Induction of NR4A Orphan Nuclear Receptor Expression in Macrophages in Response to Inflammatory Stimuli*

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Oxidized lipids and inflammatory cytokines are believed to play a causal role in atherosclerosis through the regulation of gene expression in macrophages and other cells. Previous work has implicated the nuclear receptors peroxisome proliferator-activated receptor and liver X receptor in the control of lipid-dependent gene expression and inflammation. Here we demonstrate that expression of a third group of nuclear receptors, the NR4A ligand-independent orphan receptors, is highly inducible in macrophages by diverse inflammatory stimuli. Treatment of macrophages with lipopolysaccharide (LPS), cytokines, or oxidized lipids triggers the transcriptional induction of Nur77 (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3) expression. Several lines of evidence point to the NF-κB signaling pathway as a principal mediator of inducible NR4A expression in macrophages. Analysis of the murine and human Nur77 promoters revealed two highly conserved NF-κB response elements. Mutation of these elements inhibited LPS-dependent expression of the Nur77 promoter in transient transfection assays. Furthermore, induction of Nur77 expression by LPS was severely compromised in fibroblasts lacking the three NF-κB subunits, NFκb1, c-Rel, and RelA. Consistent with its ability to be induced by oxidized lipids, Nur77 was expressed in macrophages within human atherosclerotic lesions. These results identified NR4A nuclear receptors as potential transcriptional mediators of inflammatory signals in activated macrophages.

Atherosclerosis can be viewed as both a disorder of lipid metabolism and a chronic inflammatory disease. Monocytes/macrophages play a key role in the disease from both a metabolic and inflammatory standpoint. In the early stages, monocytes are recruited into the arterial wall, differentiate into tissue macrophages, and take up oxidized low density lipoprotein (oxLDL). These lipid-loaded macrophages or “foam cells” establish a chronic inflammatory process together with Th1 and Th2 cells through the production of inflammatory molecules, cytokines, and extracellular matrix-degrading enzymes. Delineating the transcriptional processes that regulate macrophage gene expression in response to oxidized lipids and inflammatory stimuli is essential for our understanding of atherogenesis.

Nuclear hormone receptors are ligand-activated transcription factors involved in diverse physiological functions. Previous work has identified the PPAR and LXR nuclear receptor subfamilies as modulators of lipid metabolic and inflammatory gene expression in cells of the artery wall (1, 2). Macrophage lipid loading has been shown to lead to the transcriptional activation of PPARs and LXRs by providing the cell with oxidized fatty acid and oxysterol ligands for the receptors (2, 3). Genetic loss of either PPARγ or LXR expression from macrophages accelerates the development of atherosclerosis in mice (4, 5). On the other hand, synthetic ligands for both PPARs and LXRs have been shown to reduce lesion formation (6, 7), highlighting these receptors as potential therapeutic targets in cardiovascular disease. The role of other orphan nuclear receptors in the control of macrophage gene expression is not well understood.

The NR4A subfamily of nuclear receptors were first identified as early response genes for growth factors (8–10). This group includes Nur77 (NR4A1, also known as TR3 or NGFI-B), Nurr1 (NR4A2), and NOR1 (NR4A3). Subsequent studies showed that these receptors are important for apoptosis in lymphocytes (11) and other cell types (12, 13) and for the differentiation of dopaminergic neurons (14). All three proteins showed that these receptors are important mediators of inflammatory signaling in the artery wall.

Here we demonstrate that the NR4A nuclear receptors are potently induced by diverse inflammatory stimuli in macrophages. Evidence points to the NF-κB signaling pathway as a principal regulator of inducible NR4A expression. We propose that these receptors may be important mediators of inflammatory signaling in the artery wall.

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Cell Culture and Reagents—THP-1 cells (ATCC) were cultured in RPMI containing 10% FBS (Cellgro). HEK-293T and RAW264.7 cells were also obtained from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% FBS. Wild type and nfhb1−/−, c-rel−/−, relA−/− mouse embryo fibroblasts (MEF) cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine calf serum (HyClone). Mouse primary peritoneal macrophages and bone marrow-derived macrophages were prepared and cultured as described (17). Human monocyte-derived macrophages were obtained from the UCLA Lipid Core Facility. nfhb1−/−, c-rel−/−, relA−/− MEFs were generated from 12.5-day embryos resulting from nfhb1−/−, c-rel−/−, relA−/− matings. Mice with each of these individually targeted mutations have been described previously (18–20). Cells were grown in Dulbecco's medium plus 10% bovine calf serum and immortalized by repeated passage according to the 3T3 procedure (21). These cells are deficient in all three proteins associated with the canonical NF-κB signaling pathway, and they do not show detectable κB site binding activity in response to NF-κB inducing stimuli. LPS, oxidized lipoprotein, and cytochrome treatments of all cell types were carried out in media containing 1% FBS. The reagents used were: LPS (Sigma), rhIL-4 (204-ML), rhIL-6 (206-IL), rhIL-10 (206-IL), rhIL-12 (206-IL), rhM-CSF (216-MC), rhGM-CSF (215-GM, R&D Systems), vitamin D3 (BIOMOL, DM-200), TPA (Calbiochem, 524400), recombinant murine TNFα (315-01A) and rhTNFα (Peprotech, 300-01A), rhIFNγ (Endogen), rhIL-1β (BD Pharmingen), LDL (RP-032), acetylated LDL (acLDL, RP-045), and oxLDL (Intracel, RP-047), 20(S)/22(R)/25,7-b-hydroxysterol (Sigma), and SB203580, PD98059, MG-132, and LY294002 (Calbiochem).

**Transfections**—Deletions of the mouse Nur77 promoter were generated by PCR and cloned into the HindIII/Xho site of the pGL3 basic luciferase reporter vector (Promega). Two nucleotides of the NF-κB sites in Nur77 promoter were mutated using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All constructs were verified by DNA sequencing. Transient transfection of mouse Nur77 promoters into RAW264.7 cells was performed in triplicate as described (17) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Luciferase activity was normalized to internal β-galactosidase control.

**RNA and Protein Analysis**—Total RNA was extracted using Trizol reagent (Invitrogen). Procedures for Northern blotting and real-time quantitative PCR RNA analysis, as well as Western blotting for protein analysis have been described previously (17). The antibodies used were: R-α-IeBa (Santa Cruz Biotechnology, c-21), R-α-Nur77 (Santa Cruz, M-210), R-α-Nur77/Nur1/OR1 (Santa Cruz, E-20), R-α-HMG-1 (BD Pharmingen), and a goat antihuman IgG-horseradish peroxidase (Santa Cruz, sc-2301). The primer sequences used for real-time PCR SYBR Green assays were: Nur77, forward 5′-ATGCTCTCCCTCACATCTC-3′, reverse 5′-CACAGGGCTTCGAGCTGGA-3′; Nur1, forward 5′-TCACCTCGGATGTCCTGAC-3′, reverse 5′-TGGTGGATATGGTGATCTAC-3′; NOR1, forward 5′-CGCCGAAACCGATGTCA-3′, reverse 5′-TGTAGCCACATCTGTTACAA-3′.

**Immunohistochemistry**—RAW cells and HEK-293T cells were cultured in 4-well chamber slides and transfected with Nur77 or LacZ vector using Lipofectamine 2000. 24 h later, the cells were fixed with 3.8% (v/v) paraformaldehyde in PBS for 15 min and then permeabilized with 0.1% Triton-X-100 in PBS for 10 min at room temperature. Cells were stained with anti-Nur77 antibody (Santa Cruz, M-210), followed by rhodamine-conjugated or Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probe) in 10% fetal calf serum in PBS. RAW cells were treated with 500 ng/ml IFNγ and then double-stained with Nur77 antibody (Santa Cruz, M-210) and phycocyanin-conjugated anti-mouse anti-IgG (Serotec). Chamber slides were mounted in fluorescence mount with 4′,6′-diamidino-phenylindole (Vector). For immunohistochemical analysis of Nur77 in human atherosclerotic lesions, a segment of human left coronary artery containing atherosclerotic plaques was harvested from a patient at autopsy, snap-frozen, and stored at −80 °C. Frozen sections (6 μm) were fixed for 5 min in cold acetone and processed for immunohistochemical staining. After three washes with ice-cold PBS, the cells were blocked with 0.1% bovine serum albumin in PBS containing non-immune goat serum. For Nur77 staining, frozen sections were incubated with rabbit anti-Nur77 antibody (Santa Cruz, M-210) followed by Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probes). CD68 staining was performed with an anti-human CD68 monoclonal antibody (Dako) followed by Texas Red-conjugated goat anti-mouse IgG (Vector Laboratories). Staining with nonimmune rabbit IgG (Dako) served as a negative control.

**RESULTS**

In an effort to identify transcription factors involved in responses to oxidized lipids and inflammatory mediators, we screened expression of a panel of nuclear receptors in macrophages treated with a variety of stimuli. Expression of NOR1 (NR4A3) was found to be highly induced in the human monocytic cell line THP-1 in response to treatment with TPA or LPS (Fig. 1A) and the inflammatory cytokines TNFα, IFNγ, and IL-1β (Fig. 1B). NOR1 was also slightly up-regulated by M-CSF and GM-CSF in THP-1 cells. Other activators such as the retinoic acid receptor agonist AM580 or vitamin D3 had no effect. LPS also stimulated expression of the three NR4A family members in primary human monocyte-derived macrophages (Fig. 1C). We further explored the effect of LPS on expression of NR4A receptors in THP-1 cells over the time course of 1 to 24 h (Fig. 1D). All three receptors were rapidly induced (after 1 h) by LPS. Interestingly, whereas expression of Nur77 and Nur1 mRNA decreased after early induction, NOR1 mRNA increased over the course of 24 h in these cells. Note that the absolute level of Nur1 expression in macrophages was significantly lower than that of Nur77 and NOR1 (Fig. 1D and data not shown).

We next investigated the regulation of NR4A receptors in human macrophages by LPS and TNFα. A time course of LPS treatment of thioglycolate-elicited peritoneal macrophages (Fig. 1E) or RAW264.7 cells (Fig. 1F) revealed that Nur77 and NOR1 were potently induced at early time points, with RNA levels peaking at 1 and 2 h post-treatment, respectively. A similar pattern was also observed in murine bone marrow-derived macrophages (data not shown). Consistent with the results obtained in human THP-1 cells, the cytokine TNFα also induced Nur77 expression in murine RAW macrophages (Fig. 1G).

Next we examined macrophage NR4A protein expression in response to LPS. Western blot analysis using an antibody that recognizes all three NR4A receptors showed that NR4A protein accumulated in the nucleus as early as 1 h after stimulation and remained elevated over the 24-h time course (Fig. 2A). IxBα protein expression was monitored as a control. As expected, IxBα levels in the cytoplasm decreased early due to degradation but then recovered. Nur77 protein expression was further studied using immunofluorescence microscopy. The specificity of our Nur77 antibody was confirmed using 293T cells that had been transfected with an expression plasmid for Nur77 or LacZ. Strong nuclear staining was observed only in Nur77-transfected cells (Fig. 2B). Nuclear expression of Nur77 protein in RAW264.7 macrophages was confirmed by co-staining with antibodies to Nur77 and the macrophage marker F4/80 (Fig. 2C). Finally, treatment of RAW264.7 macrophages with LPS led to a marked increase in nuclear Nur77 protein expression (Fig. 2D).

To identify the upstream signaling pathways that mediate induction of Nur77 in response to LPS, we pre-incubated RAW cells with specific signaling pathway inhibitors. As shown in Fig. 3A, inhibition of the p38 MAPK pathway by SB203580 or the MAPK/extracellular signal-regulated kinase kinase (MEK) pathway by PD98059 had no effect on Nur77 induction at this time point (1 h). LY294002, a phosphatidylinositol 3-kinase inhibitor, slightly enhanced Nur77 induction. By contrast, the proteosome inhibitor MG-132 (which blocks IxB degradation and thereby inhibits NF-κB signaling) strongly inhibited the induction of Nur77 by LPS (Fig. 3A).

To further explore the signaling pathway controlling macrophage Nur77 expression, the human and mouse Nur77 promoters were compared. These promoters share a high degree of sequence identity and contain well conserved binding sites for NF-κB and AP-1 transcription factors (Fig. 3B).
Because LPS induction of Nur77 expression is very rapid, we focused our studies on the conserved NF-κB sites. NF-κB is a principal transcription factor involved in response to LPS and is known to induce gene expression very rapidly upon activation. Transient transfections into RAW macrophages were carried out using reporter constructs containing serial deletions of the Nur77 promoter. These studies confirmed the importance of the NF-κB elements, as their deletion resulted in a 50–60% reduction of reporter activity (Fig. 3C). Note that the difference between activity of p110021043 and p11002535 constructs was consistent among multiple independent experiments, suggesting the possible presence of an inhibitory element between p110021043 and p11002535. When the NF-κB sites were mutated alone or together, induction of Nur77 promoter activity by LPS (TLR4 ligand), poly(I:C) (TLR3 ligand), or lipo-teichoic acid (LTA) (TLR2 ligand) was greatly reduced (Fig. 3D).

To definitively address the role of NF-κB signaling in Nur77 promoter regulation, we employed MEFs lacking expression of the three NF-κB subunits essential for the classical NF-κB pathway (nfkb1−/−, c-rel−/−, relA−/−). These cells show no detectable κB site binding activity in response to NF-κB-inducing stimuli (data not shown). As expected, the induction of IkBα by LPS was abolished in these cells (Fig. 4). The induction of Nur77 by LPS was also severely compromised in nfkb1−/−, c-rel−/−, relA−/− cells, confirming the central role of the NF-κB pathway in this response. Similar impaired induction of NOR1 was also observed in nfkb1−/−, c-rel−/−, relA−/− cells (data not shown).

Considerable evidence has linked oxidized lipids to the pathogenesis of atherosclerosis. We therefore explored the effect of oxLDL and various oxysterols on macrophage expression of NR4A receptors. Note that under the conditions used here, oxidized lipids did not induce obvious apoptosis in treated cells (data not shown). When THP-1 cells were treated with different forms of LDL for 48 h, a robust induction of NOR1 and Nur77 was observed in response to oxLDL but not to native LDL or acLDL (Fig. 5A). We also observed a strong induction of NR4A receptor expression by 25-hydroxycholesterol (25-HC) and 7β-cholesterol. These oxysterols have been reported to be major species present in human atherosclerotic plaques and are atherogenic in animal models (22–24). Oxysterols also induced the expression of members of the NR4A family in TPA-differentiated THP-1 cells (Fig. 5B), as well as in primary human macrophages (Fig. 5E).

2 A. Hoffmann, manuscript in preparation.
monocyte-derived macrophages (Fig. 5C). Some cell type specificity in the response to oxysterols was noted. For example, although Nur77 was consistently induced in all cell types, NOR1 was induced in THP-1 cells but not human monocyte-derived macrophages.

The ability of oxidized lipids to activate NR4A expression did not correlate with their ability to activate PPAR or LXR. For example, the specificity of oxidized lipid induction of NOR1 in THP-1 macrophages was distinct from ABCA1 induction (Fig. 5D). Whereas oxLDL induced expression of both genes, 25-HC was the most effective inducer of NOR1, and the LXR agonist 22(R)-HC was the most effective inducer of ABCA1. Furthermore, synthetic high affinity ligands for PPAR or LXR (GW7845, 1M) did not induce NR4A expression (data not shown), indicating that oxidized lipids regulate NR4A receptor expression through an LXR- and PPAR-independent mechanism.

Finally, to address whether transcriptional regulation by NR4A receptors might be relevant to artery wall macrophages, we examined Nur77 expression in human coronary artery atherosclerotic lesions. As shown in Fig. 6A, Nur77 antibody showed positive staining throughout the lesion. This pattern of expression was overlapping with that of the macrophage-specific marker CD68 (Fig. 6B). Co-staining with antibodies to both Nur77 and CD68 confirmed macrophage expression of Nur77 (Fig. 6C and shown at higher magnification in Fig. 6E). However, Nur77 expression was not macrophage-specific. Several other cell types within the lesion, notably smooth muscle cells, also expressed Nur77 (Fig. 6C).

**DISCUSSION**

The NR4A proteins are among the most evolutionarily ancient nuclear receptors. Higher eukaryotes possess three closely related receptors, Nur77, Nurr1, and NOR1, whereas *Drosophila* has a single homolog, DHR38. Recent structural studies have shown that the ligand binding domains of these receptors do not contain a pocket large enough to support the binding of a small molecule (25, 26). Thus, these proteins appear to be ligand-independent transcription factors for which activity is controlled at the level of protein expression and/or post-translational modification. Indeed, the mammalian NR4A receptors were identified originally as early response genes induced by growth factors such as nerve growth factor (NGF) (8). Previous studies of Nur77 and NOR1 have focused primarily on their roles in T cell apoptosis (11), whereas Nurr1 has been implicated in the development of dopaminergic neurons (14). The function of these receptors in other cell types, however, is poorly understood. In the present work we have shown that expression of Nur77, NOR1, and Nurr1 is highly inducible in macrophages by diverse inflammatory stimuli, including cytokines, TLR ligands, and oxidized lipids. These observations point to a previously unrecognized role for NR4A receptors in macrophage inflammatory responses.

Previous work has identified the PPARs and LXRs as nuclear receptors responsive to oxidized lipids present in the atherosclerotic artery wall. Both PPARγ and LXRα are induced during macrophage differentiation, and their transcriptional activity is activated by the binding of specific oxidized lipids. Oxidized fatty acids such as 9-hydroxyoctadecadienoic acid are direct ligands for PPARγ, whereas oxysterols are direct ligands...
for LXR (2). The relevance of these observations for the pathophysiology of atherosclerosis is underscored by the observations that synthetic ligands for PPARγ or LXR inhibit the development of atherosclerosis in mice (6, 7). In the present work, we have identified NR4A receptors as a novel pathway for the control of macrophage gene expression by inflammatory stimuli. In contrast to PPAR and LXR, oxidized lipids regulate Nur77 activity not through direct binding to the receptor but by increasing the level of receptor expression within the cell. Although the molecular mechanism involved in Nur77 induction by oxidized lipids remains to be elucidated, we have shown that the rapid induction by inflammatory stimuli such as LPS requires the action of NF-κB proteins on the Nur77 promoter.

Despite the fact that the NR4A receptors were among the first orphan nuclear receptors cloned, our knowledge of their biological function is still limited. In particular, very few direct target genes for these transcription factors have been identified. In the brain, the tyrosine hydroxylase and the proopiomelanocortin genes are targets (27, 28). The apoptotic effects of Nur77 in T cells have been proposed to involve the target genes FasL and CD30 (29, 30). However, Nur77-mediated apoptosis is intact in gld/gld (FasL mutant mice) or CD30−/− mice.
NR4A Receptors Are Macrophage Inflammatory Response Genes

excluding both as major downstream effectors. Previous studies have reported that expression of Nurr1 is elevated in rheumatoid arthritis synovial tissue; however, the functional consequence of receptor induction in this context has not been explored (31). In contrast to the mitochondrial Nur77 localization seen in some apoptotic circumstances (12, 32), inducible expression of Nur77 proteins in our studies localized to the nucleus (Fig. 2). This observation suggests that NR4A proteins function as classical transcription factors in this context. An important goal of future work will be to identify genes that are controlled by NR4A proteins in macrophages. Because all three NR4A members are strongly induced by inflammatory stimuli and bind to the same response elements, it seems likely that they may regulate expression of some of the same genes. Thus, these receptors may play redundant roles in macrophage inflammatory responses as well as in T cell apoptosis (33).

Given the causal role of oxidized lipids in atherogenesis, our results raise the possibility that NR4A-dependent gene expression may be involved in the pathogenesis of atherosclerosis. Recent work from other groups has investigated the function of NR4A receptors in both smooth muscle and endothelial cells and their relevance to cardiovascular disease. Badimon and colleague (34) have reported that Nur77 expression is induced in smooth muscle cells by LDL treatment. Gruber et al. (35) have reported that Nur77 regulates PAI-1 expression in endothelial cells in response to TNFα. Another recent study employed a carotid artery ligation model to show that neointimal formation is inhibited in vascular smooth muscle cell-specific Nur77 transgenic mice and increased in smooth muscle cell-

Fig. 6. Nur77 protein is expressed in macrophages within human coronary artery atherosclerotic lesions. Frozen sections of human left coronary artery containing atherosclerotic plaques were stained with anti-Nur77 (A), anti-CD68 (B), anti-Nur77 and anti-CD68 (C and E), or control IgG and anti-CD68 (D). Nur77 protein is highly expressed in macrophages but is also present in other cell type within the lesion. Objective magnification: ×10 for A–C and ×40 for D and E.
specific Nur77 dominant-negative transgenic mice (36). However, the contribution of macrophage Nur77 to cardiovascular disease has not yet been addressed. In the future it will be important to test directly the contributions of NR4A-dependent gene expression for the development of atherosclerosis in apoE or LDLR null mice. Because of the redundancy of these receptors, however, it is likely that a strategy to knock down two or all three receptors will be required.

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