The Orphan Nuclear Receptor Nur77 Suppresses Endothelial Cell Activation Through Induction of IκBα Expression

Bei You, Yuan-Ying Jiang, Shaoping Chen, Guijun Yan, Jianxin Sun

Abstract—Endothelial inflammation plays a critical role in the development and progression of cardiovascular disease, albeit the mechanisms need to be fully elucidated. Nur77 is highly expressed in vascular endothelial cells (ECs) and plays a role in the regulation of cell proliferation and angiogenesis; its role in vascular inflammation, however, remains unknown. Treatment of human umbilical vein ECs (HUVECs) with tumor necrosis factor (TNF)-α substantially increased the transcription and protein expression of Nur77 in a dose and time-dependent manner, as determined by Northern blot and Western blot analysis. Adenovirus mediated overexpression of Nur77 markedly increased the intracellular levels of IκBα by approximately 4-fold, whereas overexpression of dominant negative Nur77 (DN-Nur77), which lacks its transactivation domain, had no effect on IκBα expression, suggesting that Nur77 is an important transcriptional factor in controlling IκBα expression in ECs. Furthermore, overexpression of Nur77 significantly increased IκBα promoter activity via directly binding to a Nur77 response element in the IκBα promoter. Importantly, overexpression of Nur77, but not DN-Nur77, protected ECs against the TNF-α- and interleukin-1β-induced endothelial activation, as characterized by attenuation in the nuclear factor κB activation, expression of adhesion molecules ICAM-1 and VCAM-1, and monocyctic adherence to ECs. These results indicate that Nur77 negatively regulates the TNF-α- and interleukin-1β-induced vascular EC activation by transcriptionally upregulation of IκBα expression. (Circ Res. 2009;104:742-749.)

Key Words: Nur77  ■  endothelial cells  ■  nuclear factor κB  ■  IκBα  ■  atherosclerosis

Arterial recruitment of inflammatory cells from the circulation and their transendothelial migration are key events in the early phase of atherosclerosis.1 In response to several inflammatory stimuli, such as tumor necrosis factor (TNF)-α, interleukin-1β (IL)-1β, and interferon-γ, endothelial cells (ECs) undergo inflammatory activation, resulting in an increased surface expression of cell adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin, which contributes to the recruitment of inflammatory cells to arterial wall and their transmigration across the wall.2 Genetic deficiencies of adhesion molecules in mice are associated with decreased atherosclerosis.3 Therefore, modulation of the expression of adhesion molecules on ECs may be an important target for the prevention and treatment of atherosclerosis.

The cytokine-induced expression of cell adhesion molecules is regulated predominantly by activation of the transcription factor nuclear factor (NF)-κB.3 The promoter regions of the genes for VCAM-1, ICAM-1, and E-selectin all have binding sites for NF-κB.3 NF-κB is an inducible dimeric transcription factor of the Rel/NF-κB family.4 In resting ECs, NF-κB resides inactive in the cytoplasm by forming complexes with its inhibitors, the IκB proteins (IκBs). Of the different IκB proteins, the best described is IκBα. On stimulation by proinflammatory cytokines, IκBα is phosphorylated by IκB kinase (IKK), ubiquitinated, and proteolytically degraded by 26S proteasomes, which allows NF-κB to translocate to the nucleus, where it binds to the promoter DNA and induces the transcription of NF-κB target genes, including adhesion molecules, monocyte chemoattractant protein-1, and several cytokines, which are important mediators of inflammation.5,6 Notably, several therapeutic approaches to inflammation are based on the inhibition of the nuclear translocation of NF-κB, thus attenuating the expression of adhesion molecules.5,6

NR4A receptors are immediate-early genes that are regulated by many physiological stimuli including growth factors, hormones, and inflammatory signals and are involved in a wide array of important biological processes, including cell apoptosis, brain development, glucose metabolism, and vascular remodeling.7 The NR4A subfamily consists of 3 well-conserved members, Nur77 (NR4A1), Nur1 (NR4A2), and...
NOR-1 (NR4A3), respectively. Like other nuclear receptors, NR4A nuclear receptors consist of an N-terminal transactivation domain, a central 2-zinc-finger DNA-binding domain, and a C-terminal ligand-binding domain. So far, no ligands have been identified for these receptors and therefore they are classified as orphan receptors. Recently, there has been much attention paid to the function of these receptors in cardiovascular system. In vascular smooth muscle cells, the expression of Nur77 and NOR-1 was significantly induced by atherogenic stimuli, such as platelet-derived growth factor-BB, epidermal growth factor, and α-thrombin. Overexpression of Nur77 has been shown to inhibit cell proliferation and attenuate vascular injury-induced neointimal formation in vivo. NR4A nuclear receptors are also induced in ECs by several stimuli, such as hypoxia, TNF-α, and vascular endothelial growth factor, and modulate EC growth, survival, and angiogenesis. At present, whether or not NR4A nuclear receptors modulate inflammatory responses in ECs, however, is unknown. In this study, we showed that Nur77 expression is induced by inflammatory cytokines TNF-α and IL-1β and that overexpression of Nur77 suppresses cytokine-induced expression of VCAM-1 and ICAM-1, as well as monocyte adhesion via induction of IkBα expression in human ECs.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture
U937 Cells were purchased from ATCC. Human umbilical vascular ECs (HUVECs) were cultured in EGM EC complete medium (Cambrex). Ad293 cells were cultured in DMEM supplemented with 10% FBS. U937 cells were cultured in RPMI medium supplemented with 10% FBS.

Northern Blot Analysis
Total RNA was isolated using TRizol reagent (Invitrogen) according to the instructions of the manufacturer. Total RNA (20 μg per lane) was resolved on a 1% formaldehyde–agarose gel and transferred onto a positively charged nylon membrane (Ambion) in transfer buffer (1 mol/L NaCl and 10 mmol/L NaOH). The probes were labeled with [α-32P]dCTP (3000 Ci/mmol; PerkinElmer) using a random primed DNA labeling kit (Ambion) and purified by NucAway spin columns (Ambion). The blots were prehybridized and hybridized at 68°C in hybridization buffer (Sigma). After washing twice in 2× SSC, 0.1% SDS at 68°C for 20 minutes and once in 0.1× SSC, 0.1% SDS at 68°C for 30 minutes, the blots were exposed to autoradiography film at −80°C using 2 intensifier screens.

Adenovirus Construction
Adenoviruses harboring wild-type Flag-tagged Nur77 (Ad-Nur77) and dominant-negative Flag-tagged Nur77 lacking the transactivation domain (Ad-DN-Nur77) were made using AdMax (Microbix) as previously described. Briefly, pBH GloXΔE1,3Cre, including the ΔE1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into Ad293 cells using FuGene 6 Transfection Reagent (Roche, Indianapolis, IN). The viruses were propagated in Ad293 cells and purified using CsCl2 banding, followed by dialysis against 20 mmol/L Tris-buffered saline with 10% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the instructions of the manufacturer.

Transient Transfection and Luciferase Assay
The human IkBα promoter was amplified by PCR on human genomic DNA (Clontech) with primers 5′- ATTCA GTCCTCGTG CTTGAGGCTT-3′ and 5′-TGCTTCCTGCTGTTGGGCCT-3′ using the GC-Rich PCR system (Roche). The PCR product was cloned into the luciferase reporter plasmid pGL3-Basic (Promega). HUVECs were seeded in 12-well plates and incubated overnight. The cells were transfected with 100 ng of NF-κB firefly luciferase reporter plasmid p(NF-κB)3-Lac (Stratagene) and 10 ng of Renilla luciferase reporter plasmid pRL-RSV (Promega), in the presence or absence of indicated expression vectors using FuGene 6 transfection reagent. Thirty-six hours after transfection, cells were treated with or without 20 ng/mL TNF-α or 10 ng/mL IL-1β for indicated time points, then directly lysed in the lysis buffer (Promega). Cell lysates (20 μL) were assayed for luciferase activity with a dual-luciferase reporter assay system (Promega) and determined with a luminescence counter (PerkinElmer) according to the instructions of the manufacturer. Firefly luciferase activity was normalized for transfection efficiency by the corresponding Renilla luciferase activity. All transfection experiments were performed at least 5 times in duplicate.

Immunofluorescence Staining
HUVECs plated on 18-mm microcover glasses (Matsunami) were transduced with Ad-LacZ, Ad-Nur77, or Ad-DN-Nur77 (multiplicities of infection [moi], 100) for 48 hours and then treated with or without 20 ng/mL TNF-α for another 1 hour. For immunostaining, fixed HUVECs were sequentially incubated with anti–NF-κB p65 polyclonal antibody (1:25 dilution; Cell Signaling) and fluorescein-5-isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody (1:200 dilution, Invitrogen), or incubated with Cy3-conjugated anti-Flag antibody (1:100 dilution, Sigma). Images were visualized using an Olympus IX70 epifluorescence microscope.

Subcellular Fractionation
HUVECs were transduced with Ad-LacZ, Ad-Nur77, or Ad-DN-Nur77 for 48 hours as described above. After 1 hour of treatment with or without 20 ng/mL TNF-α, subcellular fractions were prepared by differential centrifugation of cell homogenates. Briefly, cells were homogenized manually in hypertonic buffer (10 mmol/L Tris-HCl at pH 7.5, 1 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin). The nuclear fraction was obtained from low-speed centrifugation (500g), followed by 3 washes with PBS. For electrophoretic mobility-shift assay (EMSA), the nuclear fraction was dissolved in hypertonic buffer (20 mmol/L Hepes at pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 25% glycerol and the above protease inhibitors). After high-speed centrifugation at 12,800g for 10 minutes, the supernatants (nuclear extracts) were stored at −80°C until use.

Fluorescence-Activated Cytometry
Sorting Analysis
Ad-LacZ, Ad-Nur77, or Ad-DN-Nur77 transduced HUVECs were treated with either 20 ng/mL TNF-α or 10 ng/mL IL-1β for 8 hours to induce the expression of VCAM-1 and ICAM-1. HUVECs were then trypsinized with 0.05% trypsin (Gibco) and resuspended in a fluorescence-activated cytometry sorting (FACS) buffer (PBS containing 1% bovine serum albumin, 0.1% Na2CO3, 1 mmol/L EDTA). HUVECs were incubated with anti-VCAM-1 (1:100 dilution, R&D Systems), anti-ICAM-1 (1:100 dilution, R&D Systems), or mouse IgG as negative control for 30 minutes on ice, washed twice with FACS buffer, and incubated with FITC-conjugated donkey anti-mouse antibody for 30 minutes on ice in the dark. Then samples were washed again, fixed in 1% paraformaldehyde. For each sample, 10,000 cells were ana-
lyzed by a FACSCalibur flow cytometer (Becton-Dickson Medical Systems, Sharon, MA).

Electrophoretic Mobility-Shift Assay
The oligonucleotides corresponding to the consensus sequence of NF-κB (5′−AGTTGAGGGACTTTCCCAGGC−3′) and Nur77 binding site in IκBα promoter (5′−GTCTTTCCAAAGATCAA AAACG−3′) were labeled with [γ-32P]dCTP. EMSA was performed as described previously.16,17

Monocyte Adhesion Assay
U937 cells were labeled with calcein-AM (Invitrogen) according to the instructions of the manufacturer. After the HUVECs were stimulated and washed, 2.5 × 10⁵ calcein-labeled U937 cells were added to each well and allowed to interact for 60 minutes at 37°C. Unbound cells were removed by gently washing with complete medium, and the number of attached U937 cells was counted on an inverted fluorescent microscope.

Small Interfering RNA of Nur77
Two pairs of small interfering (si)RNA oligonucleotides for human Nur77 (sense strand, 5′−GAA GUU GUC CGA ACA GAC AGC CUG A−3′, and 5′−CAA GGU CCC UGC ACA GCU UGC UUG T−3′) and a pair of control siRNA oligonucleotides (5′−CAG AGA GGA GGA AAG GAG ACG CAG G−3′) were synthesized by Integrated DNA Technologies (Coralville, Iowa). HUVECs grown to ~50% confluence were transfected with Gene Silencer (Gene Therapy System, San Diego, Calif) transfecting agent with target-specific siRNA (20 nmol/L) and control siRNA (20 nmol/L) in serum-free EGM medium according to the recommendations of the manufacturer.

Figure 1. TNF-α−induced expression of Nur77 in HUVECs. A, Confluent HUVECs were treated with TNF-α at the indicated concentrations for 1 hour. Total RNA was extracted, and Northern blot analysis was performed as described in Materials and Methods. B, Time-dependent effect of TNF-α (10 ng/mL) on Nur77 expression as determined by Northern blot analysis. C, Time-dependent effect of TNF-α (10 ng/mL) on Nur77 expression, as determined by Western blot analysis. D, HUVECs were treated with 10 ng/mL IL-1β, 10 ng/mL TNF-α, 20 ng/mL interferon-γ (INF-γ), or 1 μg/mL lipopolysaccharide (LPS) for 1 hour, and the expression of Nur77 was determined by Northern blot analysis. *P<0.05 vs untreated control or time at 0 hour. The data represent 3 independent experiments.

Figure 2. Nur77 inhibits TNF-α−induced NF-κB activation in ECs. A, HUVECs were transfected with 100 ng of p(NF-κB)3−Luc, 10 ng of pRL-RSV, and 200 ng of either empty vector (EV) or pFlag-Nur77 or pFlag-DN-Nur77. Thirty-six hours after transfection, luciferase assays were performed 6 hours after treatment with or without 20 ng/mL TNF-α or 10 ng/mL IL-1β (n=5). *P<0.05 vs EV or DN-Nur77. B, HUVECs were transfected with 100 ng of p(NF-κB)3−Luc, 10 ng of pRL-RSV, and increasing amounts of pFlag-Nur77. Thirty-six hours after transfection, luciferase assays were performed 6 hours after treatment with or without 20 ng/mL TNF-α or 10 ng/mL IL-1β (n=5). *P<0.05 vs EV or DN-Nur77. B, HUVECs were transfected with 100 ng of p(NF-κB)3−Luc, 10 ng of pRL-RSV, and increasing amounts of pFlag-Nur77. Thirty-six hours after transfection, luciferase assays were performed 6 hours after treatment with or without 20 ng/mL TNF-α (n=5). *P<0.05 vs HUVECs treated with TNF-α alone. C, HUVECs were transduced with Ad-LacZ, Ad-Nur77, or Ad-DN-Nur77 (moi, 100) and then treated in the presence or absence of 20 ng/mL TNF-α for 1 hour. Nuclear protein was extracted, and EMSA was performed. The NF-κB complex was partially supershifted by anti-NF-κB p65 antibody or blocked by cold probe. D, HUVECs were transfected with 100 ng of p(NF-κB)3−Luc, 10 ng of pRL-RSV, or 200 ng of pCMV4−p65 and/or pFlag-Nur77. Thirty-six hours after transfection, luciferase assays were performed (n=5).
Statistical Analyses
Data are expressed as means±SEM. The statistical significance of differences was assessed by Student’s t tests or ANOVA, as appropriate; a value of P<0.05 was considered statistically significant.

Results
Upregulation of Nur77 by Inflammatory Cytokines in Human Endothelial Cells
To investigate the role of NR4A nuclear receptors in vascular inflammation, we examined the expression of Nur77 in HUVECs in response to TNF-α stimulation. In agreement with recent reports,13,18,19 we found that TNF-α strongly upregulated Nur77 expression in HUVECs, as determined by Northern blot analysis. At the concentration of as low as 1 ng/mL, TNF-α induced Nur77 mRNA expression by 5-fold. The maximal induction of TNF-α on Nur77 mRNA expression was observed at the concentration of 10 ng/mL, with increased Nur77 expression by 7-fold (Figure 1A). The time course of Nur77 expression, when incubated with 10 ng/mL TNF-α, showed a maximal induction of Nur77 expression 1 hour after TNF-α addition (Figure 1B). Likewise, Nur77 protein was upregulated in HUVECs in a time-dependent fashion by TNF-α, as determined by Western blot analysis (Figure 1C). Furthermore, the expression of Nur77 was also induced by inflammatory cytokine IL-1β but not by lipopolysaccharide and interferon-γ in HUVECs (Figure 1D).

Figure 3. Nur77 inhibits TNF-α–induced translocation of NF-κB p65 in HUVECs. HUVECs were transduced with Ad-LacZ, Ad-Nur77, or Ad-DN-Nur77 (moi, 100) for 48 hours. A, HUVECs were treated with or without 20 ng/mL TNF-α for 1 hour and then fixed and sequentially incubated with anti-NF-κB p65 polyclonal antibody and FITC-conjugated donkey anti-rabbit antibody or directly incubated with Cy3-conjugated anti-Flag antibody. Images were visualized using epifluorescence microscope. Magnification, ×20. B, Nuclear and cytosolic proteins were extracted from HUVECs treated with or without 20 ng/mL TNF-α for 1 hour and then processed for Western blot analysis using anti-NF-κB p65 polyclonal antibody. C, HUVECs were treated with 20 ng/mL TNF-α and the expression of IkBα was then detected by Western blot and quantitated by densitometric analysis. *P<0.05 vs Ad-LacZ at time 0.

Figure 4. Nur77 induces the expression of IkBα in HUVECs. A, HUVECs were infected with indicated moi of Ad-Nur77. Forty-eight hours after transduction, the expression of IkBα and Flag-Nur77 was detected using Western blot analysis (n=5). *P<0.05 vs 0 moi. B, HUVECs were transduced with Ad-Nur77 or Ad-DN-Nur77 at the indicated moi. Forty-eight hours after transduction, total RNA was extracted and IkBα mRNA was detected using Northern blot analysis. The data represent at least 3 individual experiments. *P<0.05 vs 0 moi. C, HUVECs were transfected with various IkBα promoter fragments (100 ng) and pRL-RSV (10 ng) in the presence of pFlag-Nur77 or empty vector (pSG5, 300 ng). Thirty-six hours after transfection, luciferase assays were performed and normalized by constitutive Renilla luciferase (n=5). *P<0.05 vs empty vector or mutated −1240 +50 or −420 +50. D, EMSA was performed using nuclear extracts from Flag-Nur77–overexpressing cells and a radiolabeled probe (−458, −437 bp). Fifty times of competitor wild-type (AAAGATCA) or mutated (AACCCTCA) oligonucleotides were used to demonstrate the specificity of the shifted complex. To supershift the complex, 3 μL of Flag antibody was added to the binding reaction. S indicates shifted complex; SS, supershifted complex.
observation that Nur77 can be induced in cultured ECs by TNF-α or IL-1β suggests that it may regulate cellular responses to these stimuli.

Nur77 Attenuates NF-κB Activation

To examine whether Nur77 plays a role in the regulation of vascular inflammation, we examined the effect of Nur77 overexpression on NF-κB promoter activity at the transcriptional level. HUVECs were transiently transfected with a plasmid containing a heterologous promoter driven by NF-κB elements upstream of the luciferase gene. Overexpression of Nur77 significantly inhibited both basal and TNF-α- and IL-1β-stimulated NF-κB promoter activity. In contrast, overexpression of dominant negative Nur77 (DN-Nur77), which is defective in its transactivation domain, had no effect (Figure 2A). Furthermore, Nur77 overexpression dose-dependently reduced TNF-α-induced NF-κB transcriptional activity (Figure 2B). Likewise, overexpression of Nur77, but not DN-Nur77, markedly suppressed TNF-α-induced NF-κB activation, as indicated by EMSA (Figure 2C). Because Nur77 has been shown to interact with p65,20 we examined whether the interaction of Nur77 with p65 is involved in the inhibitory effect of Nur77 on NF-κB activation. As shown in Figure 2D, p65-induced NF-κB promoter activity was not affected by overexpression of Nur77. Furthermore, p65 did not coprecipitate with the anti-Flag antibody in cell lysates from Flag-Nur77–overexpressing HUVECs (data not shown). Taken together, these findings indicate that Nur77 attenuates the NF-κB activation by targeting the NF-κB signaling pathway upstream from p65 in ECs.

Nur77 Inhibits Nuclear Translocation of NF-κB p65

The activation of NF-κB occurs via nuclear translocation of Rel family proteins, and this is preceded by the phosphorylation and degradation of IκBs by IKK. Therefore, to investigate the mechanism responsible for the inhibitory effect of Nur77 on NF-κB activation, we examined the effect of Nur77 overexpression on p65 nuclear translocation. Overexpression of wild-type Nur77, but not DN-Nur77, substantially inhibited TNF-α-induced p65 nuclear translocation, as determined by both immunofluorescence and Western blot analysis (Figure 3A and 3B). Furthermore, we examined the effect of Nur77 on the TNF-α–induced degradation of IκBα by Western blot analysis. In control cells overexpressing LacZ, IκBα was degraded following 20 ng/mL TNF-α treatment within 15 minutes, then rapidly resynthesized, and returned to normal level with 60 minutes (Figure 3C). Likewise, the time-dependent degradation and recovery of IκBα protein was not significantly altered by Nur77 overexpression. However, the basal levels of IκBα expression were markedly increased by ~4-fold in HUVECs overexpressing Nur77 as compared to cells overexpressing LacZ. As a result, after treatment with 20 ng/mL TNF-α for 15 minutes, IκBα in cells overexpressing Nur77 was degraded to the levels that are comparable to the levels in resting ECs. Furthermore, overexpression of Nur77 and DN-Nur77 had no effect on the viability of HUVECs (data not shown). Taken together, these results suggest that Nur77 inhibits the translocation of NF-κB p65 to the nucleus mainly by upregulating IκBα expression but not by influencing TNF-α signaling to IκBα.

Nur77 Transcriptionally Upregulates IκBα Expression

To determine the molecular mechanism underlying the induction of IκBα by Nur77, we examined the effect of Nur77 overexpression on IκBα expression in HUVECs by Western blot and Northern blot analysis. Adenovirus-mediated overexpression of Nur77 dose-dependently increased IκBα expression at both protein and mRNA levels (Figure 4A and 4B). The induced
expression of VCAM-1 (B) and ICAM-1 (C) was detected by flow cytometry using anti–VCAM-1 and anti–ICAM-1 monoclonal antibodies, respectively. Results are shown as the means±SEM.

Figure 6. Nur77 knockdown augments the cytokine-induced expression of adhesion molecules in ECs. A, Western blotting showing the expression of Nur77 in HUVECs transfected with Nur77-specific siRNA and control siRNA. B and C, Seventy-two hours after transfection of siRNA, HUVECs were treated with or without 20 ng/mL TNF-α or 10 ng/mL IL-1β for 8 hours. The expression of VCAM-1 (B) and ICAM-1 (C) was detected by flow cytometry using anti–VCAM-1 and anti–ICAM-1 monoclonal antibodies, respectively. Results are shown as the means±SEM of 5 individual experiments.

IκBα protein is mainly localized in the cytoplasmic fraction of human ECs (data not shown). Overexpression of Nur77 significantly increased IκBα promoter activity (−1240 +50 bp) (Figure 4C). Nur77 has been shown to regulate gene transcription through binding to the NFκB response element (NBRE, AAAGGTCA).21 Sequence analysis further revealed the presence of a putative Nur77 response element with one point mutation (AAAGATCA) located between −445 and −452 base pairs (Figure 4C) in human IκBα promoter. Mutation of this putative NBRE site abolished the responsiveness of the human IκBα promoter to Nur77 (Figure 4C), indicating that this site mediates IκBα transcriptional induction by Nur77. Furthermore, Nur77 binding to the identified NBRE site was verified by EMSA using nuclear extracts obtained from HUVECs overexpressing Flag-Nur77 (Figure 4D). Competition experiments using wild-type and mutated cold oligonucleotides, as well as supershift experiments using anti-flag antibody, further demonstrated that Nur77 binds specifically to the Nur77 responsive element located between −445 and −452 in the human IκBα promoter (Figure 4D).

Nur77 Suppresses Cytokine-Induced VCAM-1 and ICAM-1 Expression in HUVECs

Activation of NF-κB has been implicated in the cytokine-induced expression of adhesion molecules, such as VCAM-1 and ICAM-1, which mediate the adhesion of monocytes to inflamed ECs. Thus, we examined the effects of Nur77 on cytokine-induced VCAM-1 and ICAM-1 expression in HUVECs. Overexpression of Nur77 markedly attenuated both TNF-α- and IL-1β-induced expression of VCAM-1 and ICAM-1, as determined by Northern blot (Figure 5A). Similarly, as determined by FACS analysis, the TNF-α- and IL-1β–induced cell surface expression of VCAM-1 was suppressed by 85% and 80%, respectively, by Nur77 overexpression (Figure 5B). In addition, Nur77 overexpression completely inhibited the TNF-α- and IL-1β–induced cell surface expression of ICAM-1 (Figure 5C). In contrast, DN-Nur77 overexpression slightly increased both TNF-α- and IL-1β–induced expression of VCAM-1 and ICAM-1. To further substantiate the significance of Nur77 in endothelial activation, we performed loss-of-function studies, using the RNA interference technique. Transfection of Nur77 siRNA substantially inhibited Nur77 expression by ≈80% (Figure 6A). Furthermore, silencing of Nur77 expression significantly enhanced the TNF-α- and IL-1β–induced cell surface expression of VCAM-1 (Figure 6B) and ICAM-1 (Figure 6C), further suggesting the involvement of Nur77 in the regulation of cytokine-induced endothelial activation.

Nur77 Attenuates U937 Cell Adhesion to the Activated HUVECs

Monocyte adhesion to ECs is an important event in the initiation of atherosclerosis. Therefore, we examined the effect of Nur77 on U937 cell adhesion to the activated HUVECs. When HUVECs were stimulated with TNF-α (20 ng/mL) and IL-1β (10 ng/mL), U937 cell adhesion was substantially increased and the increased adhesion of U937 cells to the TNF-α- and IL-1β–stimulated HUVECs was suppressed by 58% and 50%, respectively, by Nur77 overexpression, but not by DN-Nur77 (Figure 7). Taken together, these results further suggest that Nur77 functions as a negative regulator of the cytokine-induced inflammatory responses in vascular ECs.

Discussion

In this study, we have found that Nur77, an orphan nuclear receptor, has antiinflammatory properties in human ECs. We demonstrated that Nur77 expression is upregulated in response to inflammatory stimuli, such as TNF-α and IL-1β, and acts as a potent inhibitor of EC activation by inhibiting the nuclear translocation of NF-κB via induction of IκBα expression at the transcriptional level. Thus, Nur77 overexpression inhibits the NF-κB–dependent expression of VCAM-1 and ICAM-1 and reduces monocyte adhesion to both TNF-α- and IL-1β–activated human ECs.

Vascular disease such as atherosclerosis involves predominantly macrophages, T cells, ECs, and smooth muscle cells that...
interact with each other in the vessel wall. Recently, the cardiovascular function of NR4A receptors has been investigated in these cells. In response to atherogenic stimuli, such as oxidized LDL, lipopolysaccharide and TNF-α, the expression of NR4A nuclear receptors are markedly induced in monocytes and macrophages. Furthermore, the expression of NR4A receptors in atherosclerotic lesion macrophages underlines the importance of these transcription factors in plaque progression. Indeed, overexpression of Nur77 in macrophages has been shown to inhibit inflammatory cytokine expression and reduce oxidized LDL uptake in these cells. Moreover, 6-mercaptopurine, which is used clinically in the treatment of leukemia and inflammatory bowel disease, has been shown to enhance the transcriptional activity of Nur77, attenuate smooth muscle cell proliferation, and prevent cuff injury-induced neointimal formation in a mouse model in a Nur77-dependent manner. In ECs, Nur77 overexpression has been shown to increase cell survival and angiogenesis. However, the function of endogenous Nur77 in endothelial activation has not been previously studied. Here, we demonstrate that Nur77 may exert additional activities of potential benefit for the vessel wall through suppressing NF-κB activation and monocyte adhesion to the activated endothelium, which is a key process in the initiation of atherosclerosis. Thus, we conclude that NR4A nuclear receptors such as Nur77 may contribute to the resolution of inflammation by constituting a negative feedback loop in proinflammatory activation.

Ample evidence suggests that proinflammatory cytokines such as TNF-α and IL-1β induce NF-κB activation in ECs, which mediates their proatherogenic effects. These include induction of adhesion molecules such as VCAM-1 and ICAM-1, thus promoting monocyte adhesiveness to the endothelium. Disruption of the cytokine-induced NF-κB signaling pathway has been shown to delay or prevent atherogenesis in animal disease models. Modulation of IkBα function and expression plays an essential role in the regulation of NF-κB activation. For example, the phosphorylation of IkBα is a key regulatory step in the activation of NF-κB. Indeed, some antiinflammatory agents, such as salicylates and antioxidants, have been shown to inhibit NF-κB and EC activation by preventing IkBα phosphorylation and subsequent degradation. In addition, nitric oxide (NO) and glucocorticoids have been shown to elicit their inhibitory effects on NF-κB via induction of IkBα expression. Indeed, Nur77 has been reported to inhibit NF-κB activation by an as yet unknown mechanism in HEK293 cells and Jurkat cells. In the present study, our results suggest that Nur77, an early responsive gene in ECs, inhibits NF-κB activation via induction of IkBα expression, thereby increasing the cytoplasmic levels of IκBα. Higher levels of IκBα protein in the cytoplasm, therefore, could supplement the initially degraded IκBα and help retain and stabilize the NF-κB/IκBα complex in the cytoplasm, thus limiting VCAM-1 and ICAM-1 gene transcription.

A recent report shows that the p65 subunit of NF-κB interacts with Nur77 and represses Nur77 transactivation in TNF-α-stimulated R2C cells. However, this interaction is unlikely to occur in human ECs. First, we observed the predominant localization of Nur77 in the nuclear fraction of HUVECs when it is overexpressed, whereas p65 remains in the cytoplasm in both resting and TNF-α-stimulated ECs overexpressing Nur77. Furthermore, the p65-induced NF-κB activation is not affected by Nur77, thus excluding the possibility that Nur77 inhibits NF-κB activation through its interaction with p65. Because Nur77 remains in the nucleus in response to TNF-α and is not translocated to the mitochondria, where it was reported to mediate apoptosis, it would be interesting to investigate whether Nur77 functions as a survival effector protein in the context of TNF-α-mediated signaling in ECs, as has recently been shown in mouse embryonic fibroblasts.

Overall, the data reported herein provide the evidence that the orphan nuclear receptor Nur77 is a potent negative regulator of the TNF-α- and IL-1β-induced EC activation via a novel mechanism involving the induction of IκBα expression. Thus, specifically modifying Nur77 transcriptional activity may have potential application in the treatment of inflammatory vascular disorders.

Sources of Funding
This work was supported by American Heart Association Scientist Development Grant 0630047N (to J.S.) and National Natural Science Foundation of China grants 30472024 and 30371648 (to J.S.).

Disclosures
None.

References


The Orphan Nuclear Receptor Nur77 Suppresses Endothelial Cell Activation Through Induction of IκBα Expression

Bei You, Yuan-Ying Jiang, Shaoping Chen, Guijun Yan and Jianxin Sun

*Circ Res.* 2009;104:742-749; originally published online February 12, 2009;
doi: 10.1161/CIRCRESAHA.108.192286

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 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/104/6/742

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/02/12/CIRCRESAHA.108.192286.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/
Supplement Material

Materials and Methods

Cell culture

U937 Cells were purchased from ATCC. Human umbilical vascular endothelial cells (HUVECs) were cultured in EGM endothelial cell complete medium (Cambrex). Ad293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). U937 cells were cultured in RPMI 1640 supplemented with 10% FBS.

Northern blot analysis

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer’s instruction. Total RNA (20 µg/lane) was resolved on a 1% formaldehyde-agarose gel and transferred onto a positive charged nylon membrane (Ambion) in transfer buffer (1 M NaCl and 10 mM NaOH). The probes were labeled with [\(\alpha^{-32}\)P]dCTP (3000 Ci/mmol; PerkinElmer) using a random primed DNA labeling kit (Ambion) and purified by NucAway spin columns (Ambion). The blots were prehybridized and hybridized at 68 °C in hybridization buffer (Sigma). After washing twice in 2× SSC, 0.1% SDS at 68 °C for 20 min and once in 0.1× SSC, 0.1% SDS at 68 °C for 30 min, the blots were exposed to autoradiography film at -80 °C using two intensifier screens. The bands’ intensities were normalized to the 28S, and the graphs were generated with the relative values obtained after normalization.

The cDNA probes for Nur77, IκBα, VCAM-1, and ICAM-1 were generated by RT-PCR using the following cDNA-specific primers: Nur77: 5’-ACGGCTCACACAGGAGGAGTTGGACA-3’ (forward); 5’-AGTCCTTGGTGTTAGCCAGGCAG -TGT-3’ (reverse); IκBα: 5’-AGACCTGGCTTTCCTCAACTTCCA-3’ (forward); 5’-TGGCCTCCAACACAGACACAGTCAAC-3’ (reverse); VCAM-1: 5’-TAAGCTGGGAAGGGTCCCATAGCGTGT -3’ (forward); 5’-TCAACTGGGCCT
TTCGGATGGTAT-3' (reverse); ICAM-1: 5'-AGCCCAAGTTGTTGGGCATAGAGA-3' (forward); 5'-AAGGAGTCGTTGCCATAGGTGACT-3' (reverse).

**Adenovirus Construction**

Adenoviruses harboring wild-type Flag-tagged Nur77 (Ad-Nur77), and dominant-negative Flag-tagged Nur77 lacking the AF-1 domain (Ad-DN-Nur77) were made using AdMax (Microbix) as previously described. Briefly, pBHGloxE1,3Cre, including the E1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into Ad293 cells using FuGene 6 Transfection Reagent (Roche, Indianapolis, IN). The viruses were propagated in Ad293 cells and purified using CsCl banding followed by dialysis against 20 mmol/l Tris-buffered saline with 10% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the instructions of the manufacturer.

For adenovirus-mediated Lac Z, wild-type or dominant-negative Nur77 gene transduction in vitro, HUVECs were grown to ~60% confluence and infected with adenovirus at indicated multiplicity of infection (MOI). 12 hr after transduction, the viral suspension was removed and the cells were incubated for another 36 hr.

**Western blotting**

Proteins were prepared and separated on SDS-PAGE as described previously. Immunoblotting was performed using antibodies to Nur77 (1:1,000 dilution; Santa Cruz Biotechnology), to NF-κB p65 subunit (1:1000 dilution; Cell signaling), to I-κBα (1:200 dilution; Santa Cruz Biotechnology), to α-tubulin (1:4,000 dilution; Sigma), and to Flag (HRP conjugated, 1:3000 dilution, Sigma). Immunodetection was accomplished using a sheep anti-mouse secondary antibody (1:2,000 dilution) or donkey anti-rabbit secondary antibody (1:2,000 dilution) and the enhanced chemiluminescence kit (Amersham). The bands’ intensities were normalized...
to the corresponding bands of α-tubulin, and the graphs were generated with the relative values obtained after normalization.

**Transient Transfection and luciferase assay**

The human IkBα promoter was amplified by PCR on human genomic DNA (Clontech) with primers 5′- ATTCAGTCCATGGCTTGAGCAGGCTT-3′ and 5′- TGCTTCTCGCTGGGCGCT -3′ using the GC-Rich PCR system (Roche). The PCR product was cloned into the luciferase reporter plasmid pGL3-Basic (Promega). HUVECs were seeded in 12-well plates and incubated overnight. The cells were transfected with 100 ng NF-κB firefly luciferase reporter plasmid p(NF-κB)3-Luc (Stratagene) and 10 ng Renilla luciferase reporter plasmid pRL-RSV (Promega), in the presence or absence of indicated expression vectors using FuGene 6 transfection reagent. 36 hr after transfection, cells were treated with or without 20 ng/ml TNF α or 10 ng/ml IL-1β for indicated time points, then directly lysed in the lysis buffer (Promega). 20 µl of the cell lysates were assayed for luciferase activity with a dual-luciferase reporter assay system (Promega) and determined with a luminescence counter (PerkinElmer) according to the manufacturer’s instructions. Firefly luciferase activity was normalized for transfection efficiency by the corresponding Renilla luciferase activity. All transfection experiments were performed at least five times in duplicate.

**Immunofluorescence Staining**

HUVECs plated on 18-mm microcover glasses (Matsunami) were transduced with Ad-LacZ, Ad-Nur77 or Ad-DN-Nur77 (MOI=100) for 48 hours, and then treated with or without 20 ng/ml TNF α for another 1 hr. For immunostaining, fixed HUVECs were sequentially incubated with anti-NF-κB p65 polyclonal antibody (1:25 dilution, Cell signaling) and fluorescein-5-isothiocyanate-conjugated donkey anti-rabbit antibody (1:200 dilution, Invitrogen), or incubated with Cy3-
conjugated anti-Flag antibody (1:100 dilution, Sigma). Images were visualized using an Olympus IX70 epifluorescence microscope.

**Subcellular fractionation**

HUVECs were transduced with Ad-Lac Z, AD-Nur77 or Ad-DN-Nur77 for 48 h as described above. After 1 h of treatment with or without 20ng/ml TNF-α, Subcellular fractions were prepared by differential centrifugation of cell homogenates. Briefly, cells were homogenized manually in hypotonic buffer (10 mM Tris-HCl at pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 5 µg/ml aprotinin). The nuclear fraction was obtained from low-speed centrifugation (500 g), followed by three washes with phosphate-buffered saline. For Western blot analysis, the nuclear fraction was re-suspended in RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the above protease inhibitors). For electrophoretic mobility shift assay, the nuclear fraction was dissolved in hypertonic buffer (20 mM Hepes at pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol and the above protease inhibitors). After high-speed centrifugation at 12,800 g for 10 min, the supernatants (nuclear extracts) were stored at –80 °C until use. The cytosolic fraction was collected as the supernatant resulting from the ultracentrifugation (100,000 g) of the homogenates.

**Fluorescence-activated Cytometry Sorting (FACS) Analysis**

Ad-Lac Z, Ad-Nur77, or Ad-DN-Nur77 transduced HUVECs were treated with 20 ng/ml TNFα or 10 ng/ml IL-1β for 8 h to induce the expression of VCAM-1 and ICAM-1. HUVECs were then trypsinized with 0.05% trypsin (Gibco) and resuspended in a FACS buffer (PBS containing 1% bovine serum albumin, 0.1% Na₃N, 1 mM EDTA). HUVEC were incubated with anti-VCAM-1 (1:100 dilution, R&D Systems), anti-ICAM-1 (1:100 dilution, R&D Systems), or mouse IgG as
negative control for 30 min on ice, washed twice with FACS buffer, and incubated with fluorescein-5-isothiocyanate-conjugated donkey anti-rabbit antibody for 30 min on ice in the dark. Then samples were washed again, fixed in 1% paraformaldehyde. For each sample, 10,000 cells were analyzed by a FACSCalibur flow cytometer (Becton-Dickson Medical Systems, Sharon, MA). Cell surface expression of VCAM-1 and ICAM-1 was shown as mean fluorescence intensity (MFI).

**Electrophoretic mobility shift assay**

The oligonucleotides corresponding to the consensus sequences of NF-κB (5' - AGTTGAGGGGACTTTCCCAGGC-3') and Nur77 binding site in IκBα promoter (5' - GTTTTCAAAAGATCAAAAAACG-3') were labeled with [α-32P]dCTP. Electrophoretic mobility shift assay was performed as described previously 4, 5. Briefly, nuclear extracts (10 µg) were incubated with 32P-labeled oligonucleotide (1 ng) for 30 min at room temperature in a buffer containing 2 µg of poly(dI·dC) (Roche), 10 µg of bovine serum albumin, 10 mM Tris-HCl (pH7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol (total volume of 40 µl). DNA-protein complexes were resolved on 4% native polyacrylamide gel. The gel was exposed to autoradiography film at -80 °C using intensifier screens. For supershift assays, the anti-NF-κB p65 antibody (0.6 µg, Santa Cruz Biotechnology) or anti-Flag antibody (1 µg, Sigma) was added to the nuclear extracts for 60 min prior to the addition of radiolabeled probe. For competition experiments, a 50-fold excess of unlabeled oligonucleotides or mutant oligonucleotides was added to the nuclear extracts for 30 min prior to the addition of radiolabeled probe.

**Monocyte adhesion assay**

Ad-Lac Z, Ad-Nur77, or Ad-DN-Nur77 transduced HUVECs were treated with 20 ng/ml TNF-α or 10 ng/ml IL-1β for 8 h to induce the expression of adhesion molecules before adhesion assay.
U937 cells were labeled with calcein-AM (Invitrogen) according to the manufacturer’s instruction. After the HUVECs were stimulated and washed, 2.5x10^5 calcein-labeled U937 cells were added to each well and allowed to interact for 60 min at 37°C. Unbound cells were removed by gently washing with complete medium, and the number of attached U937 cells was counted on an inverted fluorescent microscopy. Ten fields were counted for each well.

**Small interfering RNA (siRNA) of Nur77**

Two pairs of siRNA oligonucleotides for human Nur77 (sense strand, 5’-GAA GUU GUC CGA ACA GAC AGC CUG A -3’, and 5’-CAA GGU CCC UGC ACA GCU UGC UUG T -3’) and a pair of control siRNA oligonucleotides (5’-CAG AGA GGA GGA AAG GAG ACG CAG G-3’) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). HUVECs grown to ~50% confluence were transfected with Gene Silencer (Gene Therapy System, San Diego, CA, USA) transfecting agent with target-specific siRNA (20 nM) and control siRNA (20 nM) in serum-free EGM medium according to the manufacturer’s recommendation. Approximately 3 h post-transfection, fresh EGM endothelial cell complete medium was added, and the cells were cultured for an additional 72 h for the detection of the expression of adhesion molecules and cell adhesion.

**Statistical Analyses**

Data are expressed as mean ± SEM. The statistical significance of differences was assessed by Student’s t tests or ANOVA, as appropriate; a value of P<0.05 was considered statistically significant.
References


