the presence of an unlabeled, identical oligonucleotide and nonspecific oligonucleotide, respectively. The specific NF-κB complexes are as indicated.

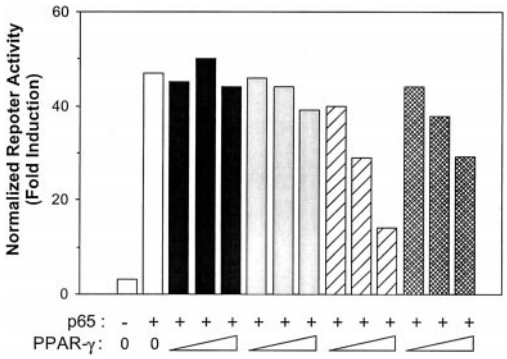

FIG. 6. **Transcriptionally inhibitory complex of NF-κB and PPAR-γ.** HeLa cells were transfected with p65 (50 ng) and increasing amounts of PPAR-γ (10, 100, and 200 ng each) expression vectors along with a reporter gene *κB-LUC* in the absence or presence of LDL, oxLDL, or 9-HODE. *Open*, *black closed*, *gray closed*, *striped*, and *checked boxes* indicate no PPAR-γ added, PPAR-γ added, PPAR-γ plus 10 μg/ml LDL, PPAR-γ plus 10 μg/ml oxLDL, and PPAR-γ plus 10 μg/ml 9-HODE, respectively. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions, and the standard deviations are less than 5%.

also inhibited the oxLDL-dependent transactivation by PPAR-γ (data not shown). These results suggest that the interactions of NFκB-PPARγ may lead to a formation of transcriptionally inactive complex *in vivo*, regardless of the nature of DNA binding sites.

The NF-κB-inhibitory Actions of OxLDL Independent of κB Sites—Next, we tested whether the NFκB-inhibitory actions of oxLDL require κB site binding. We expressed a Gal4 fusion protein to p65 (Gal4/p65) in CV-1 cells, along with a reporter construct controlled by upstream Gal4 sites. Consistent with previous finding (46), Gal4/p65 directed a strong activation of the reporter gene expression (Fig. 7). Cotransfection of increasing amount of PPAR-γ-expression vector was without any significant effects in the presence of native LDL. In contrast, PPAR-γ in the presence of oxLDL or 9-HODE directed inhibition of the Gal4/p65 transactivation in a PPAR-γ-dose dependent manner (Fig. 7). These results suggest that the inhibitory actions of oxLDL can also operate without κB site binding.

Ciglitazone, a Selective PPAR-γ Agonist, Also Inhibits IL-12 Production in Activated Mouse Macrophages—Next, we determined whether PPAR-γ agonists inhibited IL-12 production in LPS-activated macrophages, as like oxLDL. As shown in Fig. 8*A*, ciglitazone, a selective PPAR-γ agonist, significantly inhibited IL-12 production in a dose-dependent manner and also

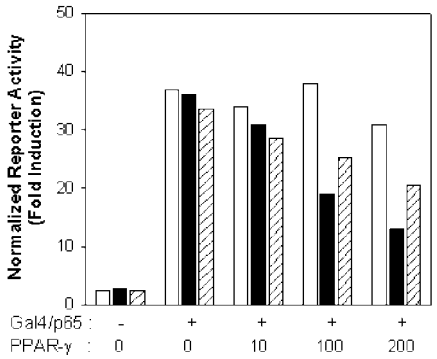

FIG. 7. **OxLDL-mediated transrepression of p65 in the absence of κB binding.** CV-1 cells were transfected with Gal4/p65 (100 ng) and PPAR-γ (10, 100, and 200 ng each) expression vectors along with a reporter gene *Gal4-LUC* either in the absence or presence of LDL (or oxLDL), as indicated. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions and the standard deviations are less than 5%. *Open*, *closed*, and *striped boxes* indicate 10 μg/ml LDL added, 10 μg/ml oxLDL, and 10 μg/ml 9-HODE, respectively.

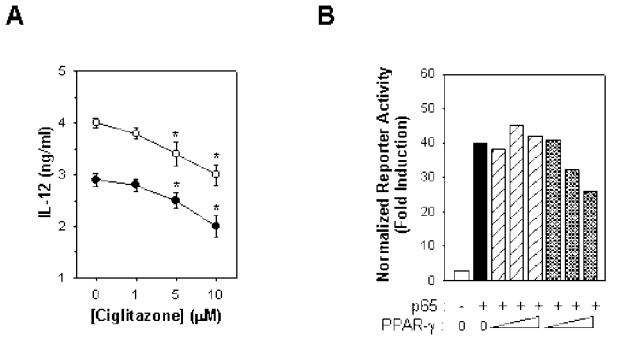

FIG. 8. **Effects of ciglitazone on IL-12 production in mouse macrophages and on PPAR-γ-dependent transactivation of p65 promoter.** *A*, macrophages were stimulated with 5 μg/ml LPS alone (*open*) or in combination with 10 μg/ml oxLDL (*closed*) in the absence or presence of varying concentrations of ciglitazone. The levels of IL-12 in culture supernatants were determined by an IL-12 p40 ELISA. The results are presented as mean ± standard deviations of triplicate determinations. *, $p < 0.01$, relative to ciglitazone-untreated group in the absence or presence of oxLDL. *B*, HeLa cells were transfected with p65 (50 ng) and increasing amounts of PPAR-γ (10, 100, and 200 ng each) expression vectors along with a reporter gene *κB-LUC* in the absence or presence of ciglitazone. *Open*, *closed*, and *striped boxes* indicate no ciglitazone added, and *checked boxes* indicate the presence of 10 μM ciglitazone. Normalized luciferase expressions from triplicate samples are presented relative to the *LacZ* expressions, and the standard deviations are less than 5%.

showed additive effect of inhibition on IL-12 production when combined with oxLDL. In addition, like oxLDL, increasing amount of cotransfected PPAR-γ in HeLa cells inhibited the p65 reporter gene expression in a PPAR-γ dose-dependent manner in the presence of ciglitazone (Fig. 8*B*).

DISCUSSION

Oxidized LDL has been hypothesized to play a causative role in atherosclerotic plaque formation (reviewed in Ref. 47). Oxidative modification of cholesterol, fatty acid, and protein components of LDL results in the formation of a lipoprotein particle with distinct biologic activities. In this study we have shown that oxLDL inhibited IL-12 production in LPS-activated mouse macrophages in a dose-dependent manner. The inhibitory effect is, at least in part, via down-regulation of NF-κB activation and binding to the p40-κB site by physical interactions of PPAR-γ and NF-κB. Oxidative modification of the LDL particle is of importance for the inhibition of IL-12 production in LPS-activated mouse macrophages. Several lines of evidence support this point. First, treatment of LPS-activated macrophages with oxLDL significantly suppressed the production of IL-12 in a dose-dependent manner while treatment with native LDL did not (Fig. 1). Furthermore, oxLDL inhibited the activation of IL-12 p40 promoter and NF-κB binding to the κB sites while LDL did not.

Exposure of monocytes and macrophages to oxLDL has a range of biological consequences, including enhanced monocyte maturation, increased adhesive properties, and production of cytokines and growth factors (15). As one might expect, the influence of oxLDL on macrophage gene expression is complex and context-dependent. Exposure of resting macrophages to oxLDL leads to increased expression of CD11b, CD18 (48), the scavenger receptors SR-A and CD36 (49), and cytokines such as IL-1α and IL-1β (50, 51). At the same time, oxLDL has been reported to suppress the IFNγ or LPS-induced expression of tumor necrosis factor-α, IP-10, and IL-1β (17, 52, 53). Interestingly, two groups have recently reported that PPAR-γ ligands can also function to suppress cytokine production in activated macrophages (24, 26). In addition, in contrast to our results, oxLDL was reported to induce IL-10 and IL-12 release from resting human monocytes *in vitro* (54). Thus, the effects of both oxLDL and PPAR-γ ligands on macrophage gene expression are dependent on the activation state of the cell. These context-dependent effects may help to explain why oxLDL-exposed macrophages maintain a chronic rather than fulminant inflammatory reaction at sites of atherosclerosis. In addition, the results of this study might indicate that oxLDL could inhibit or minimize a normal inflammatory response. It has been speculated that the suppression of cytokine expression in foam cells may be an important contributing factor of the pathophysiologic process in the formation of fatty streaks before they convert to mature atheromas (52). The capacity of macrophages in lesions to be activated might also be crucial for the stability of the atherosclerotic plaque as well as for the thrombosis formation on the plaque surface.

Although the mechanisms that transduce signals from oxLDL to the nucleus are not well defined, previous studies have suggested that isolated protein and lipid components can each recapitulate some of the effects of the intact particle. For example, 9-HODE and 13-HODE have been implicated in the induction of IL-1β (55) and function as activators and endogenous ligands of PPAR-γ (25). In this report 9-HODE inhibited IL-12 production in LPS-activated mouse macrophage and the IL-12 p40 promoter activation in a dose-dependent manner (Fig. 3).

Inhibiting the action of IL-12 has been shown to prevent development and progression of disease in experimental models of autoimmunity (56). These findings have raised great interest in identifying inhibitors of IL-12 production for the treatment of Th1-mediated diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and acute graft-*versus*-host disease. Recently, corticosteroids have been shown to enhance the capacity of macrophages to induce IL-4 synthesis in $CD4^+$ T cells by inhibiting IL-12 production (57). In addition, captopril and lisinopril, angiotensin-converting enzyme inhibitors, were also shown to suppress IL-12 production from human peripheral blood mononuclear cell (58). A phosphodiesterase inhibitor pentoxifylline (59) and thalidomide (60) inhibited IL-12 production from human monocytes by a mechanism independent of known endogenous inhibitors of IL-12 production such as IL-10, transforming growth factor-β, or prostaglandin E_2. β_2-Adrenergic compounds including salbutamol inhibited IL-12 production from human monocytes or dendritic cells by increasing intra-

cellular cAMP levels, leading to inhibition of the development of Th1 cells while promoting Th2 cell differentiation (61). Interestingly, 1,25-dihydroxyvitamin D_3 was also shown to inhibit IL-12 production, presumably by down-regulating the NF-κB activities from human IL-12 p40 gene (62). In this report, we added oxLDL to the list of compounds that inhibit production of IL-12 through specific nuclear receptors, together with corticosteroids (57), 1,25-dihydroxyvitamin D_3 (62) and retinoids (40) (Fig. 1). As was the case with corticosteroids and 1,25-dihydroxyvitamin D_3, this inhibition was also mapped to a region in the p40 promoter containing a binding site for NF-κB (Fig. 2) and may involve direct physical interactions of PPAR-γ with NF-κB (Fig. 4). However, it is interesting to note that NF-κB constitutively interacted with PPAR-γ (Fig. 3), whereas the inhibitory actions were absolutely oxLDL-dependent (Figs. 1, 2, and 6). Thus, NF-κB may exist constitutively associated with PPAR-γ *in vivo* and this complex becomes transcriptionally inactive upon addition of oxLDL. In addition, transcription coactivators such as SRC-1 and p300/CBP may play important roles since these cofactors were known to directly interact with PPAR-γ and could regulate the transcriptional activities (63, 64). In addition, oxLDL also inhibited the κB binding activities of NF-κB *in vitro* (Fig. 5), suggesting that the PPAR-γ/NF-κB complex is unable to recognize κB-sites. However, it is not currently known why this liganded PPAR-γ/NF-κB complex loses its ability to bind κB sites. It is possible that conformational change brought into this complex, upon addition of oxLDL may become propagated to the Rel homology domain of NF-κB, resulting in inability to bind κB sites. The inhibitory actions of oxLDL can also operate in the absence of κB site binding by NF-κB, as demonstrated by the results shown in Fig. 7, in which transactivation mediated by Gal4/p65 was shown to be inhibited by oxLDL. Overall, these results are similar to previously described results with steroid receptors (29–34), in which the mutual inhibitions between GR and RelA involved the DNA and the ligand binding domains of the GR (Fig. 4). The PPARγ-NFκB interactions are likely to have wide implications in various aspects of oxLDL and NF-κB biology, not limited to the regulation of IL-12 production in macrophages described in this study.

In conclusion, we have shown that PPAR-γ forms a transcriptionally inhibitory complex with NF-κB. With the NF-κB transactivation, in particular, this oxLDL-mediated inhibitory action appeared to involve inhibition of the NF-κB-DNA interactions as well as physical interactions between NF-κB and PPAR-γ. This transrepression between NF-κB and PPAR-γ could play an important role in a large variety of biological processes.

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REFERENCES

1. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. (1989) *J. Exp. Med.* **170,** 827–846
2. Stern, A. S., Podlaski, F. J., Hulmes, J. D., Pan, Y. C., Quinn, P. M., Wolitzky, A. G., Familletti, P. C., Stremlo, D. L., Truitt, T., Chizzonite, R., and Gately, M. K. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6808–6812
3. Kang, K., Kubin, M., Cooper, K. D., Lessin, S. R., Trinchieri, G., and Rook, A. H. (1996) *J. Immunol.* **156,** 1402–1407
4. Benjamin, D., Sharma, V., Kubin, M., Klein, J. L., Sartori, A., Holliday, J., and Trinchieri, G. (1996) *J. Immunol.* **156,** 1626–1637
5. Trinchieri, G. (1998) *Int. Rev. Immunol.* **16,** 365–396
6. Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U., and Presky, D. H. (1998) *Annu. Rev. Immunol.* **16,** 495–521
7. McDryer, J. F., Wu, C. Y., and Seder, R. A. (1998) *J. Allergy Clin. Immunol.* **102,** 11–15
8. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996) *J. Exp. Med.* **184,** 747–752
9. Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., and Schuler, G. (1996) *J. Exp. Med.* **184,** 741–746
10. Murphy, T. L., Cleveland, M. G., Kulesza, P., Magram, J., and Murphy, K. M. (1995) *Mol. Cell. Biol.* **15,** 5258–5267
11. Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R., and Trinchieri, G. (1996) *J. Exp. Med.* **183,** 147–157
12. Gri, G., Savio, D., Trinchieri, G., and Ma, X. (1998) *J. Biol. Chem.* **273,** 6431–6438
13. Tone, Y., Thompson, S. A., Babik, J. M., Nolan, K. F., Tone, M., Raven, C., and Waldmann, H. (1996) *Eur. J. Immunol.* **26,** 1222–1227
14. Yoshimoto, T., Kojima, K., Funakoshi, T., Endo, Y., Fujita, T., and Nariuchi, H. (1996) *J. Immunol.* **156,** 1082–1088
15. Quinn, M. T., Parthasarathy, S., Fong, L. G., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2995–2998
16. Hamilton, T., Ma, G., and Chisolm, G. (1990) *J. Immunol.* **144,** 2343–2350
17. Fong, L., Fong, T., and Cooper, A. (1991) *J. Lipid Res.* **3,** 1899–1910
18. Malden, L., Chait, A., Raines, E., and Ross, R. (1991) *J. Biol. Chem.* **266,** 13901–13907
19. Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7355–7359
20. Lemberger, T., Desvergne, B., and Wahli, W. (1996) *Annu. Rev. Cell. Dev. Biol.* **12,** 335–363
21. Tontonoz, P., Hu, E., and Spiegelman, B. (1994) *Cell* **79,** 1147–1156
22. Forman, B. M., Tontonoz, P., Chen J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* **83,** 803–812
23. Kliewer, S. A., Sundseth, S., Johns, S., Brown, P., Wisely, G., Koble, C., Devxhand, P., Wahli, W., Willson, T., Lenhard, J., and Lehman, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4318–4323
24. Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998) *Nature* **391,** 79–82
25. Nagy, L., Tontonoz, P., Alvarez, J. G. A., Chen, H., and Evans, R. M. (1998) *Cell* **93,** 229–240
26. Jiang, C., Ting, A. T., and Seed, B. (1998) *Nature* **391,** 82–86
27. Baldwin, A. S. (1996) *Annu. Rev. Immunol.* **14,** 649–681
28. Gilmore, T. D., and Morin, P. J. (1993) *Trends Genet.* **9,** 427–433
29. Ray, A., and Prefontaine, K. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 752–756
30. Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S. (1995) *Mol. Cell. Biol.* **15,** 943–953
31. Ray, A., Prefontaine, K. E., and Ray, P. (1994) *J. Biol. Chem.* **269,** 12940–12946
32. Stein, B., and Yang, M. K. (1995) *Mol. Cell. Biol.* **15,** 4971–4979
33. Kalkhoven, E., Wissink, S., van der Saag, P. T., and van der Burg, B. (1996) *J. Biol. Chem.* **271,** 6217–6224
34. Palvimo, J. J., Reinkainen, P., Ikonen, T., Kallio, P. J., Moilanen, A., and Janne, O. A. (1996) *J. Biol. Chem.* **271,** 24151–24156
35. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) *Current Protocols in Molecular Biology*, Greene Associates, New York
36. Wysocka, M., Kubin, M., Vieira, L. Q., Ozmen, L., Garotta, G., Scott, P., and Trinchieri, G. (1995) *Eur. J. Immunol.* **25,** 672–676
37. Hoff, H., Whitaker, T. E., and O'Neil, J. (1992) *J. Biol. Chem.* **267,** 602–609
38. Jougasaki, M., Kugiyama, K., Saito, Y., Nakao, K., Imura, H., and Yasue, H. (1992) *Circ. Res.* **71,** 614–619
39. Schuh, J., Fairclough, G. F., and Haschemeyer, R. H. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 3173–3178
40. Na, S.-Y., Kang, B. Y., Chung, S. W., Han, S.-J., Ma, X., Trinchieri, G., Im, S.-Y., Lee, J. W., and Kim, T. S. (1999) *J. Biol. Chem.* **274,** 7674–7680
41. Kim, T. S., DeKruyff, R. H., Rupper, R., Maecker, H. T., Levy, S., and Umetsu, D. T. (1997) *J. Immunol.* **158,** 4137–4144
42. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
43. Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995) *Nature* **374,** 91–94
44. Goldfeld, A. E., Doyle, C., and Maniatis, T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9769–9773
45. Kim, C. H., Song, Y.-H., Park, K., Oj, Y., and Lee, T. H. (1995) *J. Inflamm.* **45,** 312–322
46. Bull, P., Morley, K. L., Hoekstra, M. F., Hunter, T., and Verma, I. M. (1990) *Mol. Cell. Biol.* **10,** 5473–5485
47. Steinberg, D. (1997) *J. Biol. Chem.* **272,** 20963–20966
48. Lehr, H. A., Krober, M., Hubner, C., Vajkoczy, P., Menger, M. D., Nolte, D., Kohlschtter, A., and Messmer, K. (1993) *Lab. Invest.* **68,** 388–395
49. Han, J., Hajjar, D. P., Febbraio, M., and Nicholson, A. C. (1997) *J. Biol. Chem.* **272,** 21654–21659
50. Thomas, C., Jackson, R., Ohlweiler, D., and Ku, G. (1994) *J. Lipid Res.* **35,** 417–427
51. Lipton, B., Parthasarathy, S., Ord, V., Clinton, S., Libby, P., and Rosenfeld, M. (1995) *J. Lipid Res.* **36,** 2232–2242
52. Hamilton, T., Major, J., and Chisolm, G. (1995) *J. Clin. Invest.* **95,** 2020–2027
53. Ohlsson, B. G., Englund, M. C., Karlsson, A. L., Knutsen, E., Erixon, C., Skribeck, H., Liu, Y., Bondjers, G., and Wiklund, O. (1996) *J. Clin. Invest.* **98,** 78–89
54. Uyemura, K., Demer, L., Castle, S. C., Jullien, D., Berliner, J. A., Gately, M. K., Warrier, R. R., Pham, N., Fogelman, A. M., and Modlin, R. L. (1996) *J. Clin. Invest.* **97,** 2130–2138
55. Ku, G., Thomas, C., Akeson, A., and Jackson, R. (1992) *J. Biol. Chem.* **267,** 14183–14188
56. Caspi, R. R. (1998) *Clin. Immunol. Immunopathol.* **88,** 4–13
57. DeKruyff, R. H., Fang, Y., and Umetsu, D. T. (1998) *J. Immunol.* **160,** 2231–2237
58. Constantinescu, C. S., Goodman, D. B., and Ventura, E. S. (1998) *Immunol. Lett.* **62,** 25–31

59. Moller, D. R., Wysocka, M., Greenlee, B. M., Ma, X., Wahl, L., Trinchieri, G., and Karp, C. L. (1997) *Immunology* **91,** 197–203
60. Moller, D. R., Wysocka, M., Greenlee, B. M., Ma, X., Wahl, L., Flockhart, D. A., Trinchieri, G., and Karp, C. L. (1997) *J. Immunol.* **159,** 5157–5161
61. Panina-Bordignon, P., Mazzeo, D., Lucia, P. D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C., and Sinigaglia, F. (1997) *J. Clin. Invest.* **100,** 1513–1519
62. D'Ambrosio, D., Cippitelli, M., Gabriella, C., Mazzeo, D., Lucia, P. D., Lang, R., Sinigaglia, F., and Panina-Bordignon, P. (1998) *J. Clin. Invest.* **101,** 252–262
63. Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395,** 137–143
64. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J.-C., and Auwerx, J. (1999) *J. Biol. Chem.* **274,** 7681–7688