Involvement of Neuron-Derived Orphan Receptor-1 (NOR-1) in LDL-induced Mitogenic Stimulus in Vascular Smooth Muscle Cells: Role of CREB

Jordi Rius, José Martínez-González, Javier Crespo, Lina Badimon

Objective—Low density lipoproteins (LDLs) modulate the expression of key genes involved in atherogenesis. Recently, we have shown that the transcription factor neuron-derived orphan receptor-1 (NOR-1) is involved in vascular smooth muscle cell (VSMC) proliferation. Our aim was to analyze whether NOR-1 is involved in LDL-induced mitogenic effects in VSMCs.

Methods and Results—LDL induced NOR-1 expression in a time- and dose-dependent manner. Antisense oligonucleotides against NOR-1 inhibit DNA synthesis induced by LDL in VSMCs as efficiently as antisense against the protooncogene c-fos. The upregulation of NOR-1 mRNA levels by LDL involves pertussis-sensitive G protein–coupled receptors, Ca²⁺ mobilization, protein kinases A (PKA) and C (PKC) activation, and mitogen-activated protein kinase pathways (MAPK) (p44/p42 and p38). LDL promotes cAMP response element binding protein (CREB) activation (phosphorylation in Ser133), and in transfection assays a dominant-negative of CREB that inhibits NOR-1 promoter activity, while mutation of specific (cAMP response element) CRE sites in the NOR-1 promoter abolishes LDL-induced NOR-1 promoter activity.

Conclusions—In VSMCs, LDL-induced mitogenesis involves NOR-1 upregulation through a CREB-dependent mechanism. CREB could play a role in the modulation by LDL of key genes (containing CRE sites) involved in atherogenesis.

Key Words: atherosclerosis ■ lipoproteins ■ smooth muscle cells ■ gene expression ■ proliferation

Vascular smooth muscle cell (VSMC) migration and proliferation play key roles in the pathophysiology of vascular remodeling associated with atherosclerotic diseases. Low density lipoproteins (LDLs) are key factors in the onset and development of atherosclerosis, their effects on VSMCs have been investigated. Indeed, LDLs induce VSMC mitogenesis, increase intracellular free calcium concentration, activate mitogen-activated protein kinases (MAPK), and regulate the expression and activity of different transcription factors, including c-fos, egr-1, and sterol regulatory element binding protein-2 (SREBP-2). Recently, 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 Nurr1, forms the growth factor–induced protein-B (NGFI-B) family of orphan nuclear receptors within the steroid/thyroid receptor superfamily. These genes have been involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic stimuli in different cell types. NOR-1 is upregulated by coronary angioplasty, and both NOR-1 and Nur77 are overexpressed in atherosclerotic lesions from patients with coronary artery disease (CAD).

In the present study, we show that NOR-1 expression is transiently induced by LDL and is linked to LDL-induced VSMC mitogenic effects. NOR-1 induction by LDL involved pertussis-sensitive G protein–coupled receptors, Ca²⁺ mobilization, the activation of different protein kinases [protein kinases A (PKA) and C (PKC) and MAPK (p44/p42 and p38)], and cAMP response element binding protein (CREB) activation. Finally, analysis of NOR-1 promoter activity revealed a key role of cAMP response elements (CRE) in the upregulation of NOR-1 by LDL. These results suggest that NOR-1 may play a key role in the molecular mechanisms underlying VSMC activation by LDL.

Methods

Lipoprotein Isolation

Human LDLs and very low density lipoproteins (VLDLs) were isolated from pooled sera of healthy blood donors of the Barcelona area as described. The content of protein (BCA protein assay, Pierce) and cholesterol (Cholesterol assay kit, RefLab) in the lipoproteins was determined by colorimetric assays. The absence of contamination by other lipoproteins was determined by electrophoresis on agarose gels (Paragon Electrophoresis kit, Beckman Lipoproteins). Lipoproteins were endotoxin-free, as determined by the
Amplification was carried out by 24 cycles: denaturation at 94°C for 1 minute; and polymerization, 72°C.

Results.16

Alkaline phosphatase, and proprietary chemiluminescent substrate cleic acids was performed with an antidigoxigenin antibody linked to Blots were UV cross-linked. Detection of digoxigenin-labeled nu-

Electrophoretic Mobility Shift Assay Nuclear extracts (3 μg) from VSMCs and a double-stranded probe corresponding to the sequence of human NOR-1 promoter (from −34 to −41; Nor/3CRE) containing three putative CRE motifs were used in an electrophoretic mobility shift assay (EMSA) as described.16 Supershift experiments were performed with antibodies against CREB, ATF-2, c-FOS, and c-JUN (Santa Cruz Biotechnology).

Construction of NOR-1 Promoter Plasmids The plasmid pNORα −1703 containing the human NOR-1 promoter (from −1703 to +264)23 was kindly provided by Dr N. Ohkura (Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan). CRE motifs were mutated by site-directed mutagen-

RT-PCR Total RNA from VSMCs was isolated as indicated above, was reverse-transcribed, and NOR-1 mRNA levels were analyzed by polymerase chain reaction (PCR) using the PCR DIG Labeling Mix (Roche Molecular Biochemicals) as described.16 The specific NOR-1 oligonucleotides used were for human, 5'-AGGC-TGCAAGGGGTTTTCAGGAGA-3' and 5'-TGCTTTTCTACGAGACCTGT-3', and for rat, 5'-AGGGCATGAGATCGGCGTGC-3' and 5'-TCTTTCAGAAGCTCTG-3'. Amplification was carried out by 24 cycles: denaturation at 94°C for 30 seconds; annealing, 61°C for 1 minute; and polymerization, 72°C for 1 minute 30 seconds. PCR products were resolved by electro-

Transient Transfection and Luciferase Assays Rat VSMCs were transfected with luciferase expression vectors (wild pNORα −1703 or with constructs mutated in single CRE boxes) using Lipofectamine Reagent (Invitrogen) and Plus Reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were arrested for 48 hours and were then stimulated with LDL for 4 hours. Luciferase activity was measured in cell lysates using Luciferase assay Kit (Promega). pSVβ-gal (Promega) was used as an internal control. In cotransfection assays a CREB dominant-negative (Clontech) was used.

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

LDL Induces NOR-1 Expression in VSMC

To assess the effect of native LDL on NOR-1 expression in VSMC, arrested cells were stimulated with increasing concentrations of LDL, and NOR-1 mRNA levels were analyzed by Northern blot. LDL induced NOR-1 expression in a dose-dependent manner (Figure 1A). The effect produced on NOR-1 upregulation by the highest tested concentrations of VLDL and oxLDL (10 mg/dL) was lower than that of equivalent amount of native LDL. The effect of LDL (30 to 60 mg/dL) was similar to that of serum. NOR-1 expression was transiently induced by LDL following the same pattern as that of serum-induced cells, reaching peak at 1 hour and decreasing to undetectable levels 8 hours after stimulus (Figure 1B).

Antisense NOR-1 ODNs Inhibit LDL-Induced DNA Synthesis

NOR-1 upregulation by LDL was significantly reduced when human VSMCs were treated with antisense ODNs targeted against NOR-1 (AS-NOR-1), while the corresponding sense sequences (SE-NOR-1) did not produce any effect (Figure 2A). In these conditions LDL-induced VSMC DNA synthesis was significantly inhibited by AS-NOR-1 but not by SE-NOR-1 (Figure 2B). The effect of AS-NOR-1 was similar to that of antisense against c-fos (AS-FOS), a well-known gene involved in cell proliferation. Finally, neither mismatches AS-NOR-1 nor random ODNs inhibited DNA synthesis in human VSMCs stimulated with LDL.

Signaling Pathways Involved in NOR-1 Induction

LDLs activate different signal transduction pathways in VSMCs. To address which of these pathways were involved in NOR-1 induction, we used specific inhibitors of different pathways. Results shown in Figure 3A indicate that NOR-1 upregulation by LDL was dependent on the activation of Gi/o-proteins (inhibited by pertussis toxin), calcium mobilization [inhibited by a calcium chelator (BAPTA-AM)], and PKC activation (inhibited by GF-109203X, a PKC inhibitor). NOR-1 induction by LDL was also dependent on p44/p42 MAPK (inhibited by PD98059) and p38 MAPK activation (inhibited by SB203580) (Figure 3B). Finally, H-89 (a PKA inhibitor) also inhibited the LDL effect.

LDL Induces CREB Phosphorylation

Because CREB activation is a common output of the signal transduction pathways involved in NOR-1 upregulation, we analyzed the effect of LDL on CREB activation (phosphorylation in Ser133). Figure 4 shows that LDL induced early activation of CREB that was prevented by either BAPTA-AM or GF-109203X, two compounds that reduced LDL-induced NOR-1 expression. Thus, CREB could play a major role in NOR-1 upregulation by LDL.

Figure 1. LDL induced NOR-1 expression in human VSMCs. A, Northern blot showing NOR-1 expression in human coronary SMC exposed to increasing concentrations of LDL and serum (10%), and to 10 mg protein/dL of VLDL, LDL, or oxLDL. B, VSMCs were stimulated with LDL (30 mg/dL) at different times. Control: noninduced cells. Blots are representative of three independent experiments. rRNA indicates ribosomal RNA.
CREB Binds the CRE Sites Present in the NOR-1 Promoter

In gel shift assays, nuclear extracts from human VSMCs bind to a probe (Nor/3CRE) containing the three putative CRE sites present in NOR-1 promoter (from –83 to –42). Although LDL induced CREB phosphorylation, no changes in CREB binding were observed, in accordance with the ability of CREB to constitutively bind to its response element. The binding was specific (completed by an excess of cold-probe) and was supershifted by an antibody against CREB but not by anti–ATF-2, anti–c-Fos or anti–c-Jun antibodies.

CRE Sites Regulate NOR-1 Induction by LDL

In order to analyze the role of CRE sites in LDL-induced NOR-1 expression we transfected rat VSMCs. As in human VSMCs, LDLs in rat cells induced NOR-1 expression and both a calcium chelator (BAPTA-AM) and a PKC inhibitor (GF-109203X) prevented this effect (Figure II, available online at http://atvb.ahajournals.org). In transfection assays, LDLs induced NOR-1 promoter activity (∼4-fold over unstimulated cells), while either BAPTA-AM or GF-109203X inhibited such effect (Figure 5B).

The activity of the wild-type NOR-1 promoter was prevented by cotransfection with a dominant-negative of CREB (CREBβ). Finally, by site-direct mutagenesis we showed that when the CRE1 site was mutated, NOR-1 promoter activity decreased up to 35%, while the mutation of either CRE2 or CRE3 almost abolished NOR-1 promoter activity.

Discussion

Hypercholesterolemia is one of the most important risk factors in atherogenesis. LDL induces changes in vascular gene expression, leading to alterations in vascular function and promoting growth-related events; however, the transcription factors involved in LDL mitogenic effects have not...
been completely elucidated. In the present study, we show that LDL upregulates NOR-1, an orphan receptor involved in VSMC proliferation through a complex mechanism involving multiple signaling pathways and CREB activation. NOR-1 is a member of the NGFI-B family of orphan nuclear receptors. These genes are involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic stimuli in different cell types.

**Figure 3.** Signaling pathways involved in NOR-1 induction. VSMCs were pretreated with different signal pathway inhibitors, stimulated with LDL (30 mg/dL) for 1 hour, and NOR-1 mRNA levels were analyzed by using RT-PCR. A, Effect of pertussis toxin (PTX, 1 or 10 ng/mL), BAPTA-AM (10 or 50 μmol/L), or GF-109203X (GF, 5 or 15 μmol/L) on LDL-induced NOR-1 mRNA levels. B, Effect of PD98059 (PD, 10 or 50 μmol/L), SB 203580 (SB, 2 or 10 μmol/L), or of H-89 (1 or 5 μmol/L) on LDL-induced NOR-1 mRNA levels. Control: non-induced cells. Representative blots (n=3) are shown.

**Figure 4.** CREB activation by LDL in VSMC. A, Western blot showing CREB activation (phosphorylation in Ser133) induced by LDL (30 mg/dL) at different times. B, Western blot showing the preventive effect of BAPTA-AM (BAPTA, 50 μmol/L) or GF-109203X (GF, 15 μmol/L) on CREB activation produced by LDL treatment (for 10 minutes). Representative blots (n=3) are shown.
NOR-1 is essential for proliferation of the semicircular canals of the inner ear as has been recently demonstrated in NOR-1 knockout mice;27 NOR-1 also modulates VSMC response to injury in vitro and is induced by coronary angioplasty in vivo,16 a condition that promotes VSMC proliferation.25 Here, we show that LDLs induce NOR-1 expression in VSMCs through a complex network of signal transduction pathways, including pertussis-sensitive G proteins, Ca²⁺ mobilization, and the activation of PKA, PKC, and MAPK (p44/p42 and p38), pathways commonly activated in cell migration and proliferation.28,29 The inhibition of one of these apparently redundant pathways, in particular PKC and calcium mobilization, completely prevents NOR-1 upregulation promoted by LDL, in accordance with the major role of these pathways in native LDL-induced vascular cell proliferation.7,30

We previously showed that growth factors such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) are poor inducers of NOR-1, while serum is a strong inducer of NOR-1, acting through a complex web of signaling pathways.16 By nature LDLs are highly complex molecules different from growth factors such as PDGF, which promotes early gene induction and cell growth acting through its canonical PDGF receptor. Indeed, LDLs regulate gene transcription through mechanisms depending on their classical receptor (LDL-R)14,15 and on other uncharacterized receptors including G protein–coupled receptors.11 In fact, the “mitogenic” properties of LDLs seem to be related to their dual capability to induce early cell cycle events and to provide cholesterol that cells need to resume mitosis.8–10,31 The cross-talk between pathways could explain the diversity of second messengers and protein kinases involved in the upregulation of NOR-1 by LDL. Although all these pathways can potentially lead to CREB phosphorylation,26 their specific contribution could vary depending on different factors, including cell type. In any case, antisense ODN against NOR-1 inhibited LDL-induced mitogenic effect induced by LDL in VSMCs as efficiently as an antisense ODN against

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**Figure 5.** CRE motifs regulate NOR-1 induction by LDL. A; Structure of the NOR-1 promoter; the three CRE motifs present in positions from −83 to −42 are shown. CRE sites are indicated in bold, and the bases mutated are boxed. B, LDL increased NOR-1 promoter activity in cells transfected with the wild-type promoter (pNOR−1703); this effect was prevented by either BAPTA-AM or GF-109203X. NOR-1 promoter activity was also prevented by either cotransfection with a dominant negative of CREB (CREB133*) or by mutation on CRE sites (mtCRE1, mtCRE2, or mtCRE3). The black bars indicate LDL-treated cells, and the white bars indicate untreated cells. Results represent the mean ± SEM (n = 3 independent experiments performed in triplicate). P < 0.05; *, versus LDL untreated cells; †, versus cells transfected with only wild-type promoter and treated with LDL alone.
the protooncogene c-fos, a well known gene involved in VSMC proliferation.\textsuperscript{21,22,25} Therefore, NOR-1 could play a key role integrating signaling pathways involved in LDL-triggered VSMC induction.

Recent work from independent groups has demonstrated the induction of members of the NGFI-B gene family in vascular cells.\textsuperscript{19,32,33} Nur77 is induced in rat VSMCs under apoptotic stimulus,\textsuperscript{32} and its overexpression prevents vascular lesion formation in a mouse model of vascular injury.\textsuperscript{19} Although NOR-1, Nur77, and NurR1 are coexpressed in many tissues, they exhibit dissimilar abilities to bind to DNA response elements, which could lead to important functional differences. Indeed, Nur77 and NurR1, but not NOR-1, can form heterodimers with the retinoid X receptor (RXR),\textsuperscript{34} enabling it to activate transcription of vascular genes in a ligand-dependent manner via the retinoic acid response elements, and interestingly, RXR ligands exert antiproliferative effects on VSMCs.\textsuperscript{35} Finally, although NOR-1, Nur77, and NurR1 are closely related orphan receptors, their induction patterns in VSMCs are not coincident; in this regard, conditioned medium from macrophages exposed to oxLDL strongly induce Nur77 in VSMCs, but had minor effect on NOR-1 expression.\textsuperscript{19}

Because the pathways induced by LDL in our study shared CREB as a common output,\textsuperscript{26,28,36} we analyzed the role of CRE motifs present in NOR-1 promoter in the induction of NOR-1 by LDL. We show, for the first time, the LDL early induction of CREB phosphorylation in Ser\textsuperscript{133}, a key residue for CREB activity.\textsuperscript{26,37} By EMSA, we show that CREB binds to the CRE sites close to the transcription initiation point of NOR-1.\textsuperscript{23} LDLS did not change basal CRE binding; in accordance with the ability of CREB to constitutively bind to its response element,\textsuperscript{26} however, Ser\textsuperscript{133} phosphorylation promotes CREB activation via recruitment of CREB-binding protein (CBP)\textsuperscript{26,37}

Transfection experiments indicate a key role for the CRE motifs present in the NOR-1 promoter for NOR-1 upregulation by LDL. Indeed, we show that cotransfection with a dominant negative of CREB (CREB with a single mutation in Ser\textsuperscript{133}), which binds to CRE sequences but cannot be activated,\textsuperscript{37} prevented the induction of NOR-1 promoter activity by LDL. In addition, when CRE2 or CRE3 sites were mutated, the promoter activity fell down to basal levels, in accordance with previous results in nonvascular cells induced by the overexpression of a constitutive active form of calcium/calmodulin kinase kinase (CaM-KK).\textsuperscript{24}

Therefore, CREB activation seems to be key in LDL-induced NOR-1 upregulation. The activation of CREB by LDL could be relevant beyond NOR-1 upregulation because recent papers argue for a main role of this transcription factor in VSMC survival/proliferation and vascular remodeling processes,\textsuperscript{38} and CRE sites seem to be key in cyclin A and D1 transcriptional regulation.\textsuperscript{39,40} In fact, we show that VLDLs, lipoproteins that also activate CREB and promote vascular cell proliferation,\textsuperscript{8,9,41} also upregulate NOR-1 expression. Finally, oxLDL, a well-known inducer of VSMC proliferation through a general induction of cell cycle proteins mainly involving reactive oxygen species and the transcription factor nuclear factor κB (NF-κB),\textsuperscript{42–44} had only a minor effect on NOR-1 upregulation.

In summary, circulating LDL levels could modulate vascular function through complex gene programs involving downstream genes regulated by CREB, among them NOR-1. Because NOR-1 expression is rapidly and highly induced in VSMCs exposed to stimuli such as LDL, NOR-1 could be regarded as a new target in both molecular and pharmacological approaches to modulate VSMC function.

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