NR4A nuclear receptors in atherosclerosis
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Nuclear receptors Nur77, Nurr1 and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses

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Abstract
Objective: Atherosclerosis is an inflammatory disease in which macrophage activation and lipid loading play a crucial role. In this study, we investigated expression and function of the NR4A nuclear receptor family, comprising Nur77 (NR4A1, TR3), Nurr1 (NR4A2) and NOR-1 (NR4A3) in human macrophages.

Methods and Results: Nur77, Nurr1 and NOR-1 are expressed in early and advanced human atherosclerotic lesion macrophages primarily in areas of plaque activation/progression as detected by in-situ-hybridization and immunohistochemistry. Protein expression localizes to the nucleus. Primary and THP-1 macrophages transiently express NR4A-factors in response to LPS and TNF-α. Lentiviral overexpression of Nur77, Nurr1 or NOR-1 reduces expression and production of IL-1β and IL-6 pro-inflammatory cytokines and IL-8, MIP-1α and -1β and MCP-1 chemokines. In addition, NR4A-factors reduce oxidized–LDL uptake, consistent with downregulation of scavenger receptor-A, CD36 and CD11b macrophage marker genes. Knockdown of Nur77 or NOR-1 with gene-specific lentiviral short-hairpin RNAs resulted in enhanced cytokine and chemokine synthesis, increased lipid loading and augmented CD11b expression, demonstrating endogenous NR4A-factors to inhibit macrophage activation, foam-cell formation and differentiation.

Conclusion: NR4A-factors are expressed in human atherosclerotic lesion macrophages and reduce human macrophage lipid loading and inflammatory responses providing further evidence for a protective role of NR4A-factors in atherogenesis.
Introduction

Atherosclerosis is a chronic inflammatory disease, involving deregulation of both immune system and lipid metabolism (Ross, 1999; Libby, 2002). Macrophages, imperative in the innate immune system, are involved in the initiation, progression and rupture of atherosclerotic lesions, as well as in the initiation of smooth muscle cell (SMC)-rich pathologies like restenosis (Hansson, 2005; Welt and Rogers, 2002). At the onset of atherosclerosis, monocytes are locally recruited to the arterial vessel wall, where these cells differentiate into macrophages. These intimal macrophages ingest modified lipid particles and become lipid-laden foam-cells that form a so-called fatty streak. In advanced atherosclerotic lesions, macrophages are localized primarily around a central lipid core and at the shoulder region of the plaque. At the latter site, which is known to be prone to rupture, these cells may be involved in destabilization of the lesion (van der Wal et al., 1994). Throughout the progression of atherosclerosis, macrophages produce pro-inflammatory cytokines, chemokines, growth factors and matrix-degrading enzymes and are consequently crucial in the chronic inflammatory process in the diseased vessel wall (Kirii et al., 2003; Weber et al., 2004). Detailed knowledge on the molecular mechanisms involved in the inflammatory and metabolic processes in macrophages is essential to develop novel drug therapies against atherosclerosis. We hypothesized that NR4A nuclear receptors are key regulatory factors involved in modulation of these specific processes in macrophages.

The NR4A nuclear hormone receptors were first described as early response transcription factors expressed upon stimulation by growth factors (Nakai et al., 1990; Law et al., 1992; Ohkura et al., 1994). This NR4A subfamily comprises three members, notably Nur77 (NR4A1, TR3, NGFI-B, NAK-1), Nurr1 (NR4A2, NOT) and NOR-1 (NR4A3, MINOR) (A unified nomenclature system for the nuclear receptor superfamily, 1999). Like other nuclear receptors, NR4A-factors contain a central DNA-binding domain, comprising two zinc-fingers, that bind the consensus response element NBRE (AAAGGTCA) as monomers and the palindromic NurRE element (TGATATTTX6AAAGTCCA) as homo/heterodimers in promoters of specific target genes (Philips et al., 1997). Furthermore, nuclear receptors consist of an N-terminal domain mediating transactivation and a C-terminal ligand-binding domain. Specific ligands for the NR4A family of transcription factors have not been identified, classifying them as orphan nuclear receptors (Hsu et al., 2004). At the C-terminal domain both Nur77 and Nurr1 can heterodimerize with RXRs and mediate retinoid responses (Wallen-Mackenzie et al., 2003). The NR4A family members have been shown to be functionally involved in T-cell and cancer cell apoptosis (Cheng et al., 1997; Li et al., 2000; Lin et al., 2004), and in
dopaminergic differentiation of neurons (Zetterstrom et al., 1997).

In search for genes involved in SMC activation in atherogenesis, we revealed induction of Nur77 and NOR-1 expression in in vitro–activated SMCs (de Vries et al., 2000). Furthermore, we have shown expression of all three NR4A-factors in atherosclerotic lesions and in cultured human SMCs and endothelial cells (ECs) (Arkenbout et al., 2002; Arkenbout et al., 2003). We demonstrated that Nur77 overexpression in vitro inhibits proliferation of both SMCs and ECs. In vivo overexpression of Nur77 under the control of an arterial SMC-specific promoter in transgenic mice protects against SMC-rich lesion formation (Arkenbout et al., 2002). Other nuclear receptors, notably PPARs and LXR$s play an important role in both SMC and macrophage function relevant to atherosclerosis and restenosis (Blaschke et al., 2004; Joseph et al., 2003; Li et al., 2004; Marx et al., 2004). However, the function of NR4A-factors in human macrophages is unknown.

In the current study, we show for the first time expression of all three NR4A family members Nur77, Nurr1 and NOR-1 in human atherosclerotic lesion macrophages and we demonstrate that these factors reduce the uptake of oxidized low-density lipoprotein (ox-LDL) as well as the inflammatory response in human macrophages.

**Materials and methods**

**Human tissue specimens**

Human tissue samples were obtained with informed consent from organ donors, according to protocols approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam. The specimens were paraffin embedded, sectioned, and mounted on glass slides (Superfrost-Plus, Emergo). Vascular specimens were characterized by immunohistochemistry with antibodies specific for SMCs and macrophages to establish the stage of disease according to the American Heart Association classification (Stary et al., 1995).

**Immunohistochemistry and double in situ immunohistochemistry**

Macrophages were detected by monoclonal antibody Ham56 (DAKO) and SMCs by monoclonal antibody 1A4 (DAKO) directed against smooth muscle α-actin, in human vascular specimens. Anti-Nur77 (M-210, Santa Cruz Biotechnology), anti-Nurr1 (M-196, Santa Cruz Biotechnology) and anti-NOR-1 (rabbit polyclonal antibody directed against NOR-1 N-terminal domain was generated; for data on specificity, Supplemental Figure SI) were used to detect NR4A nuclear receptors. Briefly, after deparaffinization and endogenous peroxidase quenching, citrate antigen retrieval was performed, followed by blocking and permeabilization with 1% (w/vol)
bovine serum albumin, 1% (vol/vol) normal goat serum and 0.5% Triton-X 100 and primary antibody incubation overnight at 4°C. After biotin-labeled goat-anti-rabbit IgG secondary antibody (DAKO) incubation followed by streptavidin-HRP (DAKO), AEC (Sigma) detection was applied. Staining after secondary antibody incubation alone served as a negative control.

Combination of radioactive gene-specific in situ hybridization and macrophage-specific immunohistochemistry was essentially performed as described (Arkenbout et al., 2002). For in situ hybridization the following riboprobes were synthesized: Nur77, GenBank No. L13740, bp 1221 to 1905; Nurr1, GenBank No. X75918, bp 119 to 1003 and NOR-1, GenBank No. U12767, bp 1435 to 2172. After hybridization macrophages were detected using immunohistochemistry as described above, followed by emulsion radiography. Matching sense riboprobes were assayed for each gene and were shown to give neither background nor a non-specific signal. The sections were exposed for 4 to 8 weeks. All slides were counterstained with hemotoxylin and embedded in glycergel (DAKO).

Cell culture
Primary human macrophages were isolated from buffy-coats of blood donors, obtained from the Dutch central bloodbank Sanquin. After isolation by Ficoll-Paque (Pharmacia Biotech) gradient centrifugation, monocyte-negative selection kit (Dynal) and adhesion-mediated purification, cells were cultured for 48 hours at a density of 0.5-1 × 10⁶ cells/ml before experiments were performed. Human monocytic THP-1 cells (ATCC) and human monocytic U937 cells were cultured in RPMI 1640, 10% (vol/vol) fetal bovine serum and 100 U/ml penicillin/streptomycin (GIBCO-BRL). Cells were plated in 12-wells plates at a density of 0.5 × 10⁶ cells/ml, differentiated into macrophages by PMA (100 ng/ml) for 48 hours. After differentiation, cells were washed twice with PBS and grown in medium for 24 hours. Reagents used were PMA (Sigma), LPS (Sigma), recombinant human TNF-α (R&D) and DiI-labeled ox-LDL (Intracel-RP-173).

Lentiviral vector construction, infection and shRNA interference
hNur77 cDNA (GenBank D49728, bp 8-1920) was cloned into the XbaI-Ndel sites of the pRRI-cPpt-PKG-PreSIN vector (PGK-Nur77). hNurr1 cDNA (GenBank X75918, bp 73-2310) was placed into the SalI-NsiI sites of the pRRI-cPpt-PKG-PreSIN vector (PGK-Nurr1) and hNOR-1 cDNA (GenBank D78579, bp 513-2872) was ligated into the XbaI site of the pRRI-cPpt-PKG-PreSIN vector (PGK-NOR-1). PGK-EGFP-PreSIN (PGK-EGFP) was constructed by isolating the EGFP cDNA from the expression vector pEGFP-
N2 (Clontech) using SalI-XbaI digestion, subsequently ligated into the corresponding sites of the pRRI-cPpt-PGK-PreSIN vector (Seppen et al., 2002). Short hairpin (sh) Nur77 and shNOR-1 were cloned into p156RRL-sinPPT-CMV-GFP-PRE/NheI (Dekker et al., 2005). Briefly, shNur77: CAGTCCAGCCATGCTCCTC TCTCTTGAA GAGGAGC A TGGCTGGACTG and shNOR-1: GAAGATCAGACATTACTTA TCTCTTGAA_TAAGTAATGTCTGATCTTC (bold sequences are target sequences; underlined sequences represent hairpin) were coupled to the H1-promoter by PCR amplification and subsequently cloned into p156RRL-sinPPT-CMV-GFP-PRE/NheI as described (Dekker et al., 2005). Virus was produced as described (Seppen et al., 2002). Briefly, 20 µg of PGK transfer vector, 13 µg of pMDLg/pRRE, 7 μg pVSV-g, and 5 µg of pRSV-REV were co-transfected into 180 cm² HEK293T cells using the calcium phosphate co-precipitation method. Conditioned medium was harvested at 48 hours and 72 hours after transfection, filtered through 0.45µm filters and concentrated by ultra centrifugation (20,000 rpm, 2 hours, 4°C). Viral titers were determined essentially as described before (Sastry et al., 2002). In short, HEK 293 cells were transduced with serially diluted viral concentrate, 48 hours after transduction total genomic DNA was isolated from these cells and the number of vector DNA copies was determined using PCR analysis with pRRI-cPpt-PGK-PreSIN vector as calibration standard (forward primer: 5’-GTGCAGCAGCAGAACAA TTTG-3’, reverse primer: 5’-CCCCAGACTGTGAGTTGCAA-3’). THP-1 and U937 cells were transduced for 24 hours with recombinant lentivirus at a multiplicity of infection (MOI) of 3 and 9 respectively in the presence of 10μgr/ml DEAE-dextran. After transduction cells were cultured in suspension for 72 hours, differentiated into macrophages and cultured as described above. Overexpression of Nur77, Nurr1, NOR-1 and EGFP was verified by flow cytometric analyses (EGFP) and immunofluorescence (Supplemental Figure SII.1). shNur77 and shNOR-1 constructs contained CMV-GFP and transduction efficiency was verified by flow cytometric analysis (GFP). Knockdown was confirmed by RT-PCR (Supplemental Figure SII.2) and immunofluorescence (de Waard et al., 2006).

**Lentiviral infection**

THP-1 and U937 cells were transduced in the presence of 10 µg/ml DEAE-dextran with recombinant lentivirus for 24 hours at a multiplicity of infection of 3 and 9, respectively. Empty (Mock) and EGFP lentivirus were taken along as controls. After transduction cells were cultured in suspension for 72 hours, differentiated into macrophages and cultured as described. Overexpression of Nur77, Nurr1, NOR-1 and EGFP was checked by immunofluorescence and flow cytometric analyses.
Nur77, Nurr1 and NOR-1 in macrophages

(EGFP) (Supplemental Figure SII.1). shNur77 and shNOR-1 constructs contained CMV-GFP and transduction efficiency was verified by flow cytometric analysis (GFP) (data not shown). For immunofluorescence, cells were cultured on glass, fixed for 20 min with 4% (w/vol) paraformaldehyde PBS and permeabilized with 0.5% (vol/vol) Triton-X-100. Cells were stained by anti-Nur77 (M-210, Santa Cruz Biotechnology), anti-Nurr1 (M-196, Santa Cruz Biotechnology) and anti-NOR-1 for detection of Nur77, Nurr1 and NOR-1 respectively, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated donkey anti-goat IgG (Molecular Probes). Nuclei were stained with Hoechst.

RNA and protein analysis
Total RNA was extracted using RNA absolutely Miniprep kit (Stratagene). cDNA was made using iScript cDNA Synthesis kit (Biorad) and semi-quantitative real-time RT-PCR was performed using iQ SYBR-Green Super-Mix in the MyiQ RT-PCR system (Biorad). All RT-PCR data were corrected for housekeeping gene ribosomal protein P0. Protein levels of IL-1β, IL-6 and IL-8 were determined in supernatant of cell cultures by BD Cytometric Bead Array according to manufacturers’ protocol. Specific primers for Nur77, Nurr1, NOR-1, scavenger receptor-A (SR-A), CD36, CD11b, macrophage inflammatory protein-1α (MIP-1α) and-1β (MIP-1β), monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-1β, IL-6 and ribosomal protein P0 were designed as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nur77</td>
<td>5'-GTTCTCTGGAGGTGTCATCCCGAAG-3'</td>
<td>5'-GGCTAACTGAAGAAGGCAACAC-3'</td>
</tr>
<tr>
<td>Nurr1</td>
<td>5'-TATCCAGGAGGGCAAGGAAA-3'</td>
<td>5'-AGCCTGTCCCTACTTGAGGG-3'</td>
</tr>
<tr>
<td>NOR-1</td>
<td>5'-CCAAAGGCTAGCTGGCTGT-3'</td>
<td>5'-GTGAGTACGGAGTCAAGAGG-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-TGACCGAAAAAGTGAGGAGAA-3'</td>
<td>5'-GCTGCTTGGACATTTGCTGT-3'</td>
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<tr>
<td>IL-6</td>
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<td>5'-ATGCGCCGAGAGAGGAGG-3'</td>
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<tr>
<td>MIP-1α</td>
<td>5'-ACGGGACCGAGAGGAGG-3'</td>
<td>5'-ACCGAAGGAGGAGGAGG-3'</td>
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<tr>
<td>MIP-1β</td>
<td>5'-GCGGATGCAGCTTGAC-3'</td>
<td>5'-CCCAGGAGGAGGAGGAGG-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-CCTAGCTCTCCACACACAC-3'</td>
<td>5'-CCTAGCTCTCCACACACAC-3'</td>
</tr>
<tr>
<td>SR-A</td>
<td>5'-CTGGCTCAATGACAGCTTC-3'</td>
<td>5'-CGTGTACGGAGTAGTGAGGAG-3'</td>
</tr>
<tr>
<td>CD36</td>
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<td>5'-GGAGTACGGAGTAGTGAGGAG-3'</td>
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<tr>
<td>CD11b</td>
<td>5'-CAGCAACAGCAACACAGCAAGA-3'</td>
<td>5'-ATCCGTCTCCACAGAAGG-3'</td>
</tr>
<tr>
<td>P0</td>
<td>5'-TCGCAATGCGCAGCTAC-3'</td>
<td>5'-GGAGTACGGAGTAGTGAGGAG-3'</td>
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</table>

Lipid loading, quantification and microscopy
After lentiviral infection THP-1-derived macrophages were treated with Dil-labeled ox-LDL for time periods indicated, subsequently washed twice with PBS and lysed in isopropanol. After sonification followed by 10 minutes centrifugation (13000g) Dil-labeled ox-LDL content was measured by fluorometry. For confocal microscopy, cells were cultured on glass and treated with Dil-labeled ox-LDL.
Statistical analysis
The unpaired Student’s t-test was used to calculate the statistical significance of the expression ratios versus control. P values less than 0.05 were considered statistically significant.

Results

Nur77, Nurr1 and NOR-1 are expressed in human atherosclerotic lesion macrophages
In previous studies we demonstrated expression of Nur77, Nurr1 and NOR-1 in both SMCs and ECs in atherosclerotic lesions (Arkenbout et al., 2002; Arkenbout et al., 2003). In this study, we show expression of Nur77, Nurr1 and NOR-1 in atherosclerotic lesion macrophages by combining macrophage-specific immunostaining with gene-specific in-situ-hybridization. Aorta specimens of 8 different organ donors (3 males and 5 females, age 40-69 years) were characterized by immunohistochemistry according to the American Heart Association guidelines (Table 1 and Figure 1A and 1B) (Stary et al., 1995). The complexity of the lesions analyzed ranged from class II to VI. mRNA expression levels of Nur77, Nurr1 and NOR-1 in lesion macrophages and SMCs were scored and specific localization of expression in the lesion indicated. As a typical example of an early lesion, we show a type II lesion with high mRNA expression levels of all three nuclear receptors in macrophages (Figure 1C-E; † in Table 1). Protein expression of Nur77, Nurr1 and NOR-1 localizes to the nucleus in macrophage-rich areas and is comparable with the mRNA expression pattern (Figure 1F-I; ‡ in Table 1). Notably, in complex lesions, prominent macrophage-specific NR4A expression is localized especially to shoulder regions and macrophages infiltrated in the media.

Nur77, Nurr1 and NOR-1 are expressed in response to inflammatory stimuli and reduce ox-LDL lipid loading
High expression levels of NR4A-factors in atherosclerotic lesion macrophages prompted us to study whether their expression is dependent on inflammatory signaling pathways that are active at diseased areas. In addition, the functional activity of these transcription factors was determined in in vitro studies.
In line with recently published data (Pei et al., 2005), we observed robust and transient mRNA expression of all three NR4A-factors in primary macrophages and in monocytic THP-1 cells in response to LPS. In addition, we show that NR4A-factors are moderately induced by TNF-α in primary macrophages and highly induced (50-150 fold induction) in THP-1 PMA-maturated macrophages in response to LPS.
**Table 1. Donor characteristics and mRNA expression profiles of Nur77, Nurr1 and NOR-1.**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Class (AHA)</th>
<th>Lesion MØ</th>
<th>Lesion SMC</th>
<th>Area of expression in vessel wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>40</td>
<td>II</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>F</td>
<td>41</td>
<td>II</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>F†</td>
<td>56</td>
<td>II</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
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<td>II-III</td>
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<td>+++</td>
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<tr>
<td>M</td>
<td>49</td>
<td>V</td>
<td>+++</td>
<td>+++</td>
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<tr>
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<td>49</td>
<td>VI</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M</td>
<td>66</td>
<td>VI</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

M: male; F: female; yrs: years; AHA: American Heart Association Classification; +: low expression, ++: moderate expression, +++: high expression; †: shown in Fig 1A-1E; ‡: shown in Figure 1F-1I.

**Figure 1, A-E; Macrophage-specific expression of Nur77, Nurr1 and NOR-1 in human atherosclerosis.** A-E: Serial sections of a human type II-lesion (indicated (†) in Table 1), were analyzed by immunohistochemistry to detect macrophages (A) and SMCs (B). To demonstrate macrophage-specific expression of Nur77, Nurr1 and NOR-1, sections were analyzed simultaneously by macrophage-specific immunohistochemistry and in-situ hybridization with gene-specific probes (C-E). mRNA expression (black silver grains) of Nur77 (C), Nurr1 (D) and NOR-1 (E) co-localizes with a number of macrophages (in red). F-I: Protein expression of Nur77, Nurr1 and NOR-1 in human atherosclerosis. Serial sections of a human type II-lesion (indicated (‡) in Table 1), were analyzed by immunohistochemistry to detect macrophages (F), Nur77 (G), Nurr1 (H) or NOR-1 (I). NR4A proteins are expressed predominantly in neointimal cells and do localize to nuclei. The sections shown in G-I were not counterstained for nuclei. MΦ, macrophages; Neo, neointima; Lu, lumen; M, media. Arrows in C, D and E point at macrophages expressing the specific mRNAs.
Immunofluorescent analysis of NOR-1 expression revealed that this protein localizes predominantly to the nucleus after LPS stimulation (Supplemental Figure SIII).

To study the function of Nur77, Nurr1 and NOR-1 in macrophages, we infected THP-1 cells with lentiviruses that encode these factors or control Mock-virus and determined the effect on lipid loading, a hallmark of atherosclerosis. Lentiviral overexpression of NR4A nuclear receptors resulted in 80-90% transduction efficiency and nuclear localization of the encoded proteins (Supplemental Figure SII.1). Viability of NR4A overexpressing cells was comparable to control cells (data not shown). In macrophages overexpressing NR4A-factors Dil-labeled ox-LDL uptake was quantified by fluorometry and was shown to be reduced after 3 to 6 hours, with more than 30% reduction after 24 hours (Figure 2A). Confocal microscopy was performed to assess the cellular localization of Dil-labeled ox-LDL in macrophages. After 24 hours, Dil-fluorescence localizes to lipid vacuoles and

Figure 2. NR4A overexpression in human macrophages reduces Dil-labeled ox-LDL uptake and expression of SR-A, CD36 and CD11b. Uptake of Dil-labeled ox-LDL for 3, 6 and 24 hours was determined by fluorometry. Lipid loading was significantly lower in THP-1 macrophages overexpressing Nur77, Nurr1 or NOR-1 as compared to Mock (A). After 24 hours of Dil-labeled ox-LDL treatment THP-1 macrophages were analyzed by confocal microscopy showing reduced Dil-fluorescence intensity in Nur77-overexpressing macrophages, localizing to lipid vacuoles (B). mRNA expression of SR-A, CD36 and CD11b was determined by real-time RT-PCR. THP-1 macrophages overexpressing Nur77, Nurr1 and NOR-1 expressed significantly lower levels of SR-A, CD36 and CD11b (C) (A, C; n=3 ±SD, Student’s t-test; p<0.01). In U937 macrophages Nur77, Nurr1 and NOR-1 overexpression resulted in decreased CD11b mRNA expression as compared to Mock (D; n=2 ±SD, Student’s t-test; p<0.02).
fluorescence intensity is relatively low in Nur77-overexpressing macrophages as compared to Mock-lentivirus infected cells (Figure 2B). Since SR-A and CD36 are important genes involved in modified lipoprotein uptake, mRNA expression levels of these genes were determined by semi-quantitative real-time RT-PCR. THP-1 macrophages overexpressing Nur77, Nurr1 or NOR-1 express significantly lower levels of SR-A and CD36 than Mock-virus infected cells (Figure 2C). In addition, we show that CD11b expression, a general macrophage marker gene, is reduced in both THP-1 and U937 macrophages overexpressing NR4A receptors (Figure 2C and 2D). To unravel the function of endogenous NR4A-factors in foam-cell formation specific shRNAs against Nur77 and NOR-1 were designed. Knockdown of endogenous Nur77 or NOR-1 resulted in a significant increase in Dil-labeled ox-LDL uptake consistent with an approximately 2-fold increase in SR-A and CD36 mRNA expression as compared to cells transduced with control shRNA (Figure 3A, B). CD11b expression was increased 2.3-2.7-fold in shNur77 or shNOR-1 expressing THP-1 macrophages, respectively (Figure 3C).

**Lentiviral overexpression of Nur77, Nurr1 and NOR-1 reduces pro-inflammatory cytokine and chemokine expression**

Next, we assayed NR4A function in cytokine- and chemokine synthesis in human THP-1 and U937 macrophages. mRNA levels of pro-inflammatory cytokines IL-1β and IL-6 and chemokines IL-8, MIP-1α/-β and MCP-1 were determined after stimulation with LPS, TNF-α or vehicle (Figure 4A). As a control for the activity of LPS and TNF-α, mRNA levels were assayed in Mock-infected macrophages (Figure 4A).

![Figure 3](image-url)

**Figure 3.** shRNA-mediated Nur77 and NOR-1 knockdown results in increased Dil-labeled ox-LDL uptake and enhanced expression of SR-A, CD36 and CD11b. Dil-labeled ox-LDL uptake for 24 hours was determined by fluorometry. Ox-LDL uptake was significantly increased in THP-1 macrophages expressing shNur77 and shNOR-1 as compared to control shRNA (A), consistent with a significant increase in SR-A and CD36 mRNA expression as determined by RT-PCR (B) (A-B; n=2 ±SD, Student’s t-test, p<0.05). In addition, in shNur77 and shNOR-1 expressing THP-1 macrophages elevated CD11b mRNA expression levels was detected as compared to shRNA control (C; n=2 ±SD, Student’s t-test, p<0.05).
Except for IL-6 expression, which is not detectable (ND) in vehicle or TNF-α-treated cells, mRNA expression levels of these inflammatory genes are induced 20-8000 fold by LPS and 3-10 fold by TNF-α. mRNA levels of these chemokines and cytokines analyzed are robustly reduced (2-10 fold induction) in THP-1-macrophages infected cells the genes analyzed were induced 20-8000 fold after LPS and 3-10 fold after TNF-α (except for IL-6, not detectable after TNF-α or control (ND)). THP-1 macrophages overexpressing Nur77, Nurr1 and NOR-1 expressed significantly lower mRNA levels (> 2-fold reduction) of most of the genes analyzed. Protein levels of IL-8, IL-1β and IL-6 were determined in conditioned media collected at 0, 6 and 24 hours after treatment with LPS (B). Protein levels of IL-8, IL-1β and IL-6 were significantly reduced in THP-1 macrophages overexpressing Nur77, Nurr1 and NOR-1 (A-B; n=3 ±SD, Student’s t-test, p<0.05; ND: not detectable; NS: not significant). In U937 macrophages overexpression of NRA4-factors reduced mRNA expression of IL-8 and MCP-1 (C) and protein levels of IL-8 and IL-6 in conditioned media (D) was detected after 3 hours and 24 hours LPS (100ng/ml), respectively (n=2 ±SD, Student’s t-test, p<0.05). All data shown are significant as compared to Mock.
overexpressing either Nur77, Nurr1 or NOR-1 as compared to Mock-infected cells both after LPS and TNF-α stimulation. As an exception, MCP-1 mRNA expression is 2.5 fold induced by TNF-α in NOR-1 overexpressing macrophages and is not significantly different in Nurr1 overexpressing cells as compared to Mock-infected cells. In addition to the mRNA results described, we determined protein concentrations of IL-1β, IL-6 and IL-8 (Figure 4B) in the conditioned medium of lentivirus-infected THP-1 macrophages. Conditioned media were collected at 0, 6 and 24 hours after treatment with LPS and protein concentrations were determined by BD™ Cytometric Bead Array. Overexpression of Nur77, Nurr1 or NOR-1 results in a significant reduction of LPS-induced secretion of IL-1β, IL-6, and IL-8 by THP-1 macrophages.

To provide further evidence for an anti-inflammatory function of NR4A-factors in human macrophages, we analyzed cytokine and chemokine expression in human U937 cells in gain of function experiments. After stimulation with LPS NR4A-factors reduce mRNA expression of IL-8 and MCP-1 substantially as well as IL-6 and IL-8 protein levels in conditioned media of these cells (Figure 4C and 4D).

The function of endogenous NR4A-factors in inflammatory responses is substantiated by specific shRNAs against Nur77 or NOR-1. Lentivirally delivered shNur77 or shNOR-1 results in an increase of IL-1β, IL-8 and MCP-1 mRNA expression after LPS stimulation as compared to control shRNA infected cells (Figure 5A). In addition, Nur77 or NOR-1 knockdown significantly increases IL-1β and IL-8 protein concentrations in the supernatant of these cells (Figure 5B).

Discussion

Monocyte and macrophage activation together with foam-cell formation are critical events in atherogenesis and other related vascular pathologies. In this study, we demonstrate expression of the NR4A family of nuclear receptors Nur77, Nurr1 and

![Figure 5. Nur77 and NOR-1 knockdown in THP-1 macrophages augments cytokine and chemokine synthesis.](image-url) THP-1 macrophages expressing shNur77 and shNOR-1 were stimulated with LPS (100ng/ml) or control. mRNA expression of IL-1β, IL-8 and MCP-1 after 8 hours LPS was determined (A) and protein levels of IL-1β and IL-8 after 24 hours LPS in conditioned media (B). All data shown are significant (n=2 ±SD, Student’s t-test, p<0.05) as compared to control shRNA.
NOR-1 in human atherosclerotic lesion macrophages, especially in areas of plaque activation/progression. So far, co-localization with macrophage marker CD68 has only been reported for Nur77 (Pei et al., 2005). Lentiviral overexpression of NR4A-factors in human macrophages reduced uptake of modified lipid particles substantially as well as expression of pro-inflammatory cytokines and chemokines. Moreover, shRNA-mediated knockdown of Nur77 or NOR-1 resulted in increased lipid loading and augmented inflammatory responses in these cells indicating that endogenous NR4A-factors are involved in these processes. A potential mechanism for the effects observed is inhibition of macrophage differentiation, which is consistent with reduced expression of SR-A, CD36 and CD11b in human macrophages in NR4A gain of function experiments.

We demonstrate that Nur77, Nurr1 and NOR-1 are transiently induced in response to the inflammatory stimuli LPS and TNF-α in both primary and THP-1-derived macrophages. Especially LPS and, as recently shown, also ox-LDL strongly induce NR4A expression (Pei et al., 2005). Both LPS and ox-LDL promote Toll-like receptor-4 (TLR-4) signaling, which has been shown to be involved in atherogenesis and consequently these in vitro applied stimuli are relevant to atherosclerosis (Michelsen et al., 2004). Paradoxically, NR4A-factors are expressed in areas of plaque progression/activation and are induced by inflammatory stimuli but, as shown in this study, inhibit foam-cell formation and pro-inflammatory cytokine- as well as chemokine production. Similar atheroprotective mechanisms involved in controlling vascular pathologies have been described for other nuclear receptors and are known to be functional during vascular lesion development (Arkenbout et al., 2002; Blaschke et al., 2004; Joseph et al., 2003; Li et al., 2004; Marx et al., 2004).

Nur77 and NOR-1 have been implicated in apoptosis of T-cells involving the transcriptional activity of these transcription factors (Cheng et al., 1997). In macrophages, LPS in combination with the pan-caspase inhibitor zVAD was shown to induce apoptosis involving Nur77, however, the exact mechanism of Nur77 action in zVAD-mediated apoptosis has not been elucidated yet (Kim et al., 2003). In cancer cells, the apoptotic effect of Nur77 depends on the presence of pro-apoptotic agents and involves translocation of Nur77 to mitochondria (Li et al., 2000). Here, we demonstrate nuclear localization of NR4A proteins in human atherosclerotic lesions macrophages as well as in LPS-stimulated cultured macrophages, suggesting the protein to be predominantly active in this cellular compartment. Furthermore, lentiviral overexpression of NR4A nuclear receptors in both THP-1 and U937 macrophages did not result in a reduced viability of those cells.

The reduced uptake of modified LDL as revealed in this study correlates with
downregulation of scavenger receptors SR-A and CD36 expression, which have both been shown to enhance foam cell formation and atherosclerotic lesion size in vivo using macrophage-specific Msr1 and CD36 knock out mice in dedicated atherosclerosis models (Babaev et al., 2000; Febbraio et al., 2004). Although there is compelling evidence for a pro-atherogenic role of these receptors both in vitro and in vivo, recently this paradigm in atherosclerosis has been challenged (Boring et al., 1998). The expression of CD36, and a number of other genes, has been shown to be dependent on Nur77 in skeletal muscle (Maxwell et al., 2005). It is hypothesized that Nur77 enhances lipolysis in skeletal muscle cells and consequently protects against diet-induced obesity, which supports in combination with the reduced lipid uptake in macrophages shown, an anti-atherogenic function for Nur77-like factors. The underlying mechanism of the tissue-specific regulation of CD36 expression by Nur77 and possibly also its subfamily members NOR-1 and Nurr1, awaits further investigations.

The pro-inflammatory cytokines and chemokines analyzed in the current study are considered highly relevant for atherogenesis (Ross, 1999; Libby, 2002; Hansson, 2005; Welt and Rogers, 2002; Kirii et al., 2003; Weber et al., 2004), and the NF-κB pathway is vital for expression of these genes (Ghosh and Karin, 2002). In

Supplemental Figure S1. Specificity of the polyclonal anti-NOR-1 antibody. Human NOR-1 amino-acids 1-196 were extended with an N-terminal His-tag and overexpressed in E. coli. After NiTA-purification the protein-fragment was used to raise antibodies against human NOR-1 in rabbits. Serum IgG-fraction was purified by protein-A-Sepharose affinity chromatography. A: Western blotting. Lysates of COS cells (20μg/lane) transfected with control plasmid or plasmids encoding human Nur77, Nurr1 or NOR-1 under control of the SV40 early promoter were separated by SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, the blot was incubated with anti-NOR-1 antibody (1:1000), followed by goat-anti-rabbit-HRP, which was detected by ECL. The anti-NOR-1 antibody reacted specifically with NOR-1 and shows no cross-reactivity with Nur77 or Nurr1. B-G: Immunofluorescence. THP-1 cells were infected with empty lentivirus (Mock) or lentivirus encoding human Nur77, Nurr1 or NOR-1 and immunofluorescence was performed with the antibodies indicated. Immunofluorescence and nuclear Hoechst staining overlays are shown (see Materials & Methods). B; THP-1 infected with Mock-lentivirus, analyzed with anti-NOR-1. C; THP-1 infected with NOR-1-lentivirus, analyzed with anti-NOR-1. D, E; THP-1 infected with Nur77- or Nurr1-lentivirus analyzed with anti-NOR-1. F; THP-1 infected with Nur77-lentivirus analyzed with anti-Nur77. G; THP-1 infected with Nurr1-lentivirus, incubated with anti-Nurr1. From these data it can be concluded that the polyclonal anti-NOR-1 antibody specifically recognizes human NOR-1.
monocytes Nur77 has been isolated in a genome-wide screen that was designed to identify inhibitors of the NF-κB pathway (Diatchenko et al., 2005). It is demonstrated that overexpression of Nur77 in HEK293 cells potently reduces expression of an NF-κB reporter construct in response to IL-1β and TNF-α. In T-cells it was shown that Nur77 is involved in inhibition of NF-κB-mediated IL-2 and IL-8 promoter activity, probably through binding of the N-terminal activation domain of Nur77 with the p65 subunit of NF-κB (Harant and Lindley, 2004), comparable to the direct inhibitory interaction of the glucocorticoid receptor with NF-κB (Wissink et al., 1997). Taken together, our data may at least in part be explained by transrepression of the NF-κB pathway by NR4A-factors. Interestingly, it has recently been shown that overexpression of Nur77 in a mouse macrophage cell-line results in a pro-inflammatory response involving enhanced expression of inducible I-kappa-B kinase (IKKi), an NF-κB activating gene (Pei et al., 2006). The discrepancy may be explained by species difference, since in the latter study the murine promoter of IKKi was shown to contain a functional NBRE, whereas the human IKKi-promoter does not contain this NBRE.

The role of NR4A nuclear receptors in various vascular cell types has been studied in our group and by others (Arkenbout et al., 2002; Arkenbout et al., 2003; de Waard et al., 2006; Martinez-Gonzalez and Badimon, 2005; Zeng et al., 2006). Nur77 has been shown to promote angiogenesis in dedicated mouse models

Supplemental Figure SII. Transduction efficiency of lentiviral infection of THP-1 cells, nuclear localization of the encoded nuclear receptors and shNur77 and shNOR-1 efficiency. Figure SII.1 THP-1 cells infected with control lentivirus Mock (A, B) or EGFP-encoding lentivirus (C, D) were analyzed by flow cytometry (A-D). Lentiviral infection resulted in 80-90% transduction efficiency. In addition, monocytic THP-1 cells were infected with recombinant lentivirus encoding EGFP (E-G), Nur77 (I-K), Nurr1 (M-O), NOR-1 (Q-S), or with Mock-virus (H, L, P, and T) and differentiated to macrophages by PMA-treatment. Cells were analyzed for direct fluorescence (EGFP and Hoechst) or by immunofluorescence. EGFP protein localized throughout the cell, whereas nuclear receptors are predominantly detected in nuclei. IF, (immuno)fluorescence. SII.2 THP-1 cells were infected with shNur77 or shNOR-1 lentivirus containing CMV-GFP with a transduction efficiency of >90% (data not shown), which resulted in a >70% reduction of Nur77 or NOR-1 mRNA expression levels as compared to a control shRNA directed against luciferase (n=2, ±SD, Student’s t-test, p<0.05).
(Zeng et al., 2006), and to inhibit SMC-rich lesion formation in transgenic mice (Arkenbout et al., 2002). In contrast, inhibition of NOR-1 expression in cultured cells by anti-sense oligonucleotides resulted in reduced SMC and endothelial cell growth, suggesting a stimulatory effect of this NR4A member on proliferation of these vascular cells (Martinez-Gonzalez and Badimon, 2005). In the current study, we show that all three NR4A family members mediate similar downstream effects in macrophages, except for enhanced MCP-1 expression after TNF-α stimulation when NOR-1 is overexpressed. A detailed analysis of downstream gene targets for each of these three transcription factors will give further insight in gene-specific and cell-specific responses.

In summary, we demonstrate that the NR4A family of transcription factors is expressed in human atherosclerotic lesion macrophages and is functionally involved in inhibition of inflammatory responses and lipid loading. So far, our results point

Supplemental Figure SIII. Expression of Nur77, Nurr1 and NOR-1 in primary macrophages and THP-1-derived macrophages in response to LPS and TNFa. mRNA expression levels were determined by real-time RT-PCR. Primary macrophages of 2 different donors were treated with LPS (100ng/ml), TNFa (10ng/ml) or vehicle for 2 hours and substantially increased mRNA expression levels of Nur77, Nurr1 and NOR-1 were observed (A). Also in THP-1-derived macrophages mRNA expression levels of Nur77, Nurr1 and NOR-1 were significantly increased in response to LPS (250ng/ml, 2 hours) (B) and TNFa (10ng/ml, 1 hour for Nur77 and Nurr1, 3 hours for NOR-1) (C). Optimal expression is shown in the upper panels (n=3, ±SD, Student’s t-test, p<0.05) and time courses are given in the lower panels (representative experiment; n=2). Protein expression of NOR-1 was analyzed after 6 hours LPS in THP-1-derived macrophages by immunofluorescence and NOR-1 protein localized to the nucleus (D). All data shown are significant (p≤0.05) as compared to Mock.
towards atheroprotective properties of NR4A-factors in macrophages and for Nur77 in other vascular cell types as well. Future studies in vitro and in vivo models will unravel the significance of these transcription factors in atherogenesis and related vascular pathologies.
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