An Animal Model to Study Local Oxidation of LDL and Its Biological Effects in the Arterial Wall

Federico Calara, Paul Dimayuga, Audrey Niemann, Johan Thyberg, Ulf Diczfalusy, Joseph L. Witztum, Wulf Palinski, Prediman K. Shah, Bojan Cercek, Jan Nilsson, Jan Regnström

Abstract—Oxidized LDL (oxLDL) is present in atherosclerotic lesions and is believed to play a key role in atherogenesis. Mainly on the basis of cell culture studies, oxLDL has been shown to produce many biological effects that influence the atherosclerotic process. To study LDL oxidation in vivo, we have established a model in which Sprague-Dawley rats are given a single injection of unmodified human LDL (≈4 mg/kg body weight). Within 6 hours, an accumulation of apolipoprotein B and epitopes present on oxLDL are detected in the arterial endothelium and media. The presence of oxLDL is associated with the transcription factor nuclear factor-κB in the endothelium as well as endothelial expression of intercellular adhesion molecule-1. Injection of LDL enriched with the antioxidant probucol resulted in arterial accumulation of apolipoprotein B, but the expression of oxLDL-specific epitopes was reduced at 24 hours. Thus, this simple model has the potential to analyze the mechanisms behind and biological effects of LDL oxidation in vivo. (Arterioscler Thromb Vasc Biol. 1998;18:884-893.)

Key Words: atherosclerosis • oxLDL • nuclear factor-κB • intercellular adhesion molecule-1

Modification of LDL is believed to play an important role in the formation of atherosclerotic lesions. The most studied form of modification is oxidation of LDL, a lipid peroxidation process resulting in the formation of a wide range of biologically active products, such as peroxides, malondialdehyde, 4-hydroxynonenal, lyssolecithin, PAF or PAF-like substances, and oxysterols. Oxidation of LDL also leads to fragmentation of apoB. As a result, the particle loses its affinity for the LDL receptor and binds avidly to scavenger receptors on macrophages, resulting in foam cell formation. The presence of oxLDL has been demonstrated in human atherosclerotic lesions, and an inverse relation between the resistance of LDL to oxidation and the degree of atherosclerosis has been found in humans. Treatment of hypercholesterolemic animals with antioxidants, such as probucol, has been shown to reduce the formation of atherosclerotic lesions, thus supporting the concept that LDL oxidation is directly involved in atherogenesis. OxLDL exhibits a broad spectrum of biological effects that may promote development of the early atherosclerotic lesion, such as endothelial dysfunction, activation of endothelial adhesiveness, and monocyte differentiation. Most of these findings are based on cell culture studies using LDL in a wide range of concentrations and oxidized by different methods, such as exposure to cultured cells, transition metals, lipoygenases, phospholipase A2, and UV radiation. Despite extensive in vitro data, the in vivo relevance of these findings is not clearly known. LDL oxidation is believed to occur locally in the arterial wall, after an initial interaction with and entrapment by proteoglycans. The mechanisms involved in LDL oxidation in vivo are not known and may vary at different stages of atherosclerosis. Colocalization of 15-lipoxygenase with epitopes of oxLDL has been seen in macrophage-rich areas of atherosclerotic plaques, suggesting a potential role for this enzyme in the modification of LDL.

In the present study, we developed an animal model in which a single injection of unmodified, heterologous LDL resulted in an accumulation of LDL in the arterial wall, where it became oxidatively modified within 6 hours.

Methods

The following mouse monoclonal antibodies were used: anti-human apoB and anti–NF-κB (α-p65 mAb) from Boehringer Mannheim; anti-rat monocyte and macrophage antibody (clone ED-1) obtained from Serotec; anti-rat ICAM-1 supplied by R&D Systems; and anti–α-actin (HHF-35) from DAKO. Mouse monoclonal antibody NA59, specific for epitopes generated during oxidative modification of LDL, was supplied by Dr Joseph Witztum (University of California at San Diego, La Jolla, Calif). Biotinylated horse anti-mouse secondary antibody was supplied by Vector. Oligonucleotides for EMSAs were obtained from Promega. [γ-32P]dATP and [3H]-amebocyte lysate assay was performed by Microbiology Reference Laboratory (Cincinnati, Ohio).
Preparation and Oxidation of LDL

Blood samples for LDL preparation were taken from healthy volunteers and hyperlipidemic male survivors of myocardial infarction under the age of 45 years after 12 hours of fasting, during which time smokers were asked to refrain from smoking. All subjects were free from symptoms of infectious disease at the time of blood sampling. Venous blood was drawn into precooled Vacutainer tubes containing disodium EDTA (1.4 mg/mL) and placed on ice. Plasma was then recovered by low-speed centrifugation (1400g, 20 minutes, 1°C) and kept at this temperature throughout the preparation procedures. LDL was prepared by cumulative rate ultracentrifugation in a neutral density gradient as described in detail earlier. LDL was concentrated by pooling LDL prepared from at least four donors, adjusting the density to 1.065 g/L by adding NaBr containing 10 \(\mu\)mol/L EDTA, and ultracentrifuging the samples (200,000g, 20 hours, 1°C). The LDL was frozen in 10% sucrose (vol/vol) at 1°C and kept at this temperature throughout the preparation procedures. LDL was prepared by cumulative rate ultracentrifugation in a neutral density gradient as described in detail earlier. LDL was concentrated by pooling LDL prepared from at least four donors, adjusting the density to 1.050 g/L by adding NaBr containing 10 \(\mu\)mol/L EDTA, and ultracentrifuging the samples (200,000g, 20 hours, 1°C). The LDL was frozen in 10% sucrose (vol/vol) at −80°C. Cryopreservation of the samples in sucrose did not affect the biological properties of LDL, as described previously. Within 1 hour before the injection of native LDL into the animals, excess salt and sucrose were removed from the LDL by running the samples over a PD-10 column preequilibrated in PBS. The LDL was sterilized by passing it through a 0.45-μm filter and was kept on ice until injected. The protein content was determined by the method of Lowry et al. LDL (1 mg/mL) was oxidized by exposure to 5 \(\mu\)mol/L CuSO\(_4\) for 18 hours at 37°C. Compared with native LDL, the CuSO\(_4\)-oxidized LDL showed increased electrophoretic mobility on agarose gels, and higher concentrations of thiobarbituric acid-reactive substances. Endotoxin levels in both native and oxLDL were <2 ng/mg LDL protein, as determined by the Limulus amebocyte lysate assay.

Probuloc Enrichment of LDL

Plasma prepared as described above was incubated for 2 hours at 37°C with probucol (0.05 mmol/L) dissolved in ethanol. The LDL was then isolated and concentrated, and the protein concentration was determined by using the methods described above. Roughly 45% of the added probucol is incorporated into the LDL fraction when this protocol is used with plasma with a total cholesterol level of 6.5 to 7.0 mmol/L.

Radiolabeling of LDL

LDL was iodinated with \(^{125}\)I by the ICl method of McFarlane. Unbound iodine was removed by passing the labeled LDL through a PD-10 column equilibrated with PBS containing 0.27 mmol/L EDTA and 20 \(\mu\)mol/L BHT to protect against oxidation and filtered through a 0.45-μm filter. Specific activity was 400 to 600 counts per minute per nanogram LDL protein, and 96% was protein bound as determined by trichloroacetic acid (TCA) precipitation.

Animal Protocol

Male Sprague-Dawley rats (300 to 400 g) were injected intraperitoneally or intravenously in the tail vein with PBS, LPS, or human native or oxLDL while under ether anesthesia. The rats were euthanized at different times up to 48 hours under anesthesia (75 mg/kg ketamine and 3 mg/kg xylazine HCl [Rompun]) by intracardiac injection of KCl. Heparin (1000 IU/kg) was injected into the heart followed by perfusion with 0.9% saline containing 0.02 \(\mu\)mol/L BHT for 5 minutes. Rats were perfusion fixed for 5 minutes with either 4% formaldehyde for those rats to be analyzed by immunohistochemistry or 3% glutaraldehyde for those rats to be analyzed by electron microscopy. After perfusion, the aorta, iliac and femoral arteries, and the liver were removed. Tissue to be used for immunohistochemistry was placed in 4% formaldehyde for 3 hours, transferred to a 15% sucrose solution, and kept at 4°C overnight before being embedded in paraffin. The abdominal aorta and iliofemoral arteries were cut into three equal pieces and mounted together in one block. For EMSAs the aortas, iliofemoral arteries, and livers were removed rapidly without previous perfusion and kept for 5 minutes in ice-cold 0.9% NaCl containing 0.02 \(\mu\)mol/L BHT. The arteries were then stripped of adventitia and immediately frozen in LN\(_2\). The samples were kept at −80°C until assayed.

In rats injected with \(^{125}\)I-LDL (4 mg/kg IP and IV), the tip of the tail was cut and 0.5 mL of blood was collected at different times up to 24 hours and then allowed to clot at room temperature for 30 minutes. Serum was collected after centrifugation for 20 minutes at 2714 rpm at 4°C and counted in a Packard Cobra Auto-Gamma counting system.

Analysis of LDL After Intraperitoneal Injection

LDL was recovered from the peritoneal cavity 2, 6, and 12 hours after intraperitoneal injection by injecting 2 mL of ice-cold PBS containing 20 \(\mu\)mol/L BHT while the rat was under anesthesia (30 mg/kg ketamine and 3 mg/kg Rompun). They were then euthanized by an intracardiac injection of KCl, and the PBS was aspirated from the peritoneal cavity. Analysis of LDL modification was performed by aagarose gel electrophoresis. The relative mobility was expressed as a ratio between the distance migrated of the reisolated LDL divided by the distance migrated of noninjected LDL.

Preparation of Nuclear Extracts

Nuclear extracts were prepared according to Dignam et al. and the tissue from two rats was used for nuclear extraction at each data point. Tissues were cut into small pieces in ice-cold saline and homogenized in a Dounce homogenizer in 1 mL hypotonic lysis buffer. After a 10-minute incubation on ice, samples were centrifuged at 9000g for 2 minutes. Pellets were rinsed with 0.02 mol/L KCl buffer and centrifuged at 9000g for another minute. Pellets were resuspended in 115.5 
\(\mu\)L of 0.02 mol/L KCl buffer and 460 
\(\mu\)L of 0.6 mol/L KCl buffer. Nuclear proteins were extracted by gentle agitation for 30 minutes at 4°C. After centrifugation for 15 minutes at 9000g, the supernatant containing the nuclear protein was divided into aliquots and kept frozen at −70°C. Protein concentration was determined by the Coomassie Plus Assay from Pierce Chemical Co.

EMSA

Six micrograms of nuclear extract was incubated with 0.05 \(\mu\)g of poly(dI-dC) in binding buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 4% glycerol, 1 mmol/L MgCl\(_2\), 0.5 mmol/L EDTA, and 0.5 mmol/L DTT) on ice for 10 minutes. The double-stranded NF-κB oligonucleotide (5‘ ATTTGAGGGAGCTTCCAGG 3’) was labeled with [\(\gamma\)-32P]dATP, purified over a Pharmacia Nickel column, added (50,000 cpm per tube) with or without a 100× excess of unlabeled oligonucleotide, and incubated for 20 minutes at room temperature. The samples were then run on a 4% polyacrylamide gel in Tris-buffered EDTA buffer for 2 hours at 200 V at 4°C. The gels were dried under vacuum and heat and then exposed to Kodak film.
Preparation of RNA
RNA extracts were prepared and purified by using the protocol and reagents from the total RNA isolation system (Promega SDS). Aortas and iliac arteries from one rat were frozen in LN₂ and homogenized in 6 mL of denaturing solution containing citrate/sarcosine/β-mercaptoethanol and guanidine thiocyanate in a Dounce homogenizer. RNA extraction was performed by adding 0.6 mL of 2 mol/L sodium acetate, pH 4.0, and the sample was mixed. Six milliliters of phenol/chloroform alcohol mixture was added, and the sample was mixed thoroughly and chilled on ice for 15 minutes. The sample was then centrifuged at 10 000g for 20 minutes at 4°C. The top aqueous phase was removed, an equal volume of isopropanol was added, and the sample was incubated at −20°C for 30 minutes to precipitate the RNA. The RNA pellet was recovered by centrifugation at 10 000g for 15 minutes at 4°C and resuspended in 5 mL of denaturing solution. The RNA was reprecipitated in isopropanol and the pellet recovered. The RNA pellet was washed in ice-cold ethanol and the pellet recovered by centrifugation. The pellet was then resuspended in 1 mL of RNase-free water. After purification, the sample was treated with DNase by adding 0.1 U/µL DNase, 2 U/µL RNase inhibitor, 100 µL of transcription buffer, and 10 mmol/L DTT to 30 µg of RNA sample and incubated at 37°C for 30 minutes. The reaction was stopped by adding 4 mmol/L of EDTA.

RT-PCR
For the cDNA synthesis, 2 µg of RNA was added to 100 µL reagent mixture (10 mmol/L DTT, 0.5 mmol/L 4 dNTP, 1 U/µL RNase I, and 10 U/µL M-MLV reverse transcriptase) and reverse-transcribed at 42°C for 50 minutes by the random-priming method using Pd(Ni) hexamers. cDNA synthesis was performed using a Perkin-Elmer DNA thermal cycler model 9600 (Perkin-Elmer Sundbyberg) at an initial temperature of 30°C for 10 minutes, 42°C for 50 minutes, and 94°C for 2 minutes. The ICAM-1 oligonucleotide primers were designed from the cDNA sequence of rat ICAM-1. ²⁹ Primer 1 (5’-GTTGCTATGTATCGCC-3’) was complementary to cDNA sequences 1530 to 1550, and primer 2 (5’-TCAGAGGAAG CATGTTGTC-3’) was complementary to sequences 1972 to 1992. β-Actin primers were from the rat β-actin control amplimer set from Clonetech Intermedica. PCR amplification was performed by adding 2 µL of cDNA with 50 µL of reagent mixture (0.2 mmol/L of 4 dNTP, 2 U Taq polymerase, and 0.4 mmol/L of each primer) and amplified by a 35-step cycle program after denaturation at 90°C for 30 minutes. Each cycle included 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. A 15-µL aliquot of each reaction was electrophoresed on a 2% agarose gel, and the bands were amplified by ethidium bromide staining. Semiquantification of the ICAM-1 bands was performed by measuring the OD of the ICAM-1 bands. This study was approved by the Institutional Animal Care and Use Committee at the Karolinska Institute.

Results
Figure 1 shows the time course of human 125I-LDL in serum after intraperitoneal and intravenous injection (4 mg/kg). Venous blood samples were taken at 0.5, 5, 9, and 24 hours.

Electron Microscopy
Tissue for electron microscopy was postfixed in 1% cacodylate-buffered OsO₄ containing 0.7% K₄Fe(CN)₆, dehydrated in graded ethanol, and embedded in Spurr low-viscosity epoxy resin. Thin sections were cut with a diamond knife on an LKB Ultratome IV, picked up on carbon-stabilized Formvar films, stained with alkaline lead citrate, and finally examined in a JEOL EM 100CX.

Ethical Considerations
This study was approved by the Institutional Animal Care and Use Committee at the Karolinska Institute.

Immunohistochemistry
Tissue sections were deparaffinized with xylene and dehydrated with graded ethanol. The membranes were permeabilized in 0.2% Triton X-100. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% H₂O₂ and 80% methanol for 30 minutes at room temperature. After they were washed, the sections were blocked with 10% horse serum in PBS for 30 minutes. Primary antibodies were diluted in PBS (as indicated in the figure legends) and incubated with the sections for 18 hours at 4°C in a humidified chamber. The sections were washed, incubated with biotinylated secondary antibody at a dilution of 1:200 for 30 minutes, and then washed again. The sections were incubated for 30 minutes with a peroxidase-labeled avidin-biotin complex and washed again. The sections were developed with diaminobenzidine (Vector) and counterstained in hematoxylin. Negative controls included substitution of the primary antibody with either PBS or an irrelevant antibody. Immunohistochemically stained sections (n=3) were analyzed by a modified version of the technique described by Gallis et al. ¹⁰ Consistent positive staining covering >50% of the intima was recorded as 3, variable or weak staining as 1, and no staining as 0. The interobserver variability of the scoring was <20%.
With the use of antibodies against human apoB and epitopes present on oxLDL (NA59), LDL and oxLDL could be detected in the arterial endothelium and media 6 hours after intraperitoneal injection of native LDL (Figure 2). Semiquantitative evaluation of the staining showed that the accumulation of LDL peaked at 12 hours, whereas oxLDL peaked at 24 hours (Figure 3). The immunostaining for apoB and oxLDL was cell associated and within the extracellular matrix. Intravenous injection of LDL resulted in an earlier and stronger immunostaining of apoB and oxLDL than with intraperitoneal injection.

Intraperitoneal injection of LDL enriched with the antioxidant probucol also resulted in an accumulation of apoB, but at 24 hours a clear reduction of epitopes present on oxLDL was seen (Figure 4). The semiquantitative scoring of staining for oxLDL was 3.0±0.0 after injection of LDL without enrichment of probucol compared with 1.0±0.0 after probucol enrichment (n=3). After intraperitoneal injection of equal

Figure 2. Immunohistochemical staining of sections from rat aortas after intraperitoneal injection of native LDL (4 mg/kg) (×40). A through E, ApoB immunoreactivity (primary antibody 10 μg/mL); F through J, NA59 immunoreactivity (primary antibody 1/500); A and F, 0 hour; B and G, 6 hours; C and H, 12 hours; D and I, 24 hours; and E and J, 48 hours. n=3 observations per time.
amounts of oxLDL, neither apoB nor oxLDL could be detected in the media or endothelium at any time up to 48 hours. The general pattern of apoB and oxLDL immunostaining was the same in the aorta and the iliac and femoral arteries, although the staining tended to be more intense in the smaller arteries.

Electron Microscopic Observations After Injection of Native LDL

At 24 hours, the endothelial cell layer of the femoral arteries from rats injected intraperitoneally and intravenously with native LDL was intact and did not clearly differ in morphology from the PBS-injected controls. Both the luminal and abluminal surfaces were rich in caveolae, and neighboring cells were connected by junctional complexes. The smooth muscle cells of the media were normal in appearance, with numerous plasma membrane caveolae and a cytoplasm dominated by myofilaments and mitochondria. Immediately inside the internal elastic lamina and in direct association with the first layer of medial smooth muscle cells, focal collections of electron-dense material were observed in the LDL-injected rats (Figure 5A). These localized aggregates showed a spatial relationship to both the caveolae and the coated pits on the surface of the smooth muscle cells (Figure 5A and 5B). At higher magnification, aggregates were found to be composed of lipid bilayers arranged in parallel and forming membrane-like whorls (Figure 5B and 5C). Similar material was also seen in the deeper parts of the media, but in decreasing amounts. On the other hand, no structures of this type were detected outside the internal elastic membrane, ie, in the subendothelial space. In the PBS-injected control vessels, deposits of the type described above were only very occasionally noted, and if so, typically in deeper parts of the media together with small vesicular fragments derived from damaged or dying cells.

Activation of NF-κB

Intraperitoneal injection of LPS (100 μg/kg) resulted in a strong increase of active NF-κB, compared with baseline, in both the arteries and liver within 24 hours as analyzed by EMSA (Figure 6). Injection of oxLDL (4 mg/kg) was found to strongly activate NF-κB in the liver, whereas no

![Figure 3](image3.png)

**Figure 3.** Semiquantitative analysis of immunohistochemical staining for apoB and NA59. Immunohistochemically stained sections were analyzed by using a modified version of the technique described by Galis et al. Consistent positive staining covering >50% of the intima was recorded as 3, consistent positive staining covering <50% of the intima as 2, variable or weak staining as 1, and no staining as 0. Values are presented as mean and SD (n=3).

![Figure 4](image4.png)

**Figure 4.** Immunohistochemical staining of sections from rat aortas 24 hours after intraperitoneal injection of native LDL or native LDL + probucol (4 mg/kg) (×100). Top, ApoB immunoreactivity (primary antibody 10 μg/mL); bottom, NA59 immunoreactivity (primary antibody 1/500); A and C, native LDL; and B and D, native LDL + probucol. n=3 observations per time.
activation was seen in the arteries (Figure 6). After injection of equal amounts of native LDL (4 mg/kg), no activation of NF-κB was seen in the liver, whereas an activation was seen in the arteries (Figure 6). With the use of an antibody to detect the active form of NF-κB (α-p65 mAb), positive nuclear staining was seen in the arterial endothelium at 12 and 24 hours after injection of native LDL, whereas weaker staining was seen in the media localized to both the nucleus and cytoplasm (Figure 7).

### Expression of Adhesion Molecules and Monocyte Adhesion

Positive staining for the adhesion molecule ICAM-1 was restricted to the arterial endothelium beginning at 24 hours after injection of native LDL and persisting up to 48 hours (Figure 8). Positive staining for ICAM-1 was not noticed after injection of PBS or oxLDL at any time. A 463-bp band was observed after RT-PCR analysis for ICAM-1 in RNA extracted from rats at 24 hours after injection of PBS, native

![Figure 5](image-url)

**Figure 5.** Electron microscopic examination of a femoral artery 24 hours after intraperitoneal injection of native LDL (4 mg/kg). Photomicrographs demonstrate deposition of membrane-like whorls just inside the internal elastic lamina (IEL), in close contact with smooth muscle cells (SMC) in the first layer of the media. Arrowheads indicate caveolae. Arrows and CP indicate coated pits. A, ×25 000; B, ×100 000; and C, ×150,000. n=3 observations per time.

![Figure 6](image-url)

**Figure 6.** EMSAs showing NF-κB binding activity in nuclear extracts from arteries and livers 24 hours after intraperitoneal injection. Lanes 2 through 5, arteries; lanes 6 through 8, livers. Lane 1, probe only; 2, PBS control; 3, lipopolysaccharide 100 μg/kg; 4, native LDL 4 mg/kg; 5, oxLDL 4 mg/kg; 6, PBS control; 7, oxLDL 4 mg/kg; and 8, native LDL. Arrowhead indicates positions of specific complexes. n=3 experiments.
LDL, or native LDL+probucol (Figure 9). The mRNA levels, however, were lowered in the PBS- and native LDL+probucol–injected rats when compared with native LDL–injected rats (Figure 9). Semiquantitative analysis of the ICAM-1 RT-PCR bands showed a more than fivefold increase of the ICAM-1/β-actin OD ratio in the native LDL–injected rats when compared with PBS- or native LDL+probucol–injected rats. (P=0.005) (Figure 10). Monocytes could not be detected in the arterial wall with the ED-1 antibody, and no accumulation of leukocytes could be noted either on the luminal vessel surface or in the subendothelial space at 24 hours after injection of native LDL.

**Discussion**

The hypothesis that oxidative modification of LDL plays a significant role in atherogenesis is strongly supported by cell culture studies and animal models of atherosclerosis. Still, the mechanisms behind LDL oxidation in vivo have been difficult to elucidate, and our knowledge of the biologically relevant effects of oxLDL in the arterial wall is limited. Therefore, we set out to establish an animal model to study the mechanisms and biological effects of local LDL oxidation in the arterial wall.

Previous work has shown accumulation of lipoproteins in the arterial wall to be a crucial step in atherogenesis. Areas prone to develop atherosclerotic lesions have enhanced retention of lipoproteins. Retention and aggregation of LDL-sized particles can be seen in the subintimal extracellular matrix 2 hours after bolus infusion of heterologous LDL in rabbits, and transendothelial transport of LDL has been described in rats after LDL injection. The entrapment of LDL is believed to be mediated by an interaction of apoB-100 segments with proteoglycans, and these complexes form aggregates that are susceptible to oxidation in vitro. Colocalization of epitopes on apoB and oxLDL has been described in human atherosclerotic lesions, but at least some antibodies against oxLDL also detect oxidation-specific epitopes on proteins other than LDL.

Our results support and extend the previous work. The colocalization of apoB and oxLDL in the endothelium and media and the fact that probucol enrichment of LDL before injection reduced LDL oxidation without affecting apoB accumulation strongly suggest that LDL oxidation occurs locally in the arterial wall after retention. Considering the slight increase in relative electrophoretic mobility of LDL reisolated from the peritoneal cavity at 6 and 12 hours after

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**Figure 7.** Immunohistochemical staining for NF-κB p65 immunoreactivity in rat aortas 24 hours after (A) intraperitoneal injection of native LDL (4 mg/kg) and (B) intraperitoneal injection of PBS (primary antibody 3 μg/mL) (×100). Arrows indicate endothelium. n=3 observations per time.

**Figure 8.** Immunohistochemical staining for ICAM-1 immunoreactivity in rat aortas after intraperitoneal injection of native LDL (4 mg/kg). Arrowheads indicate endothelium. A, 0 hour; B, 12 hours; C, 24 hours; and D, 48 hours (primary antibody 2 μg/mL) (×40). n=3 observations per time.
intraperitoneal injection, a minor modification occurring in the peritoneal cavity cannot be excluded. Still, this is not a requirement for the accumulation and oxidation of LDL in the arterial wall, since LDL injected intravenously was also found to accumulate in the arterial wall and evoke similar effects. Furthermore, LDL injected intraperitoneally did not activate NF-κB in the liver and did not show an increase in electrophoretic mobility when reisolated from plasma up to 12 hours after injection. The relatively rapid modification of LDL and the biological effects seen in our model may be due to the fact that rats lack extracellular SOD.39 It cannot be excluded, however, that part of the staining for oxLDL is due to cross-reactivity with oxidation-specific epitopes on proteins other than LDL. The electron microscopic observation of an extracellular, focal collection of lipid bilayers forming membrane-like whorls inside the internal elastic membrane 24 hours after injection of LDL suggests that LDL may be taken up locally in large amounts in the arterial wall via the endothelium and then processed in the media to generate free lipid molecules that aggregate into membrane-like complexes. Extracellular, multilamellar liposome-like structures have been described in human and rabbit atherosclerotic lesions.40 In animal models, these structures form early in lesion development and do not require the presence of foam cells. It is not known whether these structures represent modified aggregates of lipoproteins trapped in the extracellular matrix42 or excreted lipid products from arterial wall cells.33 The close proximity of these aggregates to caveolae is of interest, in view of recent observations on cultured fibroblasts indicating that caveolae represent sites where cholesterol derived from LDL is accumulated and released from the cell.43

The absence of an accumulation of oxLDL and of an inflammatory response in the arterial wall after injection of oxLDL is not surprising. Previous work has shown that injection of oxLDL in the rat results in rapid clearance through scavenger receptors in the liver, with a t1/2 of a few minutes.44 Furthermore, plasma is rich in antioxidants.45 Native LDL, on the other hand, disappears from serum at a low rate.44 The present finding of a strong activation of NF-κB in the liver after injection of oxLDL could be explained by its rapid uptake by the liver. Activation of NF-κB in the liver has also been reported after injection of mildly modified LDL.46 In our model, injection of native LDL did not activate NF-κB in the liver, and activation in the endothelium could not be detected until epitopes present on oxLDL were seen at the same location, suggesting that NF-κB is activated as a result of LDL oxidation.

Activation of NF-κB in endothelial cells by modified LDL has been well studied in vitro. Endothelial cells under flow stress and incubated with oxLDL had an increase in superoxide production, NF-κB activation, and adhesion molecule expression.47 LDL minimally modified by lipoxygenase increased NF-κB activation in endothelial cells through a mechanism involving cAMP and protein kinase A.48 In addition, NF-κB has been shown to be associated with the increased expression of macrophage colony stimulating factor in vascular endothelial cells after exposure to oxLDL.49,50 In contrast, studies on oxLDL-induced NF-κB activation in vascular smooth muscle cells differ in their results. Ares and coworkers51 showed that Cu2+-oxidized LDL inhibited NF-κB activation and induced activator protein-1 in human smooth muscle cells in vitro, whereas Maziere and cowork-
ers52 showed activation of NF-κB. NF-κB has also been shown to be activated and ICAM-1 expressed in the neointimal and medial smooth muscle cells of rat femoral arteries after balloon injury.53

The adhesion molecule ICAM-1 mediates leukocyte adhesion to the endothelium.54 Structural and deletion analyses show that putative NF-κB binding sites in the promoter of the ICAM-1 gene are required for their induction by cytokines.55,56 From in vitro cell culture studies, ICAM-1 can be expressed in endothelial cells after stimulation from cytokines such as tumor necrosis factor.57 ICAM-1 could also be activated by lysophosphatidylcholine in cultured human and rabbit arterial endothelial cells.58 Minimally modified LDL, however, has been shown to increase P-selectin expression, but not ICAM-1 and vascular cell adhesion molecule (VCAM)-1, in vitro.59 In our model, NF-κB activation in the endothelium after injection of native LDL and its subsequent oxidation were associated with expression of ICAM-1 and an increase in mRNA levels, which could be reduced when LDL was enriched with probucol before injection.

Important knowledge of the biological in vivo effects of oxLDL has been gained from animal work. Systemic administration of oxLDL in hamsters results in leukocyte adhesion in the microcirculation and aortic endothelium and is believed to be mediated by reactive oxygen intermediates and platelet-activating factor (PAF) or PAF-like substances.60,61 The water-soluble antioxidant vitamin C, but not the lipid-soluble antioxidants vitamin E and probucol, prevents leukocyte adherence,62 indicating that this model involves the action of water-soluble reactive oxygen intermediates and may not be ideal to study the biological effects of LDL oxidation in the vessel wall. Furthermore, the presence of oxLDL in the circulation is unlikely to occur in vivo.63 The systemic injection of minimally modified LDL, but not of native LDL, into mice has been shown to activate NF-κB in the liver and stimulate the expression of inflammatory genes such as macrophage colony stimulating factor in the circulation and JE (the mice homologue of monocyte chemoattractant protein-1) in the liver.65 Mice that readily develop fatty streaks on an atherogenic diet (eg, C57BL/6j) were found to accumulate conjugated dienes in the liver, associated with NF-κB activation and expression of the inflammatory genes macrophage colony stimulating factor, JE, heme oxygenase, and members of the serum amyloid family.66 In hypercholesterolemic rabbits, oxLDL has been shown to accumulate in arteries,67 and hypercholesterolemia is associated with expression of VCAM-1 in endothelial cells overlaying fatty streaks.68 ICAM-1 and VCAM-1 have also been demonstrated in human atherosclerotic lesions.65,66

Our results support the hypothesis that LDL oxidation occurs locally in the arterial wall after retention of apoB, resulting in a mainly endothelial activation of NF-κB and endothelial expression of ICAM-1. It is highly unlikely that the findings could be explained by an immune response to human LDL, considering the short time span. Thus, this relatively simple model offers the opportunity to study mechanisms behind LDL oxidation in the vessel wall and to analyze early biological effects of LDL oxidation in vivo. Furthermore, this model could also be used to rapidly evaluate the effects of therapeutic intervention on the early events in atherogenesis.

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