Native LDL Induces Proliferation of Human Vascular Smooth Muscle Cells via Redox-Mediated Activation of ERK 1/2 Mitogen-Activated Protein Kinases

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Abstract—This study investigated mechanisms underlying native low-density lipoprotein (LDL)-stimulated proliferation of human vascular smooth muscle cells (VSMC). Experiments were performed to determine whether native LDL affects reactive oxygen species (ROS) formation and activity of extracellular signal-regulated kinase 1/2 (ERK1/2), and whether redox-sensitive pathways contribute to LDL-induced cell proliferation. Native LDL (100 μg/mL, 24 hours) increased cell proliferation (to 303 to 388% of control, P<0.0001) as determined by [methyl-3H] thymidine incorporation. This effect was completely blocked either by the antioxidants N-acetylcysteine, Tiron, or nordihydroguaiaretic acid; the flavin-inhibitor diphenylene iodonium; or superoxide dismutase (all P<0.0001), and partly blocked by ERK-inhibitor PD98059 or meclofenamate (P<0.01). Exposure of VSMC to native LDL for 20 minutes stimulated ROS formation, measured by dichlorodihydrofluorescein oxidation, and increased ERK1/2 activity by 3.1-fold (P<0.001). The latter effect was sensitive to MEK1/2 inhibitor PD98059 and Tiron (P<0.001), and in part to N-acetylcysteine or diphenylene iodonium (P<0.05). These results demonstrate that native LDL induces acute formation of ROS and subsequent activation of redox-sensitive ERK 1/2 mitogen-activated protein kinases, pathways that appear to be important for mitogenic signaling of native LDL in human vascular smooth muscle cells. (Hypertension. 2002;39[part 2]:645-650.)

Key Words: antioxidants ■ atherosclerosis ■ kinase ■ lipoproteins ■ muscle, smooth, vascular

Vascular smooth muscle cell migration from the media to the intima and subsequent proliferation importantly contributes to atherogenesis.1 Elevated plasma levels of low-density lipoprotein (LDL) cholesterol are associated with a risk for atherosclerosis,2–4 which is now believed to depend on chronic inflammatory processes leading to development of lesions characterized by vascular smooth muscle cells (VSMC), macrophages, lipids, T cells, and fibrous material.1 The mechanisms by which native LDL is thought to contribute to atherogenesis include uptake into the subendothelial space and subsequent oxidation by macrophages.5,6 In vitro, oxidized LDL has been shown to induce proinflammatory cytokines, vascular cell proliferation, and apoptosis in vitro.7,8 Moreover, most recent studies indicate that macrophage may also inhibit the oxidation of LDL (reviewed in Baoutina et al9).

Little is known about the mechanisms by which non-oxidized, native LDL affects vascular homeostasis. Previous studies have shown that native LDL induces vasoconstriction,10 impairment of endothelium-dependent relaxation,11,12 changes in intracellular calcium flux,13 and VSMC proliferation.14,15 Since the effects evoked by native LDL can be blocked by compounds inhibiting different intracellular pathways, even in the absence of the classical LDL receptor,16 it is possible that LDL signals via a rather nonspecific second messenger, such as modulation of the cellular redox balance.

Redox signaling provides an important regulatory system for VSMC growth and hypertrophy,17 involving stimulation of growth-promoting enzymes such as extracellular signal-regulated kinases (ERK) 1/2. Atherosclerosis is associated with increased intracellular oxidative stress17 as well as expression of ERK1/2,18 an important regulator of cell growth.19 Several substances which are believed to play a role in atherogenesis such as thrombin, angiotensin, or oxidized LDL, stimulate the release of reactive oxygen species (ROS) and growth of VSMC (reviewed in Thannickal and Fanburg, Griendling et al, and Berk20–22). In this study, we investigated through which mechanisms non-oxidized, native LDL exerts its effects on human vascular smooth muscle cells.

Materials and Methods

Low-Density Lipoproteins
Native low-density lipoprotein (LDL) was isolated from pooled human EDTA plasma obtained from healthy blood donors. Follow-
ing ultracentrifugation of plasma in the presence of 1 mmol/L EDTA in a KBr gradient at 200,000g for 14 hours at 4°C, the LDL fraction was decanted and dialyzed at 4°C for 24 hours against 3 changes of 1.5 L of 150 mmol/L of NaCl and 0.25 mmol/L EDTA (pH 7.4). Subsequently, LDL was concentrated (2 mg/mL, Centrisart® separation tubes), sterilized (0.22 μm filters, Millipore®), and stored in the dark at 4°C. Concentrations of LDL are given as μg LDL protein, measured by the bicinchoninic acid method. Native VSMC prepared according this method has no effects on endothelial cell apoptosis or protein expression in contrast to minimally oxidized or oxidized LDL, suggesting distinct mechanisms of action. Incubation of LDL in phenol-red free DMEM containing Cu²⁺ (10 μmol/L) for 24 hours was used to generate oxidized LDL. To determine whether exposure to VSMC oxidatively modifies native LDL in our experimental set-up, native LDL (100 μg/mL) was incubated either in DMEM (devoid of Fe²⁺ and Cu²⁺) or incubation medium (DMEM/HAM’s-F10, 1:1, vol/vol, and 0.1% FCS) in the absence or presence of VSMC at 37°C for 24 hours. The reaction was terminated with EDTA (5 mmol/L) and butyldihydroxytoluol (5 μg/mL), and 3 μL of sample (0.3 μg LDL) were subjected to agarose gel electrophoresis, subseuent FatRed® 7B staining (Titan; Helena BioSciences), and imaging with a GelDoc® system (Biorad).

**Human Vascular Smooth Muscle Cells**

Human VSMC were freshly isolated from human umbilical cord veins (n=11) using explant technique and cultured in Promocoll® smooth muscle cell growth medium (Gibco) including 5% FCS. Cells were passaged by treatment with 0.05% trypsin/0.02% EDTA (pH 7.4) and 5% FCS. Subconfluent cells from passages 3 to 6 were used. Cells were analyzed for smooth muscle cell-specific α-actin by immunofluorescence yielding >98% of α-actin positive cells.

**Formation of Reactive Oxygen Species (ROS)**

Serum-starved VSMC (0.1% FCS) were incubated with native LDL (100 μg/mL) or oxidized LDL (100 μg/mL) for 20 minutes, 60 minutes, and 240 minutes, and ROS formation was subsequently measured by dichlorodihydrofluorescein (DCFH) oxidation. Dichlorofluorescein (DCF) fluorescence was determined using a Zeiss® fluorescence microscope (OpenLab, Improvision) connected to an imaging system. Fluorescence intensity was determined over the whole field of vision to avoid selection bias. Background fluorescence in the absence of cells was subtracted. The dichlorofluorescein method can be applied as a qualitative marker of intracellular ROS formation, including generation of ONOO⁻ and hydroxyl radical derived from H₂O₂ and O₂⁻.

**Extracellular Signal-Regulated Kinase1/2 Activity**

ERK1/2 activity was measured by in vitro phosphorylation of erk-1,31,32 VSMC starved for 24 hours (0.1% FCS) were stimulated with native LDL (100 μg/mL), 10 minutes in absence of presence of inhibitors (120 minutes preincubation). The reaction was terminated by rinsing cells (PBS, 4°C), lysed (buffered), and immunoprecipitated using antibodies against phosphorylated (activated) ERK1/2. ERK1/2 activity was measured by in vitro phosphorylation of erk-1 (p44/42 MAP kinase assay kit, Cell Signaling Technology®, New England Biolabs, Inc). In this assay, erk-1 is expressed in a recombinant fusion protein consisting of erk-1 residues 307 to 428 and glutathion-S-transferase (molecular weight 41kDa) as previously described.31 Protein was separated by 10% SDS/PAGE gel electrophoresis and phosphorylated erk-1 fusion protein was immunodetected using a phospho-specific antibody against erk-1. Bands of the phosphorylated erk-1 fusion protein (41kD) were detected by enhanced chemiluminescence (Phototope®, New England BioLabs Inc). Densitometric analysis of bands was performed using NIH Image® (version 1.6.1, National Institute of Health).

**Cell Proliferation**

VSMC serum-deprived for 24 hours (0.1% FCS) were stimulated with LDL (100 μg/mL) in the absence or presence of inhibitors.

**Results**

**Formation of Reactive Oxygen Species (ROS)**

Exposure of VSMC to native LDL (100 μg/mL) for 20 minutes significantly increased intracellular ROS formation as measured by DCF fluorescence (P<0.01 versus control) (Figure 1). The effect of native LDL on ROS formation was almost as potent as observed with oxLDL (Figure 1). Native LDL-induced ROS generation was further increased after 60 and 240 minutes (P<0.01 versus control, data not shown).

**Extracellular Signal-Regulated Kinase 1/2 Activity**

In unstimulated cells starved for 24h, bands for phosphorylated erk-1 were detected, suggesting basal activity of ERK1/2 (Figure 2). Bands were also detected in cells starved for up to 3 days, however the degree of erk-1 phosphorylation decreased with time (data not shown). Compared with control experiments in cells starved for 24h (n=5), exposure of VSMC to native LDL for 10 minutes caused a marked increase in ERK1/2 activity (n=5, P<0.001 versus control) (Figure 2). The inhibitor of ERK1/2 activation PD98059 (30 μmol/L, n=4, P<0.001) and the ROS scavenger Tiron (10 mmol/L, n=4) completely blocked ERK1/2 activity.
induced by native LDL (both P<0.001 versus LDL) (Figure 2), whereas the antioxidant N-acetylcysteine (NAC, 20 mmol/L, n=3) or an inhibitor of flavin-containing enzymes, diphenylene iodonium (DPI, 10 μmol/L, n=7) only in part inhibited ERK activity (by 47±4% and 33±4%, respectively; both P<0.05 versus LDL) (Figure 2).

Cell Proliferation
Native LDL (100 μg/mL, n=6 for each set of experiments, Figures 3A to 3D) caused a 3 to 4-fold increase in cell proliferation (P<0.0001 versus control, Figure 3). Native LDL-stimulated cell proliferation was unaffected by the peroxynitrite scavenger ebelsen (10 μmol/L, n=6, n.s.) or catalase (100 U/mL, n=6, n.s.) (data not shown). NAC (20 mmol/L) completely blocked native LDL-induced proliferation (n=6, P<0.05) (Figure 3A). The inhibitory effect was even more pronounced with DPI (10 μmol/L, n=6, P<0.05 versus LDL) (Figure 3B), or polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (500U/mL). PEG-SOD completely inhibited LDL-induced cell proliferation (control: 145±10 cpm/mg protein, n=6; native LDL: 875±55 cpm/mg protein, n=6; native LDL + PEG-SOD: 73±9 cpm/mg protein, n=6, P<0.05 versus control and P<0.0001 versus native LDL). LDL-induced proliferation was also blocked by nordihydroguaiaretic acid, a potent antioxidant that is also known to inhibit lipoxygenase (25 μmol/L, n=6, P<0.001 versus LDL) (Figure 3C). Cyclooxygenase inhibition (meclofenamate, 10 μmol/L, n=6, P<0.05 versus LDL) (Figure 3C), monensin, an inhibitor of endocytosis interfering with Golgi transport (1 μmol/L, n=6, P<0.05 versus LDL) (Figure 3D), and the MEK1/2 kinase inhibitor PD98059 (30 μmol/L, n=6, P<0.001 versus LDL) only in part inhibited cell proliferation.

Lactate Dehydrogenase Activity
Treatment for 24 hours had no significant effect on LDH activity in cells treated with LDL alone or in combination with meclofenamate, NAC, Tiron, PD98059, monensin, PEG-SOD, catalase, ebelsen or NDGA. However, there was a significant increase of LDH activity in cells treated with DPI for 24 hours in control medium (increase: 2.4-fold) and cells concomitantly exposed to LDL (2.8-fold, both P<0.0001).

VSMC and Electrophoretic Mobility of Native LDL
As shown in Figure 4, compared with cell-free medium (PBS, lane 2; DMEM, lane 3, and DMEM+HAM-F10, 1:1 vol/vol, lane 4), incubation of native LDL for 24 hours in the presence of VSMC in either DMEM (devoid of Cu2+/Fe2+, lane 5) or incubation medium (DMEM/HAM F10, 1:1 vol/vol, lane 6) had no effect on LDL mobility. Copper-induced oxidation of LDL (lane 1) resulted in a marked mobility shift compared with native LDL “0” indicates gel loading slots; “+” and “−” indicate polarity. Representative example of 4 independent experiments.
Discussion

This study demonstrates that non-oxidized, native LDL acutely stimulates the formation of reactive oxygen species (ROS) in cultured human VSMC. These rapid stimulatory effects of native LDL were only slightly less potent than those evoked by oxidized LDL, indicating that oxidation of native LDL is not a prerequisite to serve as a stimulus for ROS generation in human VSMC. In line with the rapid increase in ROS, we observed that activity of extracellular signal-regulated kinase (ERK) 1/2 was markedly augmented after a brief exposure to native LDL. Moreover, experiments revealed that VSMC proliferation stimulated by native LDL was sensitive to the \( \text{O}_2^- \) scavenger superoxide dismutase and to substances acting as intracellular antioxidants such as Tiron or N-acetylcysteine.

The finding that human VSMC increase intracellular ROS formation in response to non-oxidized, native LDL was unexpected, as was the rapid nature of ROS formation within minutes of exposure. It has been previously reported that in endothelial cells\(^ {37} \) and glomeruli,\(^ {27} \) thus in the presence of endothelial NO synthase, formation of ROS in response to native LDL was observed after 2 and 96 hours, respectively. Yet, particularly in endothelial cells, ROS appear to arise from endothelial NO synthase,\(^ {38} \) which cannot be a source in VSMC. As our experiments did not identify the source of ROS generated by native LDL, this limits the interpretation of our results. However, the pronounced inhibitory effect of cell-permeable superoxide dismutase and the lack of effect of either catalase or the peroxynitrite scavenger ebselen strongly suggests that the ROS mediating VSMC proliferation in response to native LDL is \( \text{O}_2^- \). Moreover, nonspecific scavengers of ROS, such as N-acetylcysteine or Tiron, were highly efficient in inhibiting VSMC growth. In addition, inhibition of pathways known to generate \( \text{O}_2^- \) as a signaling intermediate, such as cyclooxygenase or flavin-containing enzymes (which also include the vascular NADPH oxidase), blocked LDL-induced cell proliferation. Whether and which of the NADPH oxidase subunits are involved in the proliferative effects of native LDL and ERK activation is not known. Experiments in rat VSMC revealed that angiotensin II-induced ERK1/2 activation occurs independent of DPI treatment.\(^ {39} \) As, therefore, an NADPH oxidase-dependent ERK-activation is not likely established, other oxidases including lipoxygenase or cytochrome \( p450 \) monooxidase, which are also DPI sensitive,\(^ {40} \) could account for LDL-mediated ERK1/2 activation. Alternatively, the mode of activation of the NADPH oxidase by native LDL may differ from that observed in response to angiotensin II.

It is important to note that LDH activity measurements after 24 hours of treatment with the flavin-inhibitor diphenylene iodonium revealed increased activity in supernatants of both unstimulated and LDL-stimulated cells, suggesting that DPI-mediated effects involve cellular injury and/or apoptosis. Indeed, independent experiments, using four hours of incubation of endothelial as well as vascular smooth muscle cells with DPI, demonstrated cellular damage and paradoxical expression of redox-sensitive genes VEGF, MCP-1, and tissue factor (Brandes et al, unpublished observation, 2001). The finding that prolonged exposure is associated with cellular injury limits the use of DPI as an inhibitor of flavin oxidases for proliferation experiments.

Interestingly, as shown in Figure 2, inhibition of endocytosis by monensin, an inhibitor of Golgi-mediated endocytosis,\(^ {41} \) also inhibited native LDL-induced proliferation nearly as effectivel as inhibition of redox-sensitive mechanisms. This observation further supports the notion that endocytosis is a prerequisite for cellular activity of native LDL to occur.\(^ {42} \) Indeed, the LDL receptor family consists of a number of different cell surface endocytosis receptors that function in delivering their ligands to lysosomes for degradation.\(^ {42} \) Since native LDL-induced cellular signaling and growth occurs even in the absence of the classical LDL receptor,

\( ^{16,43,44} \) an endocytic pathway, distinct from the classical LDL receptor yet to be identified, may mediate the redox-sensitive, growth-promoting effects of native LDL.

We next investigated whether activity of ERK1/2, a redox-sensitive enzyme,\(^ {45} \) is affected by native LDL. Here we report that native LDL rapidly (i.e., within minutes) stimulates activity of ERK 1/2 in human VSMC. Interestingly, and in spite of the potent inhibitory effect on cell growth, treatment with different antioxidants did not uniformly affect ERK 1/2 activity. It is therefore possible that the inhibitory effects of certain antioxidants on LDL-induced cell growth demonstrated here are independent of activity of ERK1/2, and possibly may modulate ERK1/2 protein expression,\(^ {46} \) mechanisms upstream or downstream of ERK1/2,\(^ {47} \) or both. ERK1/2 appears to play a crucial role for human VSMC growth since both LDL-induced cell proliferation and ERK1/2 activation were potently inhibited by the MEK1/2 inhibitor, PD98059. This finding is consistent with work by Augé and coworkers using oxLDL as a stimulus in bovine VSMC.\(^ {48} \) PD98059 did not completely inhibit native LDL-induced proliferation, which could be due to incomplete inhibition of the enzyme at the dose used. Alternatively, other growth-promoting protein kinases such as p38 MAPK, which contributes to human VSMC proliferation under certain conditions,\(^ {39} \) or Akt\(^ {49} \) could also be involved in the inhibitory effects observed with the antioxidants used in this study.

For our experiments, particular care was taken for the preparation of native LDL. Indeed, native LDL prepared under these conditions has cellular activities which are distinct from oxidized and even minimally oxidized LDL.\(^ {8} \) This is further supported by our experiments demonstrating rapid actions of native LDL on human VSMC on ERK activity and ROS generation within a time frame that is too short to allow sufficient oxidation of native LDL. As in previous studies,\(^ {25–27} \) we have used gel electrophoresis experiments to determine whether native LDL undergoes oxidative modification during exposure to cells. Using human endothelial cells, these previous studies reported that native LDL is indeed oxidized by endothelial cells resulting in a shift of electrophoretic mobility. In addition to endothelial cells, Morel and coworkers also investigated bovine VSMC.\(^ {25} \) In line with our experiments in human VSMC, these investigators did not observe changes in LDL mobility after 24 hours of incubation (consistent with our results using the same time point), but found changes in electrophoretic mobility and increased thiobarbituric acid reactive substances after 48
hours of exposure to VSMC, suggesting differences in the oxidative capacity between VSMC and endothelial cells.

In summary, we have demonstrated that non-oxidized, native LDL induces ROS-formation and activation of redox-sensitive ERK1/2 mitogen-activated protein kinases in human VSMC. These mechanisms importantly contribute to cellular growth induced by native LDL, and provide a potential target for antioxidant therapy in conditions associated with hypercholesterolemia.

Acknowledgments
The authors are indebted to Verena Güttinger, Luca Emmanuelle, and Philip Seba for superb technical assistance with the cell culture experiments. Supported by the Swiss National Science Foundation (No. 32.58421.99 SCORE, No. 32.58426.99/1), the Deutsche Forschungsgemeinschaft (Ba 1543/1-1 and Br 1839/1-1), the Hanne Lieberrmann Stiftung, the Kurt and Senta Herrmann Stiftung, and the Schaufler Stiftung.

References
synthase (Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. 


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Hypertension. 2002;39:645-650
doi: 10.1161/hy0202.103473

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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