Neuron-Derived Orphan Receptor-1 (NOR-1) Modulates Vascular Smooth Muscle Cell Proliferation

José Martínez-González, Jordi Rius, Ana Castelló, Claudia Cases-Langhoff, Lina Badimon

Abstract—Vascular smooth muscle cells (VSMCs) migration and proliferation play a key role in the pathophysiology of cardiovascular disease. However, the transcription factors that regulate VSMC activation are not completely characterized. By a mRNA-differential display approach, we have identified neuron-derived orphan receptor-1 (NOR-1), a transcription factor within the NGFI-B subfamily of nuclear receptors, as an immediate-early gene in VSMCs. Two NOR-1 isoforms (α and β) were identified and cloned from serum-induced porcine VSMC that shared high homology with the human isoforms. Northern blot analysis revealed a strong and transient (1 to 6 hours) upregulation of NOR-1 in both porcine and human coronary SMCs by growth factors (serum, platelet-derived growth factor-BB, and epidermal growth factor) and α-thrombin but not by cytokines. NOR-1 upregulation is processed through G protein–coupled receptors and tyrosine kinase receptors, and involves Ca²⁺ mobilization, protein kinase C activation, and the mitogen-activated protein kinase pathway. This induction was closely dependent of the cAMP response elements present in NOR-1 promoter as transfection assays indicate. Human coronary atherosclerotic lesions overexpress NOR-1, and balloon angioplasty transiently induces NOR-1 in porcine coronary arteries with a pattern similar to that observed in VSMCs in culture. Antisense oligonucleotides against NOR-1 inhibited human coronary SMC proliferation (reduced de novo DNA synthesis, cell cycle progression, and VSMC wound repair) as efficiently as antisense against the protooncogene c-fos. These results show that NOR-1 modulates VSMC proliferation, and suggest that this transcription factor may play a role in both spontaneous and accelerated atherosclerosis. (Circ Res. 2003;92:96-103.)

Key Words: smooth muscle cells || NOR-1 || atherosclerosis || angioplasty || gene expression

The understanding of the molecular mechanisms involved in vascular smooth muscle cell (VSMC) activation and differentiation requires an accurate mapping of the cascade of transcription factors induced by atherogenic stimuli. These master genes could be targets for new diagnosis and/or therapeutic strategies. Recently, different nuclear receptors, including peroxisome proliferator-activated receptors (PPARs),1,2 retinoid receptors, both retinoid X receptors (RXR) and retinoic acid receptors (RAR), and retinoid-related orphan receptors (ROR) have been identified in VSMC activation/proliferation and consequently have been implicated in the atherogenic process.3–5 Nuclear receptors comprise a large family of ligand-activated transcription factors that by regulating complex gene programs play critical roles in nearly all aspects of development and adult physiology.6,7 Moreover, currently, ligands have been identified for only half of the known nuclear receptors; the remaining members known as orphan nuclear receptors constitute a promising area especially for research and development.

In the present study, we have identified by mRNA-differential display (mRNA-DD) analysis neuron-derived orphan receptor-1 (NOR-1) as an early-response gene in VSMCs. NOR-1, together with Nur77 and Nur1, form the NGFI-B family of orphan nuclear receptors within the steroid/thyroid receptor superfamily.6,7 These genes have been involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic stimuli in different cell types.8 We observed a strong induction of NOR-1 in VSMCs by growth factors and thrombin. NOR-1 was upregulated through signaling pathways commonly activated in cell migration and proliferation. NOR-1 and Nur77 were overexpressed in atherosclerotic lesions from patients with coronary artery disease (CAD) and balloon angioplasty transiently induces NOR-1 in porcine coronary arteries. Moreover, antisense oligonucleotides against NOR-1 significantly inhibited VSMC proliferation and wound repair. These results suggest that NOR-1 may play a role in the molecular mechanisms underlying both spontaneous and accelerated atherosclerosis.

Materials and Methods

SMC Cultures

VSMCs were obtained by a modification of the explant technique from swine and human coronary arteries.9 Cells were cultured as described previously, and those used in the experiments were between the 2nd and 5th passage. Cells were seeded in multiwell plates and, at confluence, were arrested with medium containing...
acetate (PMA) (Sigma) in different experiments. When inhibitors were used, SMCs were precultivated with them for 30 minutes before stimulus. The inhibitors used were 1 μg/mL pertussis toxin (Sigma), 50 μmol/L PD98059 (Sigma), 15 μmol/L 1,2-Bis(2-aminoophenoxy)ethano-N,N',N''-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA) (Sigma), or increasing concentrations of GF-109203X (Sigma) or EGTA.

The inhibitors did not produce any effect on cell morphology, cell apoptosis (assessed by staining with Hoesch 33258 colorant) or cell viability. 

**mRNA-DD Analysis**
Total RNA from porcine coronary tunica media SMCs either arrested or stimulated for 1 hour with 10% human serum, 10 U/mL α-thrombin (Diagnostica Stago), or 5 nmol/L PDGF-BB was isolated using QuickPrep (Amersham-Pharmacia). mRNA-DD analysis was performed with the Delta RNA Fingerprinting kit (Clontech) as described. Positive clones (101) were isolated in a screening (1/10000) (Amersham-Pharmacia). 10 Differentially displayed bands were cloned into the pGEM-T vector (Promega) and sequenced with the ABI Prism dRhodamine Terminator Cycle Sequencing kit (Perkin Elmer).

**Screening of cDNA Libraries**
Band 2 identified by mRNA-DD was used as a probe in the analysis of cDNA libraries in λZAPI (Stratagene) prepared from porcine coronary SMCs (stimulated with human serum for 1 hour) mRNA. Positive clones (101) were isolated in a screening (1×105 plaques) and were characterized by restriction-enzyme mapping. Clones AqsNor32 and AqsNor83 were subcloned for further analysis. Nucleotide sequence was obtained by automatic sequencing of both cDNA strands. Multiple protein sequence alignments were performed using the ClustalW service at European Bioinformatics Institute.

**Northern Blot Analysis**
Total RNA was obtained as indicated above and was analyzed by Northern blot as described. NOR-1 cDNA, a rat c-fos cDNA, and a ribosomal cDNA (to normalize blots) were used as probes.

**Electrophoretic Mobility Shift Assay (EMSA)**
Nuclear extracts (3 μg) from human VSMCs and double stranded probes corresponding to the sequence of human NOR-1 promoter (from −84 to −41; Nor/3CRE) containing the three putative CRE motifs or a consensus NBRE sequence were used in EMSA as described. Supershift experiments were performed using antibodies against CREB, c-Fos, and c-Jun (Santa Cruz Biotechnology Inc.).

**Western Blot Analysis**
The levels of CREB (phosphorylated and unphosphorylated forms) in cell extracts from human VSMCs were analyzed by Western blot using specific antibodies (Santa Cruz Biotechnology Inc.).

**Construction of NOR-1 Promoter Plasmids**
The plasmid pNORα−1703 containing the human NOR-1 promoter (from −1703 to +264) was kindly provided by Dr N. Ohkura (Growth Factor Division, National Cancer Research Institute, Tokyo, Japan). Site-directed mutations of the three putative CRE sites present in NOR-1 promoter were performed by the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The changes introduced in the sequence of the mutant construction [pNORα−1703−M] were (−79)TGcatcAGCGTCCCATGcatgCATGTCCATCT−(−46) (changes are indicated in lower case letters). The new sequence was analyzed by different promoter analysis software to confirm that no new response elements were generated.
oxytnucleotides (ODNs) and [3H]thymidine incorporation was determined as described. The effect of ODNs on cell cycle was analyzed by flow cytometry as described. The antisense ODNs used were antisense-1 NOR-1 (AS1-NOR; 5'-TGGACGACGAGG-3') and antisense-2 NOR-1 (AS2-NOR; 5'-GCTGCCAAGGTCAT-3') complementary to nucleotides 732 to 746 and 849 to 863 of human NOR-1 mRNA (Accession No. NM-006981), respectively. As controls, the corresponding sense sequences and an antisense ODN against c-fos, previously used to assess the involvement of this protooncogene in cell proliferation, were used.

In Vitro SMC Injury

The ability of antisense NOR-1 ODNs to inhibit SMC wound repair after mechanical injury was assessed in human coronary SMCs in culture as described. Briefly, confluent growth-arrested human coronary SMCs were scraped and then incubated for 72 hours at 37°C in the presence or absence of the ODNs indicated above. The cells were fixed and stained with methylene blue. Images were digitalized by a Sony 3CCD camera, and cell number in the denuded zone was determined.

Statistics

Results are expressed as mean±SEM. A Stat View II (Abacus Concepts) statistical package for the Macintosh computer system was used for all the analysis. Multiple groups were compared by the one-factor ANOVA, followed by Fisher PLSD to assess specific group differences.

Results

DD/RT-PCR Analysis on Mitogen-Stimulated VSMCs

To identify new genes involved in VSMC activation, we stimulated porcine coronary SMCs with serum, PDGF, or thrombin and analyzed differential gene expression by mRNA-DD analysis. We isolated a total of 23 bands differentially regulated at least by one of the inducers used. Sequence analysis revealed that only two bands presented significantly identity with genes previously described. Band 23, induced by serum and PDGF, corresponded to thrombospondin-1. Band 2 (1010 bp) (Figure 1A) recognized a transcript of 5.7 kb early induced by serum, PDGF, and thrombin (Figures 1B and 1C, left panel). The nucleotide sequence of band 2 shared high homology with the 3'-untranslated region of members of the NGFI-B family of orphan nuclear receptors: NOR-1, Nur77, and Nur1.

Isolation of Full-Length cDNA Clones

The open reading frame (ORF) of the clone AStNor32 (submitted to GenBank/EMBL Accession No. AJ011767) encodes for a polypeptide of 643 amino acid residues, that among the receptors mentioned above, exhibited the highest homology with NOR-1α sequences. The long 3'-untranslated sequence (3072 bp) of this pNOR-1α cDNA contained 9 copies of the sequence ATTTA, typical of short-lived mRNAs. Clone AStNor83 (submitted to GenBank/EMBL Accession No. AJ011768) exhibits an ORF coding for a polypeptide of 446 amino acid residues (pNOR-1β) that does not contain the C-terminal region corresponding to the ligand-binding domain. Alignments of the porcine NOR-1α and NOR-1β amino acid sequence with their homologues in human, rat, and mouse revealed an extensive identity, ranging from 89% to 94% for NOR-1α and 87% for NOR-1β. The highest identity (100%) was found in the DNA binding domain. The leucine-zipper of the C-terminal region was conserved among the NGFI-B family members.

In Northern blot experiments, the pNOR-1α cDNA recognized two transcripts (5.7 kb and 4.0 kb) transiently induced by serum in both porcine (Figure 1D, left panel) and human coronary SMCs (Figure 1D). The induction of NOR-1 mRNAs did not require de novo protein synthesis and was overinduced by cycloheximide (Figure 1E), as previously described in nonvascular cells.

Figure 1. Identification of NOR-1 by mRNA-DD. A, Representative mRNA-DD analysis showing the induction by 10% human serum, 10 U/mL thrombin, and 5 nM PDGF (after 1 hour of stimulation) of band 2. B, Arrested versus stimulated porcine coronary SMC Northern blots using the differentially displayed cDNA (band 2) or c-fos as probes. rRNA indicates ribosomal RNA. C, Arrested versus stimulated (serum for 1 hour) porcine coronary SMC blot using the cDNA corresponding to band 2 (left) or pNOR-1α cDNA (right) as probes. D, Kinetics of NOR-1 induction by serum in human coronary SMCs. E, NOR-1 mRNA levels in human coronary SMCs stimulated with serum in the presence or absence of cycloheximide (CHX) for different time periods.

Effect of Growth Factors and Cytokines on NOR-1 Expression

Serum, PDGF, and thrombin were the strongest inducers of NOR-1 (Figure 2). EGF and IGF (only detectable in overexposed films) also induced NOR-1. PDGF and thrombin synergize with IGF-1 to induce NOR-1, although, in a lesser extent than c-fos. The effect of other mitogens and cytokines, including lipopolysaccharide and angiotensin II (data not shown), on NOR-1 expression was negligible.
Signaling Pathways Involved in NOR-1 Induction

The induction of NOR-1 is dependent on protein kinase C (PKC) activation: it was induced by PMA (a PKC activator), whereas GF-109203X (a PKC inhibitor) inhibited NOR-1 expression induced by serum (Figure 3A). NOR-1 upregulation is also dependent on Ca\(^{2+}\)/H\(^{1001}\) mobilization: A23187 (a calcium ionophore) induced NOR-1, whereas the calcium chelator EGTA inhibited its expression. Specific inhibitors of both G-protein receptors (pertussis toxin) and the mitogen-activated protein kinase (MAPK) kinase (PD98059) inhibited the induction of NOR-1 produced by serum (Figure 3A). Pertussis toxin completely abolished the induction of NOR-1 produced by thrombin (data not shown).

By EMSA, we observed an increase in the binding of nuclear extracts from serum-treated VSMCs to a probe containing a consensus NBRE (a NOR-1 response element) that was prevented when PKC activation (by GF109203X) or Ca\(^{2+}\) mobilization (by BAPTA) was inhibited (Figure 3B).

Involvement of CREB in NOR-1 Induction

In serum induced VSMCs, CREB was activated by phosphorylation in Ser\(^{133}\) (Figure 4A) and nuclear extracts from human VSMCs bind to a probe (Nor3CRE) containing the three putative CRE sites present in NOR-1 promoter (from −84 to −41)\(^{12}\) (Figure 4B). The effect was specific (competed by an excess of cold-probe) and was supershifted by an antibody against CREB but not by anti-c-Fos or anti-c-Jun antibodies.

The involvement of CRE sites in the upregulation of NOR-1 by serum was analyzed in NIH-3T3 cells. As in human VSMCs, in NIH3T3, serum-induced NOR-1 expression was prevented when PKC activation or Ca\(^{2+}\) mobilization was inhibited (Figure 4C). In transfection assays, the activity of NOR-1 promoter was prevented by either BAPTA (a calcium chelator) or GF109203X and was completely abolished when the three CRE sites were mutated (Figure 4D).

Expression of NOR-1 in Adult Tissues

Heart and skeletal muscle express high levels of NOR-1, whereas low levels are detected in thoracic aorta, lung, and adrenal glands, and marginal expression (only detected in overexposed films) in the rest of porcine tissues analyzed (Figure 5). The expression pattern of Nur77, the main member of NGFI-B family, was similar to that of NOR-1. However, Nur77 exhibited lower expression than NOR-1 in myocardium (relative to that found in skeletal muscle) but higher in diaphragm, thoracic aorta, lung, and in adrenal glands.

Expression of NOR-1 in Human Arteries

NOR-1 mRNA levels in human nonatherosclerotic vessels (both in coronary arteries and aorta) from either DIC or CAD patients were low or undetectable (Figure 6A). NOR-1 was overexpressed in primary atherosclerotic lesions from CAD...
patients. Nur77 expression, which was detected in aorta but not in coronary arteries from DIC patients, was upregulated in primary coronary atherosclerotic lesions from CAD patients but to a lesser extent than NOR.

Expression of NOR-1 in Porcine Balloon Dilated Arteries
Percutaneous transluminal coronary angioplasty (PTCA) transiently upregulates NOR-1 mRNA in porcine coronary arteries with a pattern similar to that observed in VSMCs in culture (Figure 6B). No expression of Nur77 could be detected. By in situ RT-PCR, significant expression of NOR-1 was detected in medial SMCs of the dilated arteries (Figure 6C).

Antisense NOR-1 ODNs Inhibit DNA Synthesis and Cell Cycle Progression
Antisense sequences (AS-NOR) were directed against the two putative ATG initiation codons of the human NOR-1.17,18 Both antisense NOR-1 ODNs efficiently inhibited [3H]thymidine uptake by human coronary SMC in a dose-dependent manner. Figure 7A shows the effect for AS1-NOR. No effect was produced by sense NOR-1 ODNs. A similar inhibitory effect was produced by increasing concentrations of an antisense ODNs against c-fos (data not shown), previously used to assess the involvement of this protooncogene in cell proliferation.15 NOR-1 mRNA levels in AS-NOR ODN-treated cells were significantly lower than in control cells or cells treated with either sense NOR sequences or anti-c-fos ODNs (Figure 7B).

Flow cytometry analysis of cell cycle phase distribution revealed a significant reduction in cell entry in S phase after serum stimulation (40% and 42% by AS1- and AS2-NOR, respectively) and a concomitant increase in cells in G1 phase. Figure 7C shows representative FACS profiles corresponding to human VSMCs with and without AS1-NOR. Sense NOR-1 ODNs did not produce any effect on cell cycle distribution. A similar effect was produced by antisense c-fos ODNs (41% reduction in cell entry in S and similar increase in cells in G1 phase).

Antisense NOR ODNs Inhibit Human SMC Growth After Injury
In a well-established in vitro model of wounding16 AS1- or AS2-NOR significantly inhibited VSMC regrowth into the
denuded zone by 45% and 46% respectively (Figure 7D). In contrast, equivalent concentration of sense NOR-1 ODNs failed to modulate this process. The effect produced by antisense ODNs against NOR-1 was similar to that produced by the ODNs against c-fos. Figure 7E shows a representative picture from human VSMCs subjected to this procedure in the presence of AS1-NOR-1.

Discussion
In this study, we used mRNA-DD analysis in a search for genes induced by mitogens in VSMCs using the porcine model, a highly recognized model of human resemblance for studying advanced SMC differentiation and atherosclerosis.22 We have identified NOR-1 as an immediate-early gene in VSMCs. NOR-1 was transiently upregulated by angioplasty in porcine coronary arteries and was overexpressed in human atherosclerotic primary lesions. Antisense NOR-1 ODNs inhibited VSMC proliferation and wound healing after mechanical injury.

Two NOR-1 isoforms were cloned from porcine VSMCs. NOR-1 is an immediate-early gene that was strongly induced in VSMCs by mitogenic stimuli signaling through pathways involving tyrosine kinase receptors (PDGF and EGF) and G protein–coupled receptors (thrombin). In contrast, other agents such as cytokines that induced NOR-1 in gingival fibroblasts,23 or angiotensin II that induced NOR-1 in fasciculata cells,24 did not induce NOR-1 in VSMCs. Intracellular calcium increase as well as PKC and MAPK activation pathways, commonly activated in cell migration and proliferation, are involved in NOR-1 induction in VSMCs. In this regard, mitogens that use more than one pathway (ie, serum) had the strongest effect on NOR-1 induction, and the association of PDGF or thrombin with IGF-I increased the effect produced by each molecule alone. Therefore, NOR-1 could play a key role integrating different signaling pathways in

\( \mu \text{mol/L oligonucleotides}. \) Cell numbers in the denuded zone were counted and represented histodiagrammatically (n=3 experiments performed in triplicate). E, Representative data corresponding to AS1-NOR–treated cells and Controls. Arrows indicate the wound edge; ODNs, oligonucleotides; AS-, antisense; SE- sense. *P<0.05 vs controls or cells treated with the corresponding sense oligonucleotides.
VSMCs, in processes such as thrombosis, which produces platelet activation/degranulation (releasing PDGF and EGF) and thrombin generation.

Transfection experiments indicate a key role for the CRE motifs present in NOR-1 promoter in NOR-1 upregulation produced by mitogenic stimuli. Indeed, CREB activation is a common output of both PKC- and calcium-dependent signaling pathways,25 the main pathways involved in NOR-1 upregulation. Serum induced CREB phosphorylation, although no changes in CREB binding were observed, according with the ability of CREB to constitutively bind to its response element.25

Although NOR-1 is transiently induced by mitogenic stimuli in cell culture, significant expression of NOR-1 was detected in several adult tissues, in particular in myocardium and skeletal muscle from both porcine and humans (data not shown), suggesting that in these muscle tissues NOR-1 could regulate genes involved in key housekeeping functions. The overlapping expression patterns of NGFI-B family genes in certain tissues and cells, the high homology among their amino acid sequences, and the ability of the three receptors to bind to a specific NGFI-B response element (NBRE)26 could explain the functional redundancy observed in certain systems.27 However, we observed dissimilar expression patterns in adult tissues and in the vascular wall under pathological conditions. Indeed, although in human primary atherosclerotic lesions NOR-1 and Nur77 were overexpressed, revascularization procedures that injure the vessel wall and induce VSMC proliferation transiently upregulated NOR-1 but not Nur77.

NGFI-B family genes have been involved in different cell functions, among them cell proliferation, cell differentiation, and cell apoptosis.9 It should be stressed that similar nonspecific roles have been reported for other early genes such as c-fos or c-myc.28,29 In this regard, it is likely that the particular outcome of NOR-1 activation will depend on the concomitant alterations in the expression of other genes taken place in a particular physiological context. In fact, the relationship between eukaryotic transcription factors, in particular the paradigmatic heterodimerization between nuclear receptors,30 increase the complexity of molecular networks that operate in vascular cells. Nur77 but not NOR-1 can form heterodimers with RXR,31 enabling it to activate transcription of vascular genes in a ligand-dependent manner via the retinoic acid response elements. Because RXR has been directly involved in VSMC phenotypic modulation and in atherogenesis,5,32 and it is a common partner for other nuclear receptors that work in vascular cells such as PPARs or RAR,15,33 a potential competition of these alternative RXR heterodimeric partners for RXR should not be excluded. This interference/synergy relationship among nuclear receptors themselves or among other transcription factors could be important in the regulation of vascular cell function.33

The expression pattern of NOR-1 is consistent with a role for this transcription factor in the molecular mechanisms underlying accelerated and spontaneous atherosclerosis. The nature of stimuli leading to NOR-1 upregulation, both in vitro and in vivo, and the intracellular signaling involved in such induction strongly suggest a role for NOR-1 in VSMC proliferation. To further support this hypothesis, we analyze the effect of two sets of antisense ODNs directed against NOR-1. Antisense NOR-1 ODNs significantly inhibited human coronary SMC proliferation (reduced de novo DNA synthesis, cell cycle progression, and VSMC wound repair). The effectiveness of antisense NOR-1 ODNs blocking these processes was similar to that produced by antisense ODNs against the well characterized protooncogene c-fos, which like NOR-1 is transiently upregulated in VSMCs and in the porcine coronary arteries after PTCA.9,28,34 We selected c-fos as a comparative partner because a proportional relationship between the degree of c-fos expression and VSMC proliferation has been established both in vivo35 and in vitro,9 and antisense ODNs against c-fos have been successfully used to inhibit cell proliferation in different systems including VSMCs.15,36 Our results strongly suggest a role for NOR-1 in VSMC growth, in agreement with different lines of evidence from other authors supporting a role for this orphan nuclear receptor in cell proliferation in different systems. Indeed, NOR-1 is essential for proliferation of the semicircular canals of the inner ear as has been recently demonstrated in NOR-1 knockout mice.37 In addition, among the NGFI-B genes only the oncogenic conversion of NOR-1 has been described.38

In summary, the list of nuclear receptors potentially involved in atherosclerosis and in CAD is growing, as a result of their major function regulating lipid metabolism39 or as players directly involved in vascular biology. In contrast to other nuclear receptors, NOR-1 expression is rapidly and highly modulated in VSMCs in response to mitogenic stimuli. In fact, recently a wide screening of genes induced by serum revealed that NOR-1 is a major serum-responsive gene in human fibroblasts.40 Although the identification of the genes downstream regulated by NOR-1 will be necessary to better understand the mechanism underlying its involvement in atherogenic processes, our results reveal NOR-1 as a potential regulatory factor in vascular cell function and a new target in future strategies for vascular drug discovery.

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