Deficiency of the NR4A Orphan Nuclear Receptor NOR1 Decreases Monocyte Adhesion and Atherosclerosis

Yue Zhao, Deborah A. Howatt, Florence Gizard, Takashi Nomiyama, Hannes M. Findeisen, Elizabeth B. Heywood, Karrie L. Jones, Orla M. Conneely, Alan Daugherty, Dennis Bruemmer

Rationale: The orphan nuclear receptor NOR1 is a member of the evolutionary highly conserved and ligand-independent NR4A subfamily of the nuclear hormone receptor superfamily. Members of this subfamily have been characterized as early response genes regulating essential biological processes including inflammation and proliferation; however, the role of NOR1 in atherosclerosis remains unknown.

Objective: The goal of the present study was to determine the causal contribution of NOR1 to atherosclerosis development and to identify the mechanism by which this nuclear receptor participates in the disease process.

Methods and Results: In the present study, we demonstrate expression of NOR1 in endothelial cells of human atherosclerotic lesions. In response to inflammatory stimuli, NOR1 expression is rapidly induced in endothelial cells through a nuclear factor κB–dependent transactivation of the NOR1 promoter. Overexpression of NOR1 in human endothelial cells increased the expression of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule-1, whereas NOR1 deficiency altered adhesion molecule expression in response to inflammatory stimuli. Transient transfection experiments and chromatin immunoprecipitation assays revealed that NOR1 induces VCAM-1 promoter activity by binding to a canonical response element for NR4A receptors in the VCAM-1 promoter. Further functional studies confirmed that NOR1 mediates monocyte adhesion by inducing VCAM-1 and intercellular adhesion molecule-1 expression in endothelial cells. Finally, we demonstrate that NOR1 deficiency reduces hypercholesterolemia-induced atherosclerosis formation in apoE−/− mice by decreasing the macrophage content of the lesion.

Conclusions: In concert, these studies identify a novel pathway underlying monocyte adhesion and establish that NOR1 serves a previously unrecognized atherogenic role in mice by positively regulating monocyte recruitment to the vascular wall. (Circ Res. 2010;107:501-511.)

Key Words: nuclear receptor ■ endothelial cell ■ adhesion ■ atherosclerosis

The transcription factor NOR1 (NR4A3) belongs to the highly conserved NR4A subfamily of orphan nuclear hormone receptors.1-2 Members of this subgroup are classified as early response genes, which are induced by a pleiotropy of stimuli, including mitogens and inflammatory signals.3-4 In contrast to other members of the nuclear receptor superfamily, NR4A receptors function as constitutively active and ligand-independent transcription factors.5-6 Therefore, the transcriptional activity of NR4A receptors is determined by the expression level and by posttranslational modifications of the receptor.7 NR4A receptors positively regulate target gene expression by binding as monomer or homodimer to different variations of the canonical 5′-A/TAAAGGTCGA NGFI-B response element (NBRE).8 In addition, Nur77 (NR4A1) and Nur1 (NR4A2) exhibit transcriptionally distinct mechanisms, because both are able to transactivate target genes as a heterodimer with RXR.9 Consistent with the pleiotropic stimuli that induce the expression of NR4A receptors, these transcription factors have been implicated in regulating key cellular functions, including inflammation, proliferation, and cell survival.1-2

NOR1 was first cloned from neuronal cells, and its deletion in mice results in hippocampal dysgenesis and...
inner ear defects. In addition to neurons, NOR1 is highly expressed in atherosclerotic lesions. NOR1 is induced by mitogens in smooth muscle cells and required for proliferative remodeling following vascular injury. In macrophages, NOR1 is induced during inflammation and represses cytokine secretion. Finally, NOR1 expression is increased by vascular endothelial growth factor in endothelial cells. However, the functional role of NOR1 in endothelial cells and its contribution to atherosclerosis remain to be investigated.

In the present study, we provide first evidence that NOR1 plays an essential role in the regulation of monocyte adhesion to the endothelium by positively regulating vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 expression. In vivo, loss of NOR1 function in apolipoprotein (apo)E-deficient mice reduces atherosclerosis formation and macrophage recruitment to the arterial wall. These studies identify NOR1 as a previously unrecognized key component of a transcriptional cascade regulating monocyte adhesion during atherogenesis.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mice

Littermate NOR1+/− and NOR1+/+ mice on a mixed C57BL/6J/129Sv background were used as previously described. ApoE−/− mice on a C57BL/6J background (N10) were obtained from The Jackson Laboratory (stock no. 002052) and interbred with NOR1+/− mice to obtain NOR1+/− apoE−/− mice. At 8 to 10 weeks of age, littermate female NOR1+/− apoE−/−, NOR1−/− apoE−/−, and NOR1−/− apoE−/− mice were fed a saturated fat enriched diet (TD8137; Harlan Teklad) for 12 weeks for atherosclerosis analysis or for 2 weeks to analyze N4R4A gene expression.

Isolation of Murine Endothelial Cells

Mouse aortic endothelial cells (MAECs) were isolated from aortae of littermate NOR1+/− and NOR−/− mice using an explant technique. Aortic segments were placed on Cultrex Basement Membrane Extract gel (R&D Systems) and incubated in low-glucose DMEM supplemented with 15% FBS, 180 μg/mL heparin, and 20 μg/mL endothelial cell growth supplement. Migrated endothelial cells were passaged using dispase (BD Sciences) and cultured for 2 days in media containing d-valine to limit fibroblast contamination. Once confluent, MAECs were incubated with tumor necrosis factor (TNFα) (R&D Systems) as indicated.

Adhesion Assay

Human umbilical cord vein cells (HUVECs) were infected with 50 plaque-forming unit (pfu) Ad-CMV-null or Ad-CMV-NOR1 for 6 hours and recovered for 48 hours. HUVECs treated with TNFα were used as a positive control. THP-1 monocytes were pretreated with 5 μM/L calcein-AM (Sigma-Aldrich) at 37°C for 30 minutes. After washing in PBS, fluorescently labeled THP-1 monocytes were added onto the HUVEC monolayers at the density of 10⁶ cells/mL. To block VCAM-1 and ICAM-1 function, HUVEC monolayers were incubated with blocking antibodies against VCAM-1 (25 μg/mL, BBA5, R&D Systems) and ICAM-1 (10 μg/mL, BBA3, R&D Systems) for 1 hour before the addition of THP-1 monocytes. Nonadherent monocytes were washed off after 30 minutes. For mouse monocyte adhesion assays, the thoracic aortae of littermate NOR1+/− or NOR1−/− mice were cut into segments, pinned on dental wax, and maintained in MAEC growth media supplemented with TNFα (1 ng/mL) or vehicle (PBS). After 6 hours, aortae were washed with PBS, and 700 μL of fluorescently labeled WEHI-274.1 cells were added at the density of 10⁶ cells/mL. Nonadherent monocytes were removed after 30 minutes by washing with PBS. For both experiments, 6 pictures were taken for each condition, and adhesion was quantified by counting fluorescent monocytes attached to the endothelium.

Results

NOR1 Is Expressed in Endothelial Cells of Human Coronary Atherosclerotic Lesions

To characterize the distribution of NOR1 expression in atherosclerosis, human coronary arteries were immunostained for NOR1. NOR1 protein was readily detectable in atherosclerotic lesions (Figure 1A) but negligible in normal arteries (data not shown). In these atherosclerotic sections, high levels of NOR1 immunostaining were observed in the endothelial cell layer and in subendothelial cells of advanced atherosclerotic lesions. Colocalization experiments using confocal microscopy confirmed a typical nuclear expression pattern of NOR1 in endothelial cells staining positive for the endothelial cell marker von Willebrand factor (Figure 1B).

NOR1 Is Induced by Inflammatory Stimuli in Endothelial Cells

In vitro, stimulation of HUVECs with the inflammatory mediators interleukin-1β, oxidized low-density lipoprotein, lipopolysaccharide, or TNFα highly induced NOR1 mRNA expression (Figure 2A). In contrast, interleukin-6 or interferon-γ did not significantly induce NOR1 transcript levels in HUVECs. Considering that TNFα elicited a maximal increase in NOR1 expression, subsequent experiments focused on the regulation of NOR1 by TNFα. Consistent with previous reports characterizing NOR1 as an early response gene, time course experiments confirmed a maximal induction of NOR1 mRNA after 3 hours of TNFα stimulation (Figure 2B). This increase in NOR1 transcript levels was followed by a maximal induction of NOR1 protein expression.
after 6 hours (Figure 2C and 2D). The observed regulation of NOR1 mRNA and protein expression by TNFα was dose-dependent, revealing a maximal increase of NOR1 expression with 1 ng/mL TNFα (Figure 2E through 2G, respectively). Finally, TNFα induced a similar induction of NOR1 mRNA expression in primary MAECS isolated from NOR1 wild-type mice (Online Figure I). In contrast, the other 2 members of the NR4A subfamily, Nur77 and Nurr1, were only modestly induced in NOR1 wild-type cells, and this induction was lost in NOR1+/− MAECS.

NOR1 Positively Regulates VCAM-1 and ICAM-1 in Endothelial Cells

To explore whether NOR1 is involved in the transcriptional control of endothelial cell responses, we next infected HUVECs with an adenovirus overexpressing human NOR1 (Figure 4A). As depicted in Figure 4B through 4E, NOR1 overexpression resulted in a prominent induction of VCAM-1 and ICAM-1 mRNA and protein expression levels, respectively.

We next used a murine model to address whether NOR1 is also required for VCAM-1 and ICAM-1 expression in endothelial cells. In these experiments, MAECS were isolated from littermate NOR1 wild-type and NOR1-deficient
mice. As depicted in Figure 5A through 5E, TNFα stimulation profoundly increased VCAM-1 and ICAM-1 mRNA and protein expression in NOR1 wild-type MAECS. In contrast, the induction of both adhesion molecules was markedly reduced in MAECS isolated from NOR1-deficient mice. Similar data were obtained when cells were stimulated with interleukin-1β or lipopolysaccharide (Online Figure II, A through C).

Figure 2. NOR1 is induced by inflammatory stimuli in endothelial cells. A, HUVECs were stimulated as indicated for 2 hours and NOR1 mRNA expression was analyzed. B and C, HUVECs were stimulated with TNFα (1 ng/mL), and NOR1 mRNA (B) and protein expression (C) were analyzed at the indicated time points. D, Densitometric analysis of NOR1 protein expression in C. E through G, HUVECs were stimulated with vehicle (PBS) or the indicated dose of TNFα. NOR1 mRNA (E) and protein (F) expression were analyzed after 2 hours or 6 hours, respectively. G, Densitometric analysis of NOR1 protein expression in F. NOR1 mRNA expression levels were normalized to hTBP (human TATA-binding protein) and presented as means ± SEM fold increase over vehicle-treated cells. *P<0.05 vs vehicle. Densitometric analysis was performed on 3 independent experiments. Cohybridization for GAPDH was performed to assess equal loading.

NOR1 Transactivates the VCAM-1 Promoter by Binding to a Canonical NBRE Consensus Site

We next performed transient transfection assays to investigate the underlying mechanism by which NOR1 regulates VCAM-1 expression in endothelial cells. NOR1 induces transcription by binding to NBRE consensus sites in target gene promoters. Interestingly, sequence analysis of the human VCAM-1 promoter identified a canonical NBRE
site at −2618 bp from the transcription initiation site (Figure 6A). Transient transfection of HUVECs with a luciferase reporter construct driven by the human 3.0-kb VCAM-1 promoter revealed that overexpression of NOR1 increases VCAM-1 promoter activity (Figure 6B). However, this transcriptional induction was significantly altered on site-directed mutagenesis of the canonical NBRE motif. ChIP assays subsequently confirmed that TNFα induced the recruitment of NOR1 to this NBRE site in the VCAM-1 promoter (Figure 6C). These experiments demonstrate that NOR1 transactivates the VCAM-1 promoter by binding to a NBRE consensus site in the promoter and characterize VCAM-1 as NOR1-regulated target gene.

NOR1 Mediates Monocyte Adhesion by Regulating VCAM-1 and ICAM-1 Expression

VCAM-1 and ICAM-1 have both been well characterized to mediate monocyte adhesion to the endothelium, leading to the infiltration of monocytes into the subendothelial area and atherosclerosis development.17 To investigate whether the transcriptional induction of VCAM-1 and ICAM-1 by NOR1 is sufficient to promote monocyte adhesion, we analyzed THP-1 monocyte adhesion to HUVECs overexpressing NOR1. Stimulation of a HUVEC monolayer with TNFα profoundly increased monocyte adhesion, confirming the validity of the assay (Figure 7A, top). Compared to HUVECs infected with an adenovirus overexpressing an empty vector as control, overexpression of NOR1 significantly increased monocyte adhesion in the absence of TNFα stimulation (Figure 7A and 7B). Furthermore, monocyte adhesion induced by NOR1 overexpression was almost completely abolished by preincubation of HUVECs with VCAM-1 and ICAM-1 blocking antibodies (Figure 7A and 7B). Conversely, acute knockdown of NOR1 in HUVECs using small interfering RNA significantly decreased monocyte adhesion (Online Figure III, A through C). Collectively, these studies indicate that NOR1 is necessary and sufficient for monocyte adhesion and that this activity is mediated primarily by inducing the expression of VCAM-1 and ICAM-1.
From these findings we infer that NOR1 expression in resident endothelial cells is necessary for mediating monocyte adhesion.

To further confirm a causal contribution of NOR1-dependent VCAM-1 and ICAM-1 expression for monocyte adhesion ex vivo, we performed adhesion assays with aortae isolated from NOR1+/+ and NOR1−/− mice. Incubation with TNFα significantly increased the adhesion of monocytes to the aortic endothelial cell layer of NOR1 wild-type mice (Figure 7C and 7D). However, this inducible adhesion was completely abolished on aortae of NOR1-deficient mice. These findings further demonstrate that NOR1 expression in resident endothelial cells is necessary for mediating monocyte adhesion.

**NOR1 Deficiency Decreases Atherosclerosis Formation and Reduces Macrophage Recruitment in ApoE−/− Mice**

Consistent with the important function of NOR1 to regulate monocyte adhesion ex vivo, homozygous deletion of NOR1 in apoE−/− mice resulted in a 52% reduction of atherosclerosis compared to their wild-type littermates (NOR1+/+apoE−/−, 14.3% [n=14]; NOR1+/+apoE−/−, 14.0% [n=15]; and NOR1−/−apoE−/−, 6.8% [n=15]) median atherosclerotic lesion area of aortic arches; P<0.05; Figure 8A and 8B). NOR1 deficiency revealed no overt effect on cholesterol distribution confirming a direct effect of NOR1 on lesion formation (Figure 8C). Immunostaining of atherosclerotic tissues and quantification of the macrophage content in atheroensclerotic tissues and quantification of the macrophage content confirmed macrophage-enriched lesions in NOR1+/+ apoE−/− mice that were considerably less with decreased macrophage content in NOR1−/−apoE−/− mice (Figure 8D and 8E; Online Figure IV). Finally, whereas VCAM-1 was readily detectable in atherosclerotic lesions from NOR1+/+ apoE−/− mice, there was a paucity of immunoreactivity for this adhesion molecule noted in the vascular wall of NOR1−/− apoE−/− mice (Figure 8F).

**Discussion**

Monocyte adhesion constitutes a critical event for the initiation of atherosclerosis\textsuperscript{17}; however, the molecular mechanisms that orchestrate monocyte–endothelial cell interactions are incompletely understood. In the present study, we report a previously unrecognized role for the nuclear receptor NOR1 to serve as a transcriptional regulator of adhesion molecule expression and monocyte recruitment during atherosclerosis. In endothelial cells, NOR1 is rapidly induced by inflammatory stimuli via an NF-κB-dependent transactivation of the NOR1 promoter. Loss- and gain-of-function studies establish that NOR1 positively regulates VCAM-1 and ICAM-1 expression in endothelial cells, leading to increased monocyte adhesion. Consistent with the key role of NOR1 to promote monocyte adhesion to the endothelium, our studies further demonstrate that NOR1 deficiency results in decreased atherosclerosis development and macrophage recruitment in apoE-deficient mice.

Consistent with recent studies, we identified abundant NOR1 expression in endothelial cells,\textsuperscript{13} as well as in cells of the subendothelial space likely representing macrophages and smooth muscle cells.\textsuperscript{4,13,14,18} However, the transcriptional mechanisms governing inducible NOR1 expression in endothelial cells remain elusive. Our data provide evidence for an NF-κB-dependent induction of NOR1 transcription during endothelial cell inflammation. Inhibition of NF-κB signaling in endothelial cells repressed inducible NOR1 expression in response to inflammatory stimulation. Using site-directed mutagenesis and ChIP assays, we identified 2 functional
NF-κB sites in the NOR1 promoter, to which p65 is recruited during inflammatory activation of endothelial cells. Earlier studies have demonstrated that NOR1 expression in endothelial cells is highly induced by mitogens through a CREB (cAMP response element binding protein)–dependent activation of the proximal region of NOR1 promoter. Furthermore, hypoxia has recently been reported to induce NOR1 in endothelial cells through a mechanism involving the hypox-

**Figure 5.** NOR1 is required for VCAM-1 and ICAM-1 expression in endothelial cells. NOR1+/− and NOR1−/− MAECs were stimulated with vehicle or TNFα (1 ng/mL) for 6 hours. VCAM-1 and ICAM-1 mRNA (A and B) and protein (C through E) expression were analyzed by real-time RT-PCR and Western blotting, respectively. C, The autoradiograms are representative of 3 independent experiments. D and E, Densitometric analysis of VCAM-1 and ICAM-1 protein expression after Western blotting. All experiments were repeated at least 3 times in duplicates with different cell preparations. Results are presented as means±SEM fold increase over vehicle-treated wild-type cells. *P<0.05 vs vehicle; #P<0.05 vs NOR1+/− cells.

**Figure 6.** NOR1 transactivates the VCAM-1 promoter by binding to an NBRE consensus site. A, Schematic structure of the human 3.0-kb VCAM-1 promoter and the NBRE consensus site at −2618 bp. B, HUVECs were infected with 50 pfu Ad-CMV-null or Ad-CMV-NOR1 for 6 hours and recovered for 24 hours. Infected cells were transiently transfected with luciferase reporter constructs driven by the VCAM-1 wild-type promoter or the similar promoter bearing a mutation in the NBRE site. Luciferase activity was analyzed after 48 hours, and data are presented as means±SEM fold induction from 3 independently performed experiments. *P<0.05 vs empty vector; #P<0.05 vs pVCAM-1-WT. C, HUVECs were stimulated with vehicle (PBS) or TNFα (1 ng/mL) for ChIP assays. PCR for an unrelated promoter fragment in the α-actin promoter served as control for specificity. The autoradiograms are representative of 3 independently performed experiments.
ia-inducible factor family of transcription factors. These studies, in concert with our data characterizing NOR1 as a NF-κB target gene in endothelial cells, point to distinct transcriptional mechanisms regulating the rapid NOR1 induction in response to various environmental cues.

Compared with the well-studied early-response genes encoding proteins of the activator protein-1 complex, little is known about the physiological function of NOR1 and its regulated target genes, yet the high degree of conservation points to an important role in the control of gene expression. A previous study has provided initial evidence to support a functional role of NOR1 in endothelial cells by demonstrating that NOR1 regulates growth of this cell type. The data presented here extend these findings and points to an unsuspected function of NOR1 to serve as a positive regulator of monocyte adhesion. In experiments using adenoviral-mediated overexpression, NOR1 induced VCAM-1 and ICAM-1 expression, resulting in increased monocyte adhesion. The observation that this inducible adhesion was abolished when VCAM-1 and ICAM-1 function were blocked suggests that the induction of both adhesion molecules by NOR1 constitutes a primary mechanism by which NOR1 induces monocyte adhesion. Conversely, the inducible expression of both adhesion molecules in response to inflammatory activation was attenuated in NOR1-deficient endothelial cells. Furthermore, consistent with these findings, TNFα-induced monocyte adhesion to HUVECs transfected with NOR1 small interfering RNA or to the endothelium of NOR-deficient mice was altered ex vivo, suggesting that vascular NOR1 expression is not only sufficient but also required for monocyte adhesion.

An intriguing question that arises from the observation that NOR1 induces the expression of VCAM-1 and ICAM-1 relates to the mechanisms by which NOR1 positively regulates these genes. Initial sequence analysis identified putative NBRE consensus sites in both the VCAM-1 and ICAM-1 promoters. Exemplified by the VCAM-1 promoter, our studies demonstrate that the molecular mechanisms underlying this novel function of NOR1 involve, at least in part, a direct transactivation of the VCAM-1 promoter by NOR1. In response to inflammatory activation NOR1 is recruited to a canonical NBRE site in the VCAM-1 promoter. Moreover, the observation that NOR1-dependent VCAM-1 promoter transactivation was attenuated on mutation of this NBRE site

Figure 7. NOR1 mediates monocyte adhesion by regulating VCAM-1 and ICAM-1 expression. A, Top, HUVECs were infected with 50 pfu Ad-CMV-NOR1 or Ad-CMV-null, and fluorescently labeled THP-1 monocytes were added onto HUVEC monolayers. TNFα-treated (1 ng/mL) HUVECs were used as a positive control. A, Bottom, Ad-CMV-NOR1–infected cells were preincubated with VCAM-1 and ICAM-1 blocking antibodies (B/N) or control IgG. After 1 hour, fluorescently labeled THP-1 monocytes were added onto HUVEC monolayers. B, Quantification is presented as means±SEM from 3 independently performed experiments in duplicates. *P<0.05 vs vehicle or Ad-CMV-null; #P<0.05 vs TNFα; §P<0.05 vs IgG. C and D, Aortae were isolated from NOR1+/+ and NOR1−/− mice and TNFα-induced monocyte adhesion was analyzed. C, Representative sections demonstrating adhesion of monocytes. D, Quantification of adhesion from 3 independent experiments performed in duplicates using different aortic preparations. Data are expressed as means±SEM. *P<0.05 vs vehicle; #P<0.05 vs NOR1+/+ cells.
Figure 8. NOR1 deficiency decreases atherosclerosis in apoE<sup>−/−</sup> mice. A, Atherosclerotic lesion size was measured on aortic arches from female NOR1<sup>+/+</sup>apoE<sup>−/−</sup> (n=14), NOR1<sup>+/−</sup>apoE<sup>−/−</sup> (n=15), and NOR1<sup>−/−</sup>apoE<sup>−/−</sup> (n=15) mice. Circles and triangles represent individual mice; diamonds represent medians (P<0.05 between group, *P< 0.05 vs NOR1<sup>+/+</sup>apoE<sup>−/−</sup>). B, Representative aortic arches from each genotype. C, Lipoprotein cholesterol distributions. Values represent the mean cholesterol content of each fraction (±SEM). D through F, Sections of atherosclerotic lesions were immunostained using antisera against macrophages or VCAM-1. D, Representative sections for macrophage staining (top) or IgG (bottom) (objective magnification, ×20). E, Macrophage accumulation was quantified in the aortic arch from NOR1<sup>+/−</sup>apoE<sup>−/−</sup> and NOR1<sup>−/−</sup>apoE<sup>−/−</sup> mice. *P<0.05 vs NOR1<sup>+/−</sup>apoE<sup>−/−</sup>. F, Representative sections for VCAM-1 staining (objective magnifications, ×20 and ×100).
confirms the functionality of this NBRE motif. However, the residual induction of the VCAM-1 promoter bearing a mutation of this NBRE site suggests that additional transcriptional mechanisms may regulate NOR1 expression. NOR1 has recently been shown to transactivate the inducible IκB kinase (IKKι/IIKKε) promoter, which phosphorylates IκBα and induces NF-κB activation. Therefore, in addition to a direct transactivation, NOR1 may function as a positive upstream regulator of NF-κB signaling and indirectly activate the VCAM-1 and ICAM-1 promoters. Alternatively, NOR1 deficiency may affect other transcriptional networks acting on these promoters, including for example the activator protein-1 complex. We have recently demonstrated that the combined deficiency of NOR1 and its sibling Nur77 decreases the expression of activator protein-1 transcription factors, which may regulate adhesion molecule expression. Clearly, the findings presented here provide justification for further investigating the transcriptional mechanisms by which NOR1 regulates endothelial cell gene expression and promotes monocyte adhesion.

The protein products of the VCAM-1 and ICAM-1 genes are well established to participate in atherogenesis by promoting macrophage accumulation in the arterial intima. Consistent with this evidence and with the observed regulation of endothelial cell adhesion molecule expression by NOR1, we provide the first evidence that NOR1 deficiency decreases atherosclerosis in apoE-deficient mice. Considering that all 3 members of the NR4A receptor subfamily bind to an NBRE site, functional redundancy in certain cell types between Nur77 and NOR1 has been suggested. However, NOR1 deficiency did not result in a compensatory upregulation of the siblings Nur77 and NOR1 in endothelial cells (Online Figure I) or in the aortae of NOR1-deficient mice (Online Figure V). Therefore, the previously reported phenotypes in NOR1-deficient mice, in concert with the decreased atherosclerosis in NOR1−/− apoE−/− mice presented in this study, point to a function of NOR1 that is distinct and not compensated by Nur77 or Nur1. As evidenced by the decreased accumulation of macrophages in the vascular wall of NOR1−/− apoE−/− mice, monocyte recruitment represents at least one plausible mechanism by which NOR1 acts atherogenic. However, it is possible, if not likely, that there are additional mechanisms by which NOR1 promotes atherosclerosis development. NOR1 induces neointimal proliferation of SMC and inflammatory gene expression in macrophages, which both could affect lesion development. Therefore, characterization of the cell-specific role of NOR1 in atherosclerosis will be necessary and will have to rely on tissue-specific gene targeting strategies. In conclusion, data presented here characterize the orphan nuclear receptor NOR1 as a novel positive regulator of monocyte adhesion by inducing VCAM-1 and ICAM-1 transcription. Continued investigation of the transcriptional networks regulated by NOR1 will provide new insights into how this orphan nuclear receptor participates in the development of vascular diseases.

Sources of Funding
This work was supported by the NIH (R01HL084611 and R01HL084611-04S1 to D.B. and R01 CA111411 to O.M.C.). F.G. and Y.Z. were supported by Fellowship Grants from the American Heart Association (0725313B and 0815514D, respectively).

Disclosures
None.

References
Novelty and Significance

What Is Known?
- NR4A orphan nuclear receptors constitute a group of conserved transcription factors that function as early response genes.
- Members of the NR4A orphan nuclear receptor subfamily, including Nur77, NOR1, and Nurr1, are highly expressed in atherosclerotic lesions.
- NOR1 expression is inducible in endothelial cells.

What New Information Does This Article Contribute?
- NOR1 expression in endothelial cells is mediated via an nuclear factor κB–dependent activation of its promoter.
- NOR1 induces monocyte adhesion through a transcriptional induction of the adhesion molecules VCAM-1 and ICAM-1 in endothelial cells.
- NOR1 transactivates the VCAM-1 promoter in endothelial cells by binding to a canonical response element for NR4A receptors.
- Deficiency of NOR1 in apoE<sup>−/−</sup> mice reduces atherosclerosis formation and macrophage content of the lesion.

Monocyte adhesion and recruitment into the subendothelial space constitute critical first events for the initiation of atherosclerosis. In this study, we introduce a novel transcriptional mechanism mediating monocyte adhesion to the endothelium and subsequent atherosclerosis development. Although NR4A orphan nuclear receptors are highly conserved transcription factors expressed in atherosclerosis, surprisingly little is known about their function, and whether these receptors play a causal role in atherosclerosis has not been investigated. Expression of the NR4A receptor NOR1 was induced in endothelial cells by proatherogenic stimuli and mediated through an nuclear factor κB–dependent transcriptional mechanism. Using loss- and gain-of-function approaches, we establish that NOR1 induces expression of the key adhesion molecules VCAM-1 and ICAM-1, resulting in increased monocyte adhesion. VCAM-1 constitutes a bona fide target gene for NOR1, which is activated by binding to a canonical response element. Consistent with this novel activity of NOR1, we demonstrate for the first time that deletion of this NR4A receptor reduces atherosclerotic lesion formation and monocyte recruitment into the arterial wall. Collectively, these experiments characterize a novel transcriptional cascade underlying atherogenesis and have important implications for our understanding of this disease.
Deficiency of the NR4A Orphan Nuclear Receptor NOR1 Decreases Monocyte Adhesion and Atherosclerosis

Yue Zhao, Deborah A. Howatt, Florence Gizard, Takashi Nomiyama, Hannes M. Findeisen, Elizabeth B. Heywood, Karrie L. Jones, Orla M. Conneely, Alan Daugherty and Dennis Bruemmer

Circ Res. 2010;107:501-511; originally published online June 17, 2010; doi: 10.1161/CIRCRESAHA.110.222083

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/107/4/501

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/06/17/CIRCRESAHA.110.222083.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
**Online Data Supplement**

**Materials and Methods**

**Immunostaining**

Immunostaining of human atherosclerotic lesions was performed as described previously using primary antibodies against NOR1 (1:100 dilution; IMG-71915; Imgenex, Inc.) or von Willebrand Factor (vWF) (1:100 dilution; 115-01, SIGNET). For immunofluorescent co-localization studies, sections were incubated with primary antibodies against NOR1 (1:50 dilution; ab56340; Abcam) and vWF (1:100 dilution; 115-01, SIGNET). Sections were subsequently incubated with Alexa 488-conjugated goat anti-mouse antibody (1:1000 dilution, A11001, Invitrogen) and Alexa 594-conjugated goat anti-rabbit antibody (1:1000, A11012, Invitrogen), respectively. All studies on human tissues were performed with the approval of the University of Kentucky Institutional Review Board.

For immunohistochemical analysis of mouse aortic atherosclerotic lesions, the ascending aorta were embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen. Transverse cryosections (10 µm) were collected from the aortic arch, fixed in cold acetone, and immunostained using macrophage anti-sera (1:1000 dilution, AI-AD 31240, Accurate Chemicals) or a VCAM-1 antibody (BD 550547). All experiments on mice were approved by the University of Kentucky Institutional Animal Care and Use Committee.

**Cell culture**

Human umbilical vein endothelial cells (HUVEC, Lonza) and human aortic endothelial cells (HAEC, Cascade Biologics) were cultured as directed by the manufacturer. Human THP-1 monocytes (ATCC) were maintained in RPMI-1640 medium supplemented with 10% FBS. Murine WEHI-274.1 premyelocytic cells (ATCC) were cultured in DMEM supplemented with 10% FBS. Confluent endothelial cells were stimulated with different cytokines or proinflammatory factors as indicated in the Figure Legends. The reagents used in this study included human TNFα (R & D), human IL-1β (R & D), human IL-6 (R & D), human IFNγ (R & D), human oxidized low-density-lipoprotein (ox-LDL) (INTRACEL), mouse TNFα (R & D), mouse IL-1β (R & D) and LPS (Sigma). All experiments were performed with cells between passages 2 to 8, and each experiment was repeated at least three times with different preparations of cells.

**Western blotting**

Western blotting was performed as described using antibodies against NOR1 (PP-H7833, R & D Systems), human VCAM-1 (BD Pharmingen), human ICAM-1 (Abcam), mouse VCAM-1 (R & D Systems), mouse ICAM-1 (Abcam), β-actin (Sigma), and GAPDH (FL 335) (Santa Cruz). 1,2

**Quantitative real-time RT-PCR**

Total RNA was isolated using TRIzol® (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). RNA expression levels of target genes were quantified using an iQ™SYBR Green Supermix (BioRad) and 5 pmol of the indicated primer pairs (Supplemental Table SI). PCR reactions were performed on an iCycler (BioRad) using the following PCR cycles: 1 cycle of 95 °C 10 min; 40 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; 1 cycle of a final extension at 72°C for 10 min. Each sample was analyzed in triplicate and normalized to expression values of the house-keeping gene TBP or TFIIB. Data were calculated using the 2^ΔΔCT method. 3

**Adenovirus-mediated overexpression in HUVEC**

The adenovirus over-expressing the dominant-negative IkBα mutant (IkBα-S32A/S36A) (Ad-CMV-IkB(DN)) was purchased from Vector Biolabs. The adenovirus over-expressing human NOR1 (Ad-CMV-NOR1) was generously provided by Dr. Peter Tontonoz (University of California, Los Angeles, CA).
Subconfluent HUVEC were infected with 25 PFU Ad-CMV-IκB(DN) for 3 hours and 50 PFU Ad-CMV-NOR1 for 6 hours, respectively. Adenoviruses over-expressing GFP (Ad-CMV-GFP) or an empty vector (Ad-CMV-null) were used as controls.

**Plasmids, Transient Transfections and Luciferase Assay**

The human NOR1 promoter constructs have previously been described. The NF-κB response elements located at -198bp to -190bp and -595bp to -496bp from the transcription initiation site were mutated from GGAGTTTCC to AGAGTTTAA and from GGGATTAGCC to ATGATTAGAA using the QuickChange II XL site-directed mutagenesis kit (Stratagene). HAEC were transiently transfected with NOR1 promoter constructs using promofectin (PromoKine). Following transfection, cells were recovered overnight and stimulated with TNFα. The human VCAM-1 promoter construct was commercially obtained from Epoch Biolabs Company. The NBRE site located at -2618 bp to -2611 bp from the transcription initiation site was mutated from TGACCTTT to TCGGAGTT. For overexpression of NOR1, HUVEC were infected with 50 PFU Ad-CMV-Null or Ad-CMV-NOR1 for 6 hours and recovered for 24 hours. Infected cells were subsequently transfected with luciferase reporter constructs driven by the VCAM-1 promoter. Following transfection, cells were recovered in growth media for 2 days. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay (Promega). Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection of 5 ng pRLCMV.

**siRNA Experiments**

siRNA experiments were performed using the SMARTpool technology (L003428, Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific against human NOR1. HUVEC were seeded at a density of 1.2 × 10^5 cells/well in 6-well plates and transiently transfected for 3 h with 30 nM NOR1 siRNA or scrambled siRNA using promofectin (PromoKine). Following transfection, cells were recovered in complete growth media overnight and subsequently stimulated with TNFα for 6 h. Fluorescently labeled THP-1 monocytes were added onto the HUVEC monolayers and quantification of adhesion was performed after 30 min as indicated in the section "Adhesion assay".

**Chromatin Immunoprecipitation (ChIP) Assays**

ChIP assays were performed using the EZ-ChIP kit (Millipore) as described. Briefly, HUVEC were stimulated with TNFα and sheared chromatin was immunoprecipitated using 5 µg antibodies directed against NF-κB p65 (sc-372x, Santa Cruz) or NOR1 (PP-H7833, R & D Systems). Target DNA product was amplified by PCR using primer pairs covering the NF-κB binding sites in the NOR1 promoter or the NBRE site in the VCAM-1 promoter (Supplemental Table SI).

**Atherosclerosis quantification**

Atherosclerosis was quantified as described and reviewed recently in detail. Briefly, after exsanguination aortic tissue was removed from the ascending aorta to the ileal bifurcation and fixed by perfusion with freshly prepared 4 % paraformaldehyde in PBS overnight at room temperature. After tissue fixation, the aorta was dissected from the adventitia. The intimal surface was exposed by a longitudinal cut through the inner curvature of the aortic arch that extended down the whole length of the aortic tree. To permit the arch region to be laid out flat, the greater curvature was cut down to the level of the subclavian artery. The tissue was laid out, and an image of the aorta was recorded. To quantify the extent of intimal surface covered by grossly discernible lesions, image analysis was performed with Image-Pro (Media Cybernetics). The extent of atherosclerotic lesions was quantified in the arch as defined from the ascending arch to 4 mm distal to the left subclavian artery. The data were presented as the percentage of lesion area on the aortic arch.
Quantification of macrophage accumulation
Macrophage content was quantified in a 2-mm segment as defined from the beginning of the ascending aortic arch using a modified technique described by Mach et al. 6 From this segment 180 serial transverse cryosections (10 µm) were collected and placed onto twenty slides per mouse. Slides were immunostained for macrophages as described above, and the macrophage content was quantified in nine sections 200 µm apart. Macrophage accumulation was determined by quantifying the total area positive for macrophage staining using computer-assisted image analysis (Image-Pro, Media Cybernetics). Each section was quantified by two observers blinded to the experimental design. The data were presented as mean area ± SEM positive for macrophage staining for each of the nine measurements and their cumulative sum.

Lipoprotein resolution and quantification
Lipoproteins were resolved using size exclusions chromatography as described. 4, 5 Briefly, serum samples were centrifuged and placed onto a single Sepharose 6 HR 10/30 column (300 x 10 mm, Pharmacia) with a mobile phase of saline/EDTA run at 0.5 ml/min. Fractions (0.5 ml) were collected and cholesterol concentrations were determined by placing 100 µl of each fraction into an equivalent volume of cholesterol reagent (Wako Chemical Company) that was diluted to half the manufacturer’s instructions. Assays were performed in a 96 well format and absorbance was determined at 600 nm.

Statistical Analysis
Results were represented as means or medians depending on the distribution of data. Unpaired Student’s t-test was utilized to compare the means between two independent groups on a single variable. One-way or Two-way ANOVA was used to compare groups. The effect of NOR1 on atherosclerosis was compared using the Kruskal-Wallis test followed by Dunn Test post-hoc analysis. P values < 0.05 were considered to be statistically significant.

References
## Supplemental Table SI: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Use</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>human NOR1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-GGGCTTTTTTCAAGAGAACAGTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-ATCTCTGGGTGTTGAGTCTGTGTT-3'</td>
</tr>
<tr>
<td>human VCAM-1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-TGAGGGGACCACAATTCCAC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-ATTCACGAGGCCACCACACT-3'</td>
</tr>
<tr>
<td>human ICAM-1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-ACCGTGAATGTGCTCTCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GGCTTGTGTTGTCGTTTT-3'</td>
</tr>
<tr>
<td>murine VCAM-1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-TCAAAGAAAGGGAGACTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCTGGAGAAGTTTACTATCC-3'</td>
</tr>
<tr>
<td>murine ICAM-1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-AGATCACATCCAGGTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CTTCAGAGGCAGGAAACAGG-3'</td>
</tr>
<tr>
<td>human TBP</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-GGAGAGTTCTGGGAATGTACCGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-ATTTCGCGTTTCCGGC-3'</td>
</tr>
<tr>
<td>murine TFIIB</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-CTCTCCCAAGAGTCATGTCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CAATAACTCGGTTCCCTACAAC -3'</td>
</tr>
<tr>
<td>murine NOR1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-GGCCGCAGCTGCACTCAGTC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCAGAGGGAAGTCAGCTG -3'</td>
</tr>
<tr>
<td>murine Nur77</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-TTAGACCTGGTGTTCTCCTTC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GGTAGGCGTATGTGCCTGTC-3'</td>
</tr>
<tr>
<td>murine Nurr1</td>
<td>Real-time RT-PCR</td>
<td>F: 5'-TCACCTCCCGGTAGCTGATC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TGCTGGATATGTTGGGTATCATCT-3'</td>
</tr>
<tr>
<td>hNOR1-NFKB</td>
<td>ChIP PCR</td>
<td>F: 5'-CCATCTGACATCCCTGTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCTGACCTTCTCTCTTGC-3'</td>
</tr>
<tr>
<td>hVCAM-1-NOR1</td>
<td>ChIP PCR</td>
<td>F: 5'-CTGTACTCAAAACTTGGGAACATT -3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CCTTAGAGATGAGAAAGCAAGA-3'</td>
</tr>
</tbody>
</table>
Supplemental Figure I. Expression of NR4A orphan nuclear receptors in endothelial cells. NOR1+/+ and NOR1-/- MAEC were incubated with TNFα (5 ng/ml) and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Experiments were repeated at least three times in duplicates with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle).
Supplemental Figure II. (A-C) NOR1+/+ and NOR1-/- MAEC were stimulated with vehicle, IL-1β (1 ng/ml), or LPS (50 ng/ml) for 6 hours. VCAM-1 and ICAM-1 protein expression were analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (A) Autoradiograms are representative of three different experiments. (B and C) Densitometric quantification of VCAM-1 and ICAM-1 protein expression from three experiments with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle, # P < 0.05 vs. NOR1+/+ cells).
Supplemental Figure II. (A-C) NOR1+/+ and NOR1−/− MAEC were stimulated with vehicle, IL-1β (1 ng/ml), or LPS (50 ng/ml) for 6 hours. VCAM-1 and ICAM-1 protein expression were analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (A) Autoradiograms are representative of three different experiments. (B and C) Densitometric quantification of VCAM-1 and ICAM-1 protein expression from three experiments with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle, # P < 0.05 vs. NOR1+/+ cells).
Supplemental Figure III. siRNA-mediated knock-down of NOR1 expression decreases monocyte adhesion. (A) Western Blotting for NOR1 in endothelial cells transfected with scrambled or NOR1 siRNA and treated with TNFα for 6 h. (B) HUVEC were transfected with scrambled or NOR1 siRNA, stimulated with TNFα (1 ng/ml), and analyzed for THP-1 monocyte adhesion as detailed in the Materials and Methods section. Representative images showing monocyte adhesion. (C) Quantification is presented as mean ± SEM from three independently performed experiments in duplicates (*P < 0.05 vs. vehicle, # P < 0.05 vs. scrambled siRNA).
Supplemental Figure III. siRNA-mediated knock-down of NOR1 expression decreases monocyte adhesion. (A) Western Blotting for NOR1 in endothelial cells transfected with scrambled or NOR1 siRNA and treated with TNFα for 6 h. (B) HUVEC were transfected with scrambled or NOR1 siRNA, stimulated with TNFα (1 ng/ml), and analyzed for THP-1 monocyte adhesion as detailed in the Materials and Methods section. Representative images showing monocyte adhesion. (C) Quantification is presented as mean ± SEM from three independently performed experiments in duplicates (*P < 0.05 vs. vehicle, # P < 0.05 vs. scrambled siRNA).
Supplemental Figure IV. Macrophage accumulation in the aortic arch of NOR1+/+ apoE-/− and NOR1-/-apoE-/− mice. Serial sections from a 2-mm segment beginning at the lesser curvature of the aortic arch were collected from NOR1+/+apoE-/− and NOR1-/-apoE-/− mice. Sections were immunostained for macrophages using antisera against macrophages or control IgG. Macrophage content was quantified in nine sections 200 µm apart using computer-assisted image analysis. Data are presented as mean area in mm² ± SEM. Statistical analysis was performed using factorial ANOVA test. No significant difference between single segments of the arch in NOR1+/+apoE-/− and NOR1-/-apoE-/− mice was detected.
Supplemental Figure V. NOR1+/+apoE-/-(n=5) and NOR1-/-apoE-/- (n=5) mice were fed a diet enriched in saturated fat for 2 weeks. Aortae were collected and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Results are presented as mean ± SEM fold increase over NOR1+/+apoE-/- mice. Note, no NOR1 transcript was detected in NOR1-/-apoE-/- mice (N.D., not detectable).