Very Low-Density Lipoprotein Activates Nuclear Factor-κB in Endothelial Cells

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Abstract—High plasma levels of VLDL are associated with increased risk for atherosclerosis. Here we show that VLDL (75 to 150 μg/mL) activates nuclear factor-κB (NF-κB), a transcription factor known to play a key role in regulation of inflammation. Oxidation of VLDL reduced its capacity to activate NF-κB in vitro, whereas free fatty acids such as linoleic and oleic acid activated NF-κB to the same extent as did VLDL. Intravenous injection of human VLDL (6 mg protein per kg) into rats resulted in arterial activation of NF-κB as assessed by electrophoretic mobility shift assay. Aortic endothelial cells showed positive nuclear staining for the activated RelA (p65) subunit of NF-κB at 6 to 24 hours after injection. There was also a parallel expression of the adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, as well as the cytokine tumor necrosis factor-α. Pretreatment of the rats with diet containing 1% of the antioxidant probucol for 8 weeks did not inhibit arterial activation of NF-κB in response to injection of VLDL. Moreover, injection of triglycerides (10% Intralipid, 5 mL/kg) activated arterial expression of NF-κB to the same extent as VLDL. Our results suggest that VLDL may promote the development of atherosclerotic lesions by activation of the proinflammatory transcription factor NF-κB. The effect appears to be mediated by a release of VLDL fatty acids but not to involve VLDL oxidation. (Circ Res. 1999;84:1085-1094.)

Key Words: VLDL ■ fatty acid ■ transcription factor ■ oxidation ■ atherosclerosis

Hypertriglyceridemia is a strong risk factor for the development of coronary heart disease (see Reference 1 for review). VLDL, the main carrier of triglycerides, has a well-established indirect atherogenic potency as the precursor of LDL. Furthermore, increased plasma VLDL levels are associated with a low HDL cholesterol, and possible thrombogenic effects of VLDL, such as the induction of plasminogen activator inhibitor-1 (PAI-1) in endothelial cells, have been described.2,3 Nevertheless, the knowledge concerning the direct atherogenic effects of VLDL in terms of effects on gene transcription in the vascular wall is limited.

The redox-sensitive transcription factor nuclear factor-κB (NF-κB) may influence most stages of atherosclerosis, from early endothelial dysfunction4 to plaque disruption. Activated NF-κB is present in the atherosclerotic plaque, whereas little activated NF-κB can be detected in vessels free of atherosclerosis.5 Blocking of NF-κB in vivo prevents myocardial infarction.6

NF-κB consists of different proteins belonging to the Rel family, such as NF-κB1 (p50), NF-κB2 (p52), RelA (p65), and c-Rel (see Reference 7 for review). In most cell types, NF-κB is a heterodimer of the p50 and p65 subunits and bound to 1 of the closely related inhibitory proteins, collectively referred to as IκB. The activation of NF-κB requires the release of IκB, usually mediated by serine phosphorylation, ubiquitination, and proteosomal degradation of IκB. Activation of NF-κB involves translocation of the transcription factor to the nucleus, where it regulates the transcription of a wide variety of genes, many of which are involved in immune and inflammatory responses. Rapid but transient activation of NF-κB is mediated through release from IκB-α, whereas degradation of IκB-β leads to long-term activation.8 A large number of agents, such as inflammatory cytokines (eg, interleukin-1 and tumor necrosis factor-α [TNF-α]), pathogenic microorganisms, reactive oxygen intermediates, and UV light, can activate NF-κB.

In the present study, we investigated both in vitro and in vivo effects of VLDL and fatty acids on NF-κB. Previous reports concerning the effects of lipoproteins on NF-κB have mostly involved oxidized LDL, whereas native LDL has been used as a control lipoprotein with very weak effects compared with oxidatively modified LDL. The present study shows that VLDL induces NF-κB activation and expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and TNF-α, all of which can contribute to inflammation and recruitment of monocytes and T lymphocytes to the vascular wall.
Materials and Methods

Reagents
Monoclonal anti-human apolipoprotein B (apoB), monoclonal anti-human apolipoprotein E (apoE), and monoclonal anti–NF-κB antibodies were obtained from Boehringer Mannheim. Monoclonal anti-rat TNF-α was purchased from Serotec Ltd. Monoclonal anti-rat ICAM-1 and monoclonal anti-mouse VCAM-1 were obtained from R&D Systems. Biotinylated horse anti-mouse and goat anti-rabbit purchased from Vector Laboratories were used as secondary antibodies. Double-stranded NF-κB oligonucleotides for electrophoretic mobility shift assay (EMSA) were obtained from Promega. Mutant NF-κB oligonucleotides and rabbit polyclonal IgG against NF-κB1 (p50) and RelA (p65) subunits were obtained from Santa Cruz Biotechnology. [γ-32P]ATP was from Amersham. Intralipid emulsion, Sephadex G-25 (model MPD-10), and Sephadex G-50 (DNA grade) were from Pharmacia. Endothelial cell growth supplement, BSA, fatty acids (16:0, 18:1, 18:2, and 18:3), and E-TOXATE (limulus amebocyte lysate [LAL] assay) were obtained from Sigma; human recombinant TNF-α from R&D Systems; and heparin (5000 IU/mL) from KabiVitrum. Male Sprague-Dawley rats were obtained from B&K Universal AB (Sollentuna, Sweden). Probucol and butylated hydroxytoluene, and put on ice, and nuclear extracts were prepared within 6 hours. For experiments with probucol, rats were put on a diet containing 1% probucol for 8 weeks, after which they typically weighed 500 to 600 g when euthanized. This study was approved by the Institutional Animal Care and Use Committee of the Karolinska Institute.

In Vivo Studies
Male Sprague-Dawley rats (280 to 380 g) were injected in a tail vein with 2 to 3 mL of PBS, 6 mg/kg VLDL, or 5 mL/kg Intralipid (10% 10) with or without heparin (100 IU/kg) while under anesthesia with ether. The rats were euthanized after 6, 12, 24, or 48 hours under anesthesia (ketamine, 20 mg/kg, and rompun, 3 mg/kg). Rats for immunohistochemical investigations were perfused with 0.9% saline for 10 to 15 minutes and fixed by perfusion for 10 minutes with 4% formaldehyde. The aorta were removed, placed in 4% formaldehyde for 3 hours, and transferred to a 15% sucrose solution; kept at 4°C overnight; and embedded in paraffin. For EMSA, the tissue sections were removed rapidly without previous perfusion, washed in cold PBS containing 20 μmol/L butylated hydroxytoluene, and put on ice, and nuclear extracts were prepared within 6 hours. For experiments with probucol, rats were put on a diet containing 1% probucol for 8 weeks, after which they typically weighed 500 to 600 g when euthanized. This study was approved by the Institutional Animal Care and Use Committee of the Karolinska Institute.

Preparation of Nuclear Extracts
Nuclear extracts were prepared essentially as described by Alksnis et al. 10 All buffers were kept on ice unless stated otherwise. 2-Mercaptoethanol (5 mmol/L) and the protease inhibitors leupeptin (0.7 μg/mL), aprotinin (16.7 μg/mL), and PMSF (0.5 mmol/L) were added to all buffers just before use. Cells cultured in 90-mm dishes were rinsed with ice-cold PBS, harvested, centrifuged at 850g (4°C) for 2 minutes, and washed in ice-cold PBS. The cells were resuspended in 60 μL of hypotonic buffer containing (in mmol/L) Tris (pH 7.3) 10, KCl 10, and MgCl2 1.5. After centrifugation (9000g, 2 minutes), the cells were lysed by resuspen- sion in 80 μL of lysis buffer containing (in mmol/L) Tris (pH 7.3) 10, KCl 10, and MgCl2 1.5, and 0.4% NP-40. After incubation at 4°C for 10 minutes, nuclei were collected by centrifugation for 1 minute at 9000g, and the pellets were washed once in 1 mL of 20 mmol/L KCl buffer, containing (in mmol/L) Tris (pH 7.3) 20, KCl 20, MgCl2 1.5, and EDTA 0.2, and 21.75% glycerol. The isolated nuclei were resuspended in 15 μL of 20 mmol/L KCl buffer, and 60 μL of 600 mmol/L KCl buffer containing (in mmol/L) Tris (pH 7.3) 20, KCl 600, MgCl2 1.5, and EDTA 0.2, and 21.75% glycerol was added. Nuclear proteins were extracted by incubation on ice for 30 minutes. After centrifugation (9000g, 15 minutes), the supernatant containing nuclear proteins was transferred to a precooled microcentrifuge tube. An aliquot of the extract was diluted 40 times with 0.484 mol/L KCl buffer (a mixture of 20 mmol/L KCl buffer and 600 mol/mol KCl buffer to give the same glycerol and salt concentrations as in the undiluted nuclear extracts) for the protein assay. Protein concentration was determined spectrophotometrically according to the following equation: concentration (μg/mL) = 183.4(A 230 nm)–75.8(A 260 nm), where A denotes absorbance. Aortic tissue was harvested as described separately, minced in ice-cold PBS, and homogenized in a Dounce glass homogenizer in ice-cold lysis buffer (see above) for 2 to 3 minutes. After centrifugation at 9000g for 1 minute, the pellet was washed in 20 mmol/L KCl buffer. The pellet was resuspended in 0.02 mol/L KCl buffer and 0.6 mol/mol KCl buffer as described above to extract nuclear proteins. Protein concentrations were measured as described above.

EMSA
Equal amounts of protein from nuclear extracts (cultured cells, 1 to 3 μg; rat aortic tissue, 5 to 10 μg) were incubated on ice with 2 μg of poly(dI-dC) and 1 μg of acetylated BSA in binding buffer (giving the final concentrations stated below) for 10 minutes. 12 The oligonucleotide probe (50 000 cpm in 5 μL) was added, and the reaction mixture (25 μL) was incubated for 25 minutes at room temperature. Final concentrations in binding reactions were as follows: 10% glycerol and (in mmol/L) HEPES (pH 7.9) 10, KCl 60, MgCl2 5, EDTA 0.5, DTT 1, and PMSF 1. DNA-protein complexes were separated from unbound DNA probe on native 7% polyacrylamide gels (acrylamide:bisacrylamide [wt/wt], 80:1) in low ionic strength buffer containing (in mmol/L) Tris 22.5, borate 22.5, and EDTA 0.5 (pH 8) by electrophoresis at 200 V for 2 hours. The sequences of the double-stranded oligonucleotide probes (labeled with T4 kinase and [γ-32P]ATP and purified using Pharmaea NICK columns) were as follows: κB consensus, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; C-κB mutant, 5′-AGT TGA GGC GCC TTT CCC AGG C-3′. For supershift analysis, 1 μg of the relevant antibodies was added after the binding buffer solution, and the incubation time on ice was increased from 10 to 30 minutes. Unlabeled competitor oligonucleotides were added in a 50-fold excess to confirm the specificity of the binding reactions.

Preparation and Oxidation of Lipoproteins
Blood samples for the preparation of VLDL were taken from fasting hypertriglyceridemic patients free of any infectious disease. Plasma was recovered by centrifugation at 1500g for 20 minutes at 4°C. VLDL was prepared by density-gradient ultracentrifugation, essentially as described by Redgrave and Carlsson. 13 Plasma was adjusted to a density of 1.10 g/mL by addition of NaCl. A density gradient consisting of 4 mL of 1.10 g/mL plasma and 3 mL each of 1.065,
Preparation of Fatty Acid–BSA Complexes

Fatty acid–BSA complexes were prepared essentially according to the method of Spector and Hoak. Briefly, 25 mg of fatty acids (16:0, 18:1, 18:2, and 18:3) were dissolved in 7.5 mL hexane, and 800 mg celite was added. The solvent was removed under N2 by continuous magnetic stirring. When the solvent had evaporated completely, BSA free of fatty acids (25 mL of 0.25 mmol/L) was added. The mixture was stirred for 1 hour at room temperature with N2 constantly passing over the surface. After centrifugation at 800 g for 5 minutes, the supernatants were carefully decanted. Samples containing fatty acid–BSA complexes were filtered through a 0.45 μm filter. For experiments comparing the effects of LDL and VLDL, plasma from 3 to 4 normal donors free of any infectious disease was pooled, and the lipoproteins were prepared as described above. The LDL fraction was isolated from the easily identifiable yellow band located between 4 and 5 cm from the bottom of the tube. The LDL was subsequently desalted with Sephadex G-25 MPD-10 columns. Half of the lipoproteins were then oxidized by incubation with 5 μmol/L CuSO4 at 37°C (5% CO2) for 18 hours or incubated at 37°C (5% CO2) for 18 hours without copper to obtain control lipoproteins. For all experiments, freshly prepared lipoproteins were used within 24 hours after ultracentrifugation. The endotoxin content in the lipoprotein preparations was tested by LAL assay (E-TOXATE, Sigma). Briefly, lipoprotein samples were diluted 1:10 in endotoxin-free water and heated at 65°C for 5 minutes to inactivate the LAL inhibitor found in plasma. E-TOXATE working solution containing the LAL was added, and samples were incubated at 37°C for 1 hour. Positive controls containing 0.06 endotoxin units per mL (derived from Escherichia coli, 0.55:B5 lipopolysaccharide) yielded the formation of a hard gel that permitted complete inversion of the tube without disruption of the gel. All samples were found to be endotoxin free as assessed by this assay.

Immunohistochemistry

Tissue sections were deparaffinized with xylene and dehydrated with graded ethanol. The membranes were permeabilized in 0.2% Triton X-100 for 10 minutes. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% H2O2 and 80% methanol for 30 minutes at room temperature, followed by washing 3 times for 5 minutes. PBS was used as washing buffer for all antibodies except apoB and apoE, for which a Tris buffer, pH 7.4, was used. After the sections were rinsed again 3 times in washing buffer, the samples were incubated with 10% goat serum (for rabbit antibodies) or 10% horse serum (for mouse antibodies) diluted in PBS or Tris/0.2% Triton X-100 for 20 to 30 minutes. Primary antibodies were diluted in PBS or Tris buffer to a final concentration of 10 μg/mL and incubated overnight at 4°C. On the following day, sections were washed and incubated with biotinylated secondary antibodies (diluted 1:200) for 30 minutes and then washed. The sections were incubated for 30 minutes with a peroxidase-labeled avidin-biotin complex and washed again. The sections were developed using 3,3′-diaminobenzidine (Vector Laboratories) and counterstained in Harris reagent. Negative controls included substitution of the primary antibody with either irrelevant IgG-antibody or washing buffer. For apoB and apoE, staining specificity was also assessed by preincubation of the antibodies with the respective protein for 2 hours at 37°C. This protocol was used for all antibodies except for VCAM-1. As this antibody was raised in rats, it was primarily biotinylated to avoid using a secondary antibody. Furthermore, the alkaline-phosphatase method with Fast Red substrate solution (Vector Laboratories) was used for color development. Blocking of endogenous peroxidase activity was not required.

Results

VLDL Activates NF-κB in EA.hy926 Cells and HUVECs

The ability of VLDL to influence endothelial NF-κB activity was first studied in the human endothelial cell line EA.hy926 (Figure 1, lanes 1 to 9) and in cultured HUVECs (Figure 1, lanes 10 to 14). Low levels of basal NF-κB binding activity were detected in unstimulated control cells (Figure 1, lanes 5, 7, and 12). Stimulation with TNF-α (10 ng/mL) led to a strong activation of NF-κB within 30 minutes in both cell types (Figure 1, lanes 1 and 10). Competition by a 50-fold excess of unlabeled κB binding oligonucleotide abolished binding of the labeled probe (Figure 1, lane 2), whereas a 50-fold excess of the mutant κB binding oligonucleotide (1 bp changed) still allowed some binding of the specific oligonucleotide probe (Figure 1, lane 3). Increased NF-κB binding activity was detected in EA.hy926 cells incubated with 150 μg/mL of VLDL for 1 hour and remained increased after 2 hours of incubation (Figure 1, lanes 4 and 6). Similar observations were made in HUVECs (Figure 1, lane 11 and data not shown). Supershift assays using specific antibodies against NF-κB subunits demonstrated the presence of NF-κB1 (p50) and RelA (p65) in most of the complexes (Figure 1, lanes 8, 9, 13, and 14).
VLDL-induced NF-κB activation was concentration dependent (Figure 2). After an incubation time of 2 hours, EA.hy926 cells exposed to 25 μg/mL VLDL contained no detectable active NF-κB, whereas 75 μg/mL VLDL led to a moderate and 150 μg/mL VLDL to a strong activation of NF-κB. The specificity of the binding reactions was demonstrated with competitor oligonucleotides (Figure 2, lanes 5 and 6).

Oxidation of VLDL Decreases NF-κB Activation

Some previous studies have reported activation of NF-κB by oxidized LDL, whereas others have demonstrated inhibitory effects. To analyze the possible involvement of oxidative modification of VLDL in endothelial NF-κB activation, EA.hy926 cells were incubated for 3 or 24 hours with 75 μg/mL of LDL, copper-oxidized LDL, VLDL, or copper-oxidized VLDL. After 3 hours of incubation, low levels of activated NF-κB were detected in cells treated with oxidized LDL (Figure 3, lane 3), whereas VLDL induced a strong activation of NF-κB (Figure 3, lane 4). Oxidation of VLDL strongly inhibited NF-κB activation (Figure 3, lane 5).

Activation of Endothelial Cell NF-κB by Free Fatty Acids

One mechanism by which VLDL could influence NF-κB is by release of fatty acids from core triglycerides. Free fatty acids are known to activate transcription factors of the PPAR family, but their effect on NF-κB is less well studied. HUVECs (Figure 4, lanes 1 to 7) and EA.hy926 cells (Figure 4, lanes 8 to 15) were incubated with 50 μmol/L of BSA-bound palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) for 3 or 6 hours. Only very low levels of activated NF-κB were detected in control cells with or without incubation with BSA (Figure 4, lanes 1, 8, and 9) and in cells after incubation with palmitic acid and linolenic acid (data not shown). In contrast, oleic acid and particularly linoleic acid induced pronounced NF-κB activation in both cell types (Figure 4, lanes 2, 3, 10, and 11).

Activation of NF-κB by VLDL in the Rat Aorta

To study whether VLDL activates endothelial NF-κB also in vivo, we used a modified version of a model previously

Figure 2. Autoradiograph showing the concentration dependence of VLDL-induced NF-κB activation. EA.hy926 cells were incubated with 25 to 150 μg/mL VLDL for 2 hours. NF-κB binding activity was determined by EMSA. Specificity tests were performed as described in Materials and Methods. Arrow indicates position of NF-κB-specific complexes. F denotes free probe. Shown is a representative EMSA from 3 different experiments. Abbreviations are as in Figure 1.

Figure 3. Autoradiograph showing the effects of LDL, copper-oxidized LDL (ox LDL), VLDL, and copper-oxidized VLDL (ox VLDL) on NF-κB activation. EA.hy926 cells were treated with lipoproteins (75 μg/mL) for 3 or 24 hours. All lipoproteins were from the same pooled plasma source obtained from 4 healthy, fasting subjects. NF-κB binding activity was determined by EMSA. Arrow indicates position of NF-κB-specific complexes. F denotes free probe. Shown is a representative EMSA from 3 different experiments.
developed to study modification of human LDL in the rat aorta. Six hours after intravenous injection of human VLDL (6 mg protein/kg) into rats, human apoB and apoE began to accumulate in the intima and in a few layers of the media located immediately below the internal elastic lamina (Figure 5). At 12 and 24 hours after injection, human apoB and apoE epitopes were present throughout the entire aortic wall. These observations demonstrate that injected VLDL particles accumulate in the rat aorta for at least 24 hours after injection.

Arterial activation of NF-κB after injection of VLDL was studied using antibodies specific for the activated RelA (p65) subunit of NF-κB. This antibody had been raised against epitopes expressed on RelA (p65) after release of the IκB subunit. Positive nuclear staining for active NF-κB was seen

Figure 4. Autoradiograph showing NF-κB activation by fatty acids. Linoleic acid (18:2; 50 μmol/L) and to a lesser extent oleic acid (18:1; 50 μmol/L) complexed to BSA induced NF-κB in both HUVECs (lanes 1 to 7) and EA.hy926 cells (lanes 8 to 15). NF-κB binding activity was determined by EMSA. Specificity tests were performed as described in Materials and Methods. Arrow indicates position of NF-κB–specific complexes. F denotes free probe. Shown is a representative EMSA from 4 different experiments. Abbreviations are as in Figure 1.

Figure 5. Immunohistochemical staining of sections from rat aortas showing the presence of apoB, apoE, and activated NF-κB in rat aortas at different time points after injection of VLDL. Male Sprague-Dawley rats were injected with 6 mg/kg of VLDL and euthanized at the indicated time points. Paraffin-embedded sections were stained with antibodies specific for human apoB, apoE, and the activated p65 (RelA) subunit of NF-κB. Secondary antibody was labeled with peroxidase (brown). Original magnification, ×40. For each time point, 3 to 5 animals were tested.
Expression of Inflammatory Molecules in the Rat Aorta After Injection of Human VLDL

NF-κB is known to regulate several molecules involved in inflammation. The arterial expression of the cytokine TNF-α and the adhesion molecules ICAM-1 and VCAM-1 after injection of VLDL was studied by immunohistochemistry. There was no detectable expression of ICAM-1, VCAM-1, or TNF-α in the aorta of control rats (Figure 7). Endothelial ICAM-1 expression was present 6 hours after injection of VLDL and endothelial VCAM-1 expression after 24 hours. At 12 and 24 hours, ICAM-1 expression was found also in the media, whereas the expression of VCAM-1 remained restricted to the endothelium. Twelve hours after injection of VLDL, epitopes specific for TNF-α were observed in the medial layers located immediately below the internal elastic lamina and at 24 hours throughout the entire media (Figure 7). After 48 hours, neither active NF-κB nor TNF-α expression could be detected in the vessel wall (data not shown).

Effects of Probucol Supplementation and a Chylomicron-Like Lipid Emulsion on Activation of Endothelial NF-κB in Rats

To study the role of oxidation in VLDL-induced activation of NF-κB in vivo, rats were fed a diet containing 1% of the antioxidant probucol for 8 weeks before injection of VLDL. This treatment did not prevent VLDL-induced NF-κB activation (Figure 8, lane 3). Weak basal NF-κB activation could be detected also in control rats given PBS (Figure 8, lane 1). This was a consistent finding in older rats both at normal and probucol-enriched diet and suggests a positive correlation between age and vascular NF-κB activation. In addition to the specific upper NF-κB band, which disappeared when unlabeled probe but not unlabeled mutant probe had been added, an additional band with higher mobility (Figure 8, small arrow) was seen that appeared to be stronger in the extracts from VLDL-injected rats. The identity of this band, which seemed to have a lower affinity for the probe than NF-κB, remains to be clarified.

To study whether a triglyceride emulsion can activate NF-κB in vivo, Intralipid (5 mL/kg; 10% solution) was injected into rats. Enhanced NF-κB binding activity was detectable in nuclear extracts prepared from rat aorta 1 hour after injection (Figure 9, lane 3). Heparin administration abolished Intralipid-induced NF-κB activation (Figure 9, lane 4), which suggests that NF-κB activation was at least partly dependent on the amount of lipoprotein lipase bound to endothelial cells mediating the release of fatty acids from the lipid emulsion. Three hours after Intralipid injection, NF-κB binding activity had returned to basal level again (Figure 9, lane 7).

Discussion

In the present study, we show that VLDL activates NF-κB in endothelial cells both in vitro and in vivo and that this is associated with an increased expression of NF-κB–regulated inflammatory molecules such as ICAM-1, VCAM-1, and TNF-α. As NF-κB is a redox-sensitive transcription factor, oxidative modification of VLDL should be considered as a possible mechanism responsible for VLDL-induced NF-κB activation.
A recent study from our laboratory has demonstrated that in vivo oxidation of accumulated LDL within the rat arterial wall is associated with NF-κB activation in the endothelium. In vitro studies have shown both stimulatory and inhibitory effects of oxidized LDL on NF-κB activation, depending on the cell type, extent of LDL oxidation, and incubation time used in the different experiments. Cultured endothelial cells are the only cell type in which an oxidized LDL–induced activation of NF-κB has been well established so far. In contrast, oxidized LDL has been found to inhibit lipopolysaccharide-induced NF-κB activation in smooth muscle cells and macrophages. In a recent study, oxidized LDL was shown to inhibit NF-κB after long (≥20 hours) incubations in THP-1 monocytic cells, whereas a stimulatory effect was reported after short treatments (4 hours).

All of these data are in accordance with our observation that short treatments with both oxidized LDL and VLDL activate NF-κB in cultured endothelial cells. At the same protein concentration and derived from the same plasma source, VLDL had a stronger potential to activate NF-κB than oxidized LDL. Interestingly, oxidation of VLDL reduced the activation of NF-κB. This finding suggests that oxidized LDL and VLDL activate NF-κB in endothelial cells through different mechanisms.

It is likely that lipoprotein oxidation causes degradation of the unsaturated fatty acids. Because oleic and linoleic acid were found to induce endothelial activation of NF-κB as effectively as VLDL, loss of fatty acids represents one possible explanation for the decreased ability of VLDL to activate NF-κB after oxidation. Alternatively, as oxidation of LDL is known to lead to impaired lysosomal degradation of LDL, lipoprotein oxidation might decrease the lysosomal release of stimulatory fatty acids from triglycerides. Furthermore, aldehydes formed during lipid oxidation are known to inhibit NF-κB activation. The notion that VLDL-induced activation of NF-κB is mediated by mechanisms other than oxidation of VLDL is also supported by the observation that a probucol-supplemented diet did not suppress VLDL-induced activation of NF-κB in rat aorta. However, this does not exclude the possibility that the induction of NF-κB may involve redox-regulated processes (eg, fatty acid–induced activation of NADPH oxidase, as shown by Cui and Douglas).

One major difference between VLDL and LDL is the high triglyceride content of the former. The notion that fatty acids are involved in VLDL-induced activation of NF-κB in the endothelium was supported by the finding that linoleic and oleic acids activated NF-κB in cultured endothelial cells as effectively as VLDL, as well as by the observation of NF-κB activation in rat aorta after injection of a triglyceride emulsion. Stimulatory effects have been reported for fatty acids, including enhancement of PAI-1 expression by n-3 polyunsaturated fatty acids, which may involve previously unknown transcription factors. In rat aortic smooth muscle cells, linoleic acid induces DNA synthesis; c-fos, c-jun, and c-myc mRNA expression; and mitogen-activated protein kinase activation. It is also known that lipoxygenase inhibitors inhibit cell proliferation. Taken together, these findings suggest that fatty acid metabolites could play an important role in many of the processes implicated in atherogenesis. Whether lipoxygenase or cyclooxygenase products of linoleic acid or arachidonic acid mediate the VLDL-induced NF-κB activation.
activation reported in the present study remains to be investigated. Oxidized LDL at high concentrations has been shown to induce interleukin-1β secretion and mRNA expression in macrophages, which may have been due to oxidized derivatives of linoleic acid, such as 9-hydroxyoctadecadienoic acid. However, expression of TNF-α was not induced in the experiments in question, which suggests that 9-hydroxyoctadecadienoic acid did not mediate the VLDL-induced TNF-α expression observed in the present study.

Much attention has been paid to the role of LDL oxidation in the development of atherosclerosis and in particular to the possibility that oxidative modification of LDL in the arterial wall activates the inflammatory process that characterizes lesion progression. Activation of the redox-regulated transcription factor NF-κB has been suggested to play a key role in this process, as it binds to the promoter of many genes involved in inflammation. The present observations suggest that VLDL is an activator of NF-κB that is at least as efficient as oxidized LDL. Previous studies with the same animal model suggest that accumulation of LDL is associated with expression of oxidized LDL–specific epitopes, activation of NF-κB, and induction of adhesion molecules, whereas HDL appears to be without effect in this respect. In addition to oxidative stress, release of fatty acids seems to be another major mechanism involved in lipoprotein-dependent activation of NF-κB. Lipoprotein lipase is known to hydrolyze lipids in Intralipid emulsions in vivo. Thus, the observation that heparin injection decreased Intralipid-induced NF-κB activation suggests that fatty acids may induce vascular cell NF-κB activation also in vivo. A high plasma level of VLDL triglycerides is part of a metabolic syndrome frequently encountered in individuals with early-onset coronary heart disease. Impaired glucose tolerance, abdominal obesity, hypertension, low HDL cholesterol, and impaired fibrinolysis due to increased plasma levels of PAI-1 are other common features of this syndrome, the etiology of which remains largely unknown.

Great care was taken to exclude the possible influence of endotoxins in this study. All lipoprotein samples were screened for endotoxin contamination using the limulus
assay, with negative results. The finding that VLDL and oxidized LDL (but not LDL) derived from the same plasma source, as well as different fatty acids, activated NF-κB also supports the notion that the effects are not mediated by endotoxins.

There is relatively little information about the influence of VLDL on inflammatory activity. β-VLDL is known to increase monocyte binding to endothelial cells. 39–41 VLDL has been reported to potentiate the tissue factor–inducing capacity of lipopolysaccharide 42 and to induce expression of tissue factor and PAI-1 in endothelial cells and monocytes. 43 In clinical studies, high levels of VLDL have been shown to be associated with increases in plasma TNF-α and PAI-1. 44 Elevated triglycerides but not total cholesterol levels were significantly correlated with soluble VCAM-1 plasma values in patients suffering from peripheral vascular disease along with essential hypertension. 45 Together with our findings, these studies suggest that VLDL and presumably other triglyceride-rich lipoproteins have important stimulatory effects that distinguish them from smaller lipoproteins and extensively oxidized lipoproteins.

In conclusion, the present investigation shows that VLDL activates NF-κB in cultured human endothelial cells and in rat aortic endothelial and smooth muscle cells in vivo. In rat aorta, VLDL induces expression of TNF-α and ICAM-1 in smooth muscle cells and VCAM-1 expression in endothelial cells. VLDL may thus contribute to inflammation and recruitment of monocytes and T lymphocytes to the vascular wall, thereby promoting the development of atherosclerotic lesions. These effects may be mediated by the release of fatty acids from triglycerides in VLDL such as linoleic acid and oleic acid.

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