Liver X Receptor Agonists Inhibit Cytokine-Induced Osteopontin Expression in Macrophages Through Interference With Activator Protein-1 Signaling Pathways

Daisuke Ogawa, Jeffrey F. Stone, Yasunori Takata, Florian Blaschke, Van H. Chu, Dwight A. Towler, Ronald E. Law, Willa A. Hsueh, Dennis Bruemmer

Abstract—Osteopontin (OPN) is a proinflammatory cytokine and adhesion molecule implicated in the chemotraction of monocytes and in cell-mediated immunity. We have recently reported that genetic OPN-deficiency attenuates the development of atherosclerosis in apoE<sup>−/−</sup> mice identifying OPN as potential target for pharmacological intervention in atherosclerosis. Synthetic agonists for the Liver X Receptor (LXR), members of the nuclear hormone receptor superfamily, prevent the development of atherosclerosis by regulating cholesterol homeostasis and suppressing inflammatory gene expression in macrophages. We demonstrate here that LXR ligands inhibit cytokine-induced OPN expression in macrophages. Two synthetic LXR ligands, T0901317 and GW3965, inhibited TNF-α, IL-1β, INF-γ and lipopolysaccharide induced OPN mRNA and protein expression in RAW 264.7 macrophages. Transient transfection experiments revealed that LXR ligands suppress cytokine-induced OPN promoter activity. Deletion analysis, heterologous promoter assays, and site-directed mutagenesis identified an activator protein-1 (AP-1) consensus site at -76 relative to the initiation site that supports OPN transcription in macrophages and mediates the effects of LXR ligands to inhibit OPN transcription. Electrophoretic mobility shift and chromatin immunoprecipitation assays indicated that LXR agonists inhibit cytokine-induced c-Fos and phospho-c-Jun binding to this AP-1 site. Cytokine-induced c-Fos and phospho-c-Jun protein expression was inhibited by LXR ligands and overexpression of c-Fos and c-Jun reversed the inhibitory effect of LXR ligands on OPN promoter activity in transactivation assays. Finally, treatment of C57BL/6J mice with LXR ligands inhibited OPN expression in peritoneal macrophages indicating that the observed effects of LXR ligands to inhibit OPN expression are applicable in vivo. These observations identify the regulation of macrophage OPN expression as a mechanism whereby LXR ligands may impact macrophage inflammatory responses and atherosclerosis.

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Osteopontin (OPN) is as an extracellular matrix protein that has recently been characterized as a proinflammatory cytokine with pleiotropic functions in inflammation. OPN promotes monocyte adhesion, migration, activation, and cytokine expression as well as macrophage survival. Additional proinflammatory functions of OPN include the ability to stimulate the activation of matrix-metalloproteinases (MMPs) promoting the invasive behavior of monocytes at sites of inflammation. OPN further induces interleukin (IL)-12 and inhibits IL-10 expression in macrophages, suggesting critical regulatory roles in inflammation and early type-1 cytokine expression.

OPN is highly expressed in atherosclerotic lesions where it is secreted by macrophages, vascular smooth muscle cells (VSMCs), and endothelial cells (ECs). We and other investigators have recently used genetic approaches to define the causal contribution of OPN to the development of atherosclerosis. Using OPN-deficient mice crossed into atherosclerosis-prone apoE<sup>−/−</sup> mice and infused with angiotensin II, we have provided evidence that OPN promotes the development of atherosclerosis and abdominal aortic aneurysms. In particular, OPN derived from hematopoietic cells contributes to atherosclerosis, indicating an important role of macrophage-derived OPN for the development of atherosclerosis. The mechanisms by which OPN contributes to atherosclerosis involve the regulation of monocyte recruitment into the arterial wall, the induction of vascular cytokine and MMP expression, and the regulation of macrophage survival in the arterial wall. Because OPN appears to specifically promote these early inflammatory mechanisms leading to atherosclerosis...
rosis, OPN expression in macrophages may potentially provide a novel pharmacological target for the treatment of atherosclerosis.

The liver X receptor (LXR) is an important regulator of cholesterol, free fatty acid, and glucose metabolism. In addition to its importance in lipid and glucose metabolism, LXR activation has recently been demonstrated to regulate immune processes and to inhibit inflammatory gene expression in macrophages. Synthetic LXR agonists have been demonstrated to prevent atherosclerosis in murine models and to inhibit inflammation. In the present study, we demonstrate that LXR ligands inhibit cytokine-induced OPN expression in macrophages. This effect is mediated by an inhibition of c-Jun/c-Fos DNA binding activities to the proximal OPN promoter, which impairs AP-1–dependent OPN transcription. These results define a novel mechanism by which LXR ligands may impact macrophage inflammatory responses and atherosclerosis.

Materials and Methods

Materials
T0901317 was purchased from Sigma-Aldrich. GW3965 was kindly provided by Dr Peter Tontonoz (University of California, Los Angeles, Calif). Primary antibodies were purchased from Santa Cruz Biotechnology: USP1 (sc-8983), USP2 (sc-861), c-Fos (sc-8047 or sc-253), FosB (sc-7203), Fra1 (sc-602), Fra2 (sc-604), jun B (sc-8051), c-Jun (sc-45), phospho-c-Jun (sc-822), jun D (sc-74), Oct1 (sc-232), and Oct-2 (sc-233). The OPN antibody (MPIII1B0) was obtained from the University of Iowa Hybridoma Bank.

Cell Culture
RAW 264.7 macrophages (American Type Culture Collection, ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as directed. THP-1 monocytes (ATCC) and HPBM (Cambrex Bio Science Walkersville) were cultured in RPMI1640 medium supplemented with 10% FBS according to the manufacturer’s instructions. Murine peritoneal macrophages were isolated and cultured as described. For ligand treatment, cells were serum-deprived by culturing in 0.5% FBS and treated with the LXR ligand T0901317 or GW3965 to inhibit inflammation. In the present study, we demonstrated to prevent atherosclerosis in murine models and to inhibit inflammation. In the present study, we demonstrated to prevent atherosclerosis in murine models and to inhibit inflammation.

Chromatin Immunoprecipitation Assays
ChIP assays were performed using a chromatin immunoprecipitation (ChIP) assay kit (Upstate) according to the manufacturer’s instructions. Soluble chromatin was prepared from RAW 264.7 macrophages treated with 5 μmol/L T0901317 for 24 hours followed by stimulation with TNF-α (20 ng/mL) for 1 hour. Chromatin was immunoprecipitated with antibodies (2 μg) 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 β2 promoter as follows: forward 5’- AACAGAGGGGAGGTAGGA-3’; antisense 5’- ACCATCAGGAGGTAGTGTG-3’.

Animal Studies
Seven-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). T0901317 was dissolved at 50 mg/mL in DMSO and further diluted 1:5 in 0.9% FBS. Mice were treated with daily intraperitoneal injections of T0901317 at 50 mg/kg/d or vehicle for 7 days. Four days after beginning of the treatment 1.5 mL thioglycollate (4% wt/vol) was injected intraperitoneally. Three days later peritoneal macrophages were isolated and analyzed for OPN mRNA expression. All animal protocols were approved by the University of California, Los Angeles Animal Research Committee and complied with all federal, state, and industrial regulations.

Statistical Analysis
Analysis of variance (ANOVA) and paired or unpaired t test were performed for statistical analysis as appropriate. Probability values less than 0.05 were considered to be statistically significant. Results are expressed as mean±SEM.

Results
LXR Agonists Suppress OPN Expression in Macrophages
Based on our previous studies demonstrating that macrophase-derived OPN contributes to the development of accelerated atherosclerosis, we first determined the regulation of OPN expression in macrophages by different proinflammatory stimuli. As depicted in Figure 1A, OPN construct driven by seven AP-1 consensus sites was commercially obtained from Stratagene.

RAW 264.7 macrophages were transfected with 1 μg DNA using LipofectAMINE 2000 (Invitrogen). Eight hours after transfection, cells were cultured in DMEM supplemented with 0.5% FBS in the presence of the LXR ligand T0901317 or GW3965 for 12 hours before cytokine stimulation. Luciferase activity was assayed 24 hours after stimulation using a Dual Luciferase Reporter Assay System (Promega). Transfection efficiency was adjusted by normalizing firefly luciferase activities to renilla luciferase activities generated by cotransfection with 10 ng pRL-CMV (Promega). All experiments were repeated at least 3 times with different cell preparations.

Electrophoretic Mobility Shift Assay
Oligodeoxynucleotides were labeled with [γ-32P]–ATP and incubated with 4 μg of nuclear extract for 20 minutes. DNA-protein complexes were analyzed by electrophoresis using a 6% nondenaturing acrylamide gel. For competitive oligonucleotide assays, 100-fold excess of unlabeled oligodeoxynucleotide was added to the nuclear extracts 20 minutes before the addition of the radiolabeled probe. Supershift experiments were performed by incubating 4 μg of nuclear extract with 0.5 μg of the indicated antibody for 20 minutes before addition of the radiolabeled probe. For the treatment studies, RAW 264.7 macrophages were cultured for 24 hours in the presence of 5 μmol/L T0901317 (0.5% FBS) and stimulated with TNF-α or IL-1β for either 1 or 6 hours as indicated.

Northern Blotting
Northern Blotting was performed as previously described. Blots were cohybridized with cDNA encoding the constitutively expressed housekeeping gene Chinese hamster ovary gene B (CHOB) to assess equal loading of samples. Quantification was performed by densitometry and normalization to CHOB mRNA expression.

Western Blot Analysis
Probed membranes were hybridized with cDNA encoding the constitutively expressed housekeeping gene Chinese hamster ovary gene B (CHOB) to assess equal loading of samples. Quantification was performed by densitometry and normalization to CHOB mRNA expression.
protein expression in RAW 264.7 macrophages was significantly upregulated by TNFα, IL-1β, INF-γ, and LPS. Northern blotting further revealed that these cytokines induced OPN mRNA, indicating that OPN regulation in macrophages occurs at the gene expression level (Figure 1B).

Because LXR agonists have recently been proposed as therapeutic approaches to treat cardiovascular disease and to suppress inflammatory gene expression in macrophages, we next investigated the effect of two different LXR ligands on macrophage OPN expression.9,14 Consistent with recent re-
ports, treatment of macrophages with both LXR agonists increased their viability as assessed by annexin V-FITC FACS analysis (data not shown). Preincubation of macrophages with the synthetic LXR agonists resulted in a substantial suppression of cytokine-induced OPN protein and mRNA levels (Figure 1A and 1B), which was dose-dependent over a concentration range of 100 nmol/L to 5 μmol/L (Figure 1C).

Similarly, LXR agonists suppressed cytokine-induced OPN mRNA and protein expression in human peripheral blood monocytes and THP-1 monocytes (Figure 2A and 2B).

**LXR Agonists Suppress Cytokine-Induced OPN Transcription**

We next transiently transfected RAW 264.7 macrophages with a 2-kb OPN promoter fragment to determine whether cytokine-induced OPN promoter activity is suppressed by LXR agonists. Cytokine-induced OPN promoter activity was completely inhibited by T0901317 (Figure 3A). Both LXR agonists T0901317 and GW3965 inhibited TNF-α–induced OPN promoter activity in a dose-dependent manner (Figure 3B). These results suggest that LXR ligands suppress cytokine-induced OPN expression by inhibiting transcription of the OPN gene.

**The Proximal OPN Promoter Confers Transcriptional Regulation of OPN in Macrophages**

The mechanism for the inhibition of cytokine-induced OPN transcription by LXR ligands is unlikely to be direct, as activation of LXR by its ligands has not been reported to function as a transcriptional repressor. Moreover, sequence analysis of the 5′-flanking region of the OPN gene did not reveal the presence of potential LXR response elements. To identify the promoter elements that regulate cytokine-induced OPN gene transcription and mediate the transcriptional suppression of OPN in macrophages by LXR agonists, we used a series of 5′-deletion constructs (Figure 4A). Whereas TNF-α–induced transcriptional activity of the −83OPN-Luc promoter activity in a dose-dependent manner (Figure 3B). These results suggest that LXR ligands suppress cytokine-induced OPN expression by inhibiting transcription of the OPN gene.
OPN(-83/-45)RSVLuc was substantially inhibited by treatment with the LXR agonist. These results suggest that the proximal OPN promoter region -83 to -45 assembles transcriptional complexes that mediate both basal transcriptional activity and the suppression of cytokine-induced OPN promoter activity by LXR ligands.

**LXR Agonists Suppress Cytokine-Induced OPN Promoter Activity by Negatively Interfering With AP-1 Transcription Activity**

Based on previous studies demonstrating an important role of the AP-1 site located at -76 for the transcriptional regulation of the OPN gene, a mutation in this AP-1 site was generated.\(^{13,17}\) Whereas treatment with TNF-\(\alpha\) resulted in an induction of the wild-type OPN promoter, the promoter containing a mutation of this AP-1 site exhibited low basal activity and was not induced by TNF-\(\alpha\) (Figure 5A). We next cotransfected macrophages with the -83OPN-Luc reporter and eukaryotic expression vectors for c-Fos and c-Jun (Figure 5B). Overexpression of c-Fos and c-Jun resulted in a complete loss of the effect of LXRs to inhibit TNF-\(\alpha\)-induced OPN transcriptional activation. Furthermore, TNF-\(\alpha\)-induced transcriptional activity of a heterologous promoter driven by multiple AP-1 response elements was dose-dependently inhibited by the LXR agonist (Figure 5C). In concert, these findings suggest that the suppression of cytokine-induced OPN promoter activity by LXR ligands is mediated through negative interference with c-Fos/c-Jun acting on the proximal OPN promoter.

**The OPN Promoter Region -80 to -65 Assembles AP-1, Oct-1, and USF-1 Protein-DNA Complexes**

In addition to the reported AP-1 site at -76,\(^{13,17}\) the OPN promoter sequence between -80 and -65 contains a CCAAT- CATGA motif resembling an atypical E-box as well as an Oct-1 (CTCATGA) cognate.\(^{13}\) As depicted in Figure 6A (lane 1 and 2), EMSA experiments revealed three specific complexes (labeled A, B, and C). Competition experiments confirmed AP-1–like protein-DNA interactions (lane 3), an Oct-1 cognate (lane 4), and an USF-1 cognate (lane 5). A duplex oligonucleotide possessing a CC→TT transition, which selectively disrupts the putative E-box (MUT1), can still compete for complex A and B, but not for complex C (lane 6). A mutant disrupting the AP-1, Oct-1, and E-box did not compete for any of the complexes formed at this promoter region (lane 7). A cold oligonucleotide MUT3 competed for the formation of all three complexes, indicating that only the intact cognate CCTCATGAC was sufficient to compete for assembly of complexes. In concert, these findings suggest that the CCTCATGAC motif between -80 and -71 relative to the transcription initiation site assembles three DNA/protein complexes consisting of AP-1, Oct-1, and USF-1 binding.

We next performed supershift experiments using nuclear extracts isolated from TNF-\(\alpha\)-stimulated RAW 264.7 macrophages. Antibodies against c-Fos and Ser-63 phosphorylated c-Jun substantially diminished complex formation and supershifted complexes formed at -80 to -65 of the OPN promoter (Figure 6C). In addition, antibodies against Fra-1 promoter construct was completely suppressed by the LXR agonist, a -61OPN-Luc construct was not inducible by TNF-\(\alpha\) and exhibited a transcriptional activity only slightly above the background signal. Comparable results were obtained after stimulation with IL-1\(\beta\), INF-\(\gamma\), and LPS (data not shown). These findings indicate that basal and TNF-\(\alpha\)-induced OPN promoter activity in macrophages is dependent on transcription factor binding sites located between -83 to -61 relative to the transcription initiation site.

We next performed heterologous promoter assays to confirm that the proximal -83 to -61 region of the OPN promoter confers the transcriptional regulation of OPN in macrophages. The basal transcriptional activity of the OPN(-83/-45)RSVLuc construct was approximately 30-fold greater than that of the minimal RSVLuc (Figure 4B). The OPN(-83/-45)RSVLuc conferred TNF-\(\alpha\) responsiveness compared to the unresponsive RSV minimal promoter. Moreover, TNF-\(\alpha\)-induced transcriptional activity of the

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**Figure 4.** OPN promoter elements encoded by nucleotides -83 to -61 convey the transcriptional activity in macrophages. A, RAW 264.7 macrophages were transiently transfected with the indicated 5'-deletion constructs of the OPN promoter. Transfected macrophages were left untreated (white bars) or incubated with vehicle (DMSO, black bars) or T0901317 (5 \(\mu\)mol/L) for 12 hours before stimulation with TNF-\(\alpha\) (20 ng/mL). Cells were harvested after 24 hours and analyzed for luciferase activity as described in Figure 3. (\(P<0.05\) vs transcriptional activity of the -83OPN-Luc construct). B, RAW 264.7 macrophages were transiently transfected with either the OPN(-83 to -45)RSVLuc or the control RSVLuc construct. After transfection, cells were pretreated for 12 hours with the synthetic LXR agonist T0901317 (5 \(\mu\)mol/L) and stimulated for 24 hours with TNF-\(\alpha\) (20 ng/mL). After stimulation, luciferase activity was assayed as described in Figure 3. All experiments were repeated at least 3 times. Data are expressed as mean±SEM (\(P<0.05\) vs vehicle).
and JunB partially supershifted the AP-1 complex, whereas an antibody directed against Fra-2 diminished the complex but did not result in a supershift of the complex. In contrast, antibodies to FosB, unphosphorylated c-Jun, and JunD had no

Figure 5. LXR agonists suppress OPN promoter activity by interfering negatively with the AP-1 signaling pathway. A, RAW 264.7 macrophages were transiently transfected with the wild-type or AP-1–mutated 2-kb OPN promoter. After transfection, macrophages were pretreated for 12 hours with the synthetic LXR agonist T0901317 (5 μmol/L) and stimulated for 24 hours with TNF-α (20 ng/mL) as indicated. B, RAW 264.7 macrophages were transfected with the −83OPN-Luc 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 pretreated for 12 hours with the synthetic LXR agonist T0901317 (5 μmol/L) and stimulated for 24 hours with TNF-α (20 ng/mL). C, RAW 264.7 macrophages were transfected with a heterologous promoter driven by multiple AP-1 sites (pAP-1-Luc). Transfected macrophages were pretreated for 12 hours with vehicle (DMSO) or the indicated concentrations of the synthetic LXR agonist T0901317 and stimulated for 24 hours with TNF-α (20 ng/mL). Luciferase activities were assayed as described in Figure 3. Data are expressed as normalized luciferase activity and presented as mean ± SEM (*P < 0.05 vs TNF-α).

Figure 6. OPN promoter region −80 to −65 assembles three protein-DNA complexes. A, DNA binding activities in nuclear extracts from serum-deprived RAW 264.7 macrophages recognizing the OPN promoter region −80 to −65 were detected by electrophoretic mobility shift assays. Specificity was confirmed by competition with cold oligonucleotides and three OPN promoter mutants as indicated in B. C, Characterization of the AP-1 complex was performed by electrophoretic mobility supershifts. Serum-deprived RAW 264.7 macrophages were stimulated with TNF-α for 1 hour and 4 μg nuclear extract were incubated with the indicated antibodies (0.5 μg) before addition of the radiolabeled OPN −80 to −65 promoter probe. Autoradiograms shown are representative of three independently performed experiments.
effect on complex formation. Finally, antibodies directed against USF-1 and Oct-1 confirmed the presence of USF-1 and Oct-1 protein/DNA complexes. This data confirms that AP-1 (mainly c-Fos and phosphorylated c-Jun), Oct-1, and USF-1 transcription factors assemble protein/DNA complexes at the OPN promoter region.

**AP-1 Binding to the −80 to −65 OPN Promoter Motif Is Suppressed by LXR Agonists**

We next examined the effect of LXR ligands on the formation of protein/DNA complexes at the −80 to −65 OPN promoter sequence. Murine RAW 264.7 macrophages were serum-deprived for 24 hours and stimulated with TNF-α (20 ng/mL) or IL-1β (20 ng/mL) for 1 or 6 hours. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6. DNA binding activities were analyzed as described in Figure 6.

To confirm that c-Fos and phospho-c-Jun bind to the endogenous OPN promoter and that LXR agonists interfere with this binding, we next performed ChIP assays. PCR amplification using primer pairs that cover the AP-1 site at −76 in the OPN promoter, demonstrated that this OPN promoter region coimmunoprecipitated with c-Fos and phospho-c-Jun after stimulation with TNF-α (Figure 7B). This induction of c-Fos and phospho-c-Jun binding to the proximal OPN promoter was substantially inhibited by the LXR agonist. In concert, these findings indicate that TNF-α-induced c-Fos and phospho-c-Jun binding to the AP-1 site at −76 of the proximal OPN promoter is inhibited by LXR agonists.

**TNF-α–Induced c-Fos and Phospho-c-Jun Protein Expression Is Inhibited by LXR Ligands**

To further determine whether the inhibition of AP-1 binding by LXR ligands reflects changes in c-Fos or phospho-c-Jun expression levels, protein levels of both transcription factors were determined by Western blotting analysis. TNF-α–Induced c-Fos (Figure 8A) and phospho-c-Jun (Figure 8B) protein expression
LXR Agonists Inhibit OPN Expression in Macrophages In Vivo

To finally determine whether the effects of LXR agonists to suppress OPN expression are applicable in vivo, T0901317 was administered to C57BL/6J mice for 7 days before isolation of peritoneal macrophages. The mRNA expression level of the LXR target gene ABCA1 was induced by 8.3-fold in peritoneal macrophages elicted from mice treated with T0901317, indicating sufficient availability of the LXR compound (data not shown). OPN protein (Figure 9A) and mRNA (Figure 9B) expression was substantially decreased in peritoneal macrophages from T0901317-treated mice compared with vehicle-treated mice (76±8% and 64±5% decrease in OPN mRNA and protein expression levels, respectively; n=4, P<0.005). Similarly, treatment of elicited murine peritoneal macrophages with the LXR agonist T0901317 dose-dependently suppressed TNF-α-induced OPN protein expression (Figure 9C). Taken together, these results demonstrate that LXR ligands inhibit OPN expression in murine peritoneal macrophages and further support an important role for LXR ligands to suppress proinflammatory gene expression in vitro and in vivo.

Discussion

LXR signaling pathways have recently been proposed as potential target for therapeutic interventions in cardiovascular diseases.9,14 Whereas the antiatherogenic effects of LXR agonists have been attributed to the modulation of cholesterol efflux from macrophages and induction of reverse cholesterol transport to the liver, recent evidence indicates that LXR ligands also modulate inflammatory gene expression.10,11 In the present study, we demonstrate that LXR agonists inhibit expression of OPN, a key inflammatory gene in macrophages. This inhibition is mediated through an inhibition of AP-1–dependent transcriptional activation of the OPN promoter. These observations further support an important role for LXR agonists to suppress macrophage inflammatory responses.

We have recently demonstrated that OPN promotes atherogenesis by modulating leukocyte chemotaxis, cytokine and MMP expression, and macrophage survival.6 However, the molecular mechanisms that regulate OPN expression in macrophages during inflammatory processes are incompletely understood. OPN expression in macrophages was substantially induced after cytokine stimulation and this induction was transcriptionally suppressed by two LXR agonists. The OPN promoter is remarkably responsive to various stimuli, including cytokines, growth factors, and hormones.1 We identified a CCTCATGAC cognate in the OPN promoter located between −80 and −71 that supports basal and cytokine-induced transcriptional activity of the OPN promoter in macrophages. Consistent with previous reports in VSMCs, cold-competition and immunological supershift assays revealed AP-1 binding to this site.13,17 A variety of inflammatory genes are under the control of AP-1–dependent signaling pathways; however, the transcriptional regulation of the OPN promoter by AP-1 in macrophages has not previously been investigated. The importance of this AP-1 site for the transcriptional activity of the OPN promoter was further demonstrated by a complete loss of basal and inducible OPN promoter activity after site-directed mutagenesis of this AP-1 site. Further characterization of the AP-1 complex binding to the OPN promoter revealed the presence of c-Fos and Ser-63 phosphorylated c-Jun binding activities. In addition to an induction of transcription from Fos and Jun genes, AP-1 activity is regulated through phosphorylation of c-Jun and phosphorylated c-Jun is the most potent transactivator of the OPN promoter. These observations further support an important role for LXR agonists to suppress macrophage inflammatory responses.

The analysis of the OPN promoter did not reveal the presence of any putative LXRE response elements, indicating that the inhibition of cytokine-induced OPN transcription by LXR ligands likely involves an indirect mechanism through regulation of other transcription factors supporting OPN transcription. Because AP-1 binding activities appeared to be
important for the transcriptional activity of the OPN promoter in macrophages, we next analyzed whether LXR agonists interfere with AP-1 binding to the proximal OPN promoter. EMSA and ChiP experiments indicated that LXR ligands suppress c-Fos and phospho-c-Jun binding to the AP-1 site at −76 of the proximal OPN promoter. Results from transactivation studies further suggested that LXR ligands inhibit transactivation of a heterologous AP-1–driven promoter and that overexpression of c-Fos and c-Jun reverses the inhibition of OPN promoter activity by LXR ligands. Finally, LXR ligands inhibit cytokine-induced c-Fos and phospho-c-Jun protein expression, which may contribute at least in part to the observed inhibition of AP-1 binding to the proximal OPN promoter.

Cross-talk between different transcription factors and a similar negative interference with AP-1 activity has been described for ligand-induced activation of other nuclear receptors, such as the retinoic acid receptor (RAR), the glucocorticoid receptor (GR), or the peroxisome proliferator–activated receptor (PPAR). Although PPARγ ligands inhibit AP-1–dependent signaling pathways, agonists for this receptor have been demonstrated to inhibit PMA-induced OPN expression in macrophages through interference with homeobox-like A/T-rich sequences between −990 and −981 of the OPN promoter. These findings suggest that the mechanisms by which LXR and PPARγ agonists suppress OPN expression are distinct and likely depend on the mechanisms of OPN induction. However, additional studies are necessary to delineate in more detail the molecular mechanism involved in the negative regulation of c-Fos/c-Jun transactivation by LXR ligands.

In conclusion, data presented in this study demonstrate the observation that cytokine-induced OPN expression in macrophages depends on AP-1 binding to the proximal OPN promoter. LXR agonists inhibit cytokine-induced OPN expression and interfere negatively with AP-1 binding to the proximal OPN promoter by inhibiting c-Fos and phospho-c-Jun protein expression. The suppression of OPN expression by LXR agonists are applicable in vivo as OPN expression is inhibited in mouse peritoneal macrophages elicited after treatment with the LXR agonist. Because OPN is a key component for the inflammatory response and the recruitment of monocytes/macrophages into the atherosclerotic lesion, inhibition of cytokine-induced OPN expression by LXR agonists could provide a potential target for therapeutic intervention in human cardiovascular disease.

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