Oxidation of Low Density Lipoprotein Enhances Its Potential to Increase Intracellular Free Calcium Concentration in Vascular Smooth Muscle Cells

Burkhard Weisser, Rudolf Locher, Thomas Mengden, and Wilhelm Vetter

There have been suggestions that oxidation of low density lipoproteins (LDL) might increase their atherogenic potential. Because changes in intracellular free calcium concentration \([\text{Ca}^{2+}]\), have been linked to atherogenesis, we compared the influence of oxidized LDL (Ox-LDL) and native LDL (N-LDL) on \([\text{Ca}^{2+}]\), in vascular smooth muscle cells cultured from rat aortas. For determination of \([\text{Ca}^{2+}]\),, fura-2 fluorescence was used. LDL was isolated by ultracentrifugation from the sera of human donors \((n=17)\). In N-LDL, oxidation was prevented by addition of antioxidants, whereas Ox-LDL was obtained by auto-oxidation. The extent of oxidation was assessed by measurement of thiobarbituric acid–reactive substances. Addition of Ox-LDL \((20 \mu\text{g}\text{ protein/ml})\) to the vascular smooth muscle cells induced a mean increase of \(129\pm13\%\) in \([\text{Ca}^{2+}]\), compared with \(81\pm7\%\) with N-LDL \((p<0.01)\). Dose–response curves from 1 to 20 \(\mu\text{g}\text{ /ml}\) (six experiments) confirmed this difference within the entire dose range. These results indicate that a more pronounced increase in \([\text{Ca}^{2+}]\), induced by Ox-LDL might be one of the cellular mechanisms responsible for the higher atherogenic potential of Ox-LDL compared with N-LDL, as \([\text{Ca}^{2+}]\), is an important second-messenger system involved in many atherogenic processes such as hypertrophy, cell migration, and cell damage. (Arteriosclerosis and Thrombosis 1992;12:231–236)

Low density lipoproteins (LDL) are known to be a major risk factor for accelerated atherosclerosis\(^1,2\) and subsequently for cardiovascular diseases such as coronary heart disease. The relation between high levels of LDL and the development of atherosclerosis is well established, and correction of hypercholesterolemia has been shown to reduce the progression of atherosclerosis and mortality from cardiovascular disease.\(^3,4\) Nevertheless, there is still controversy about the mechanisms of atherogenesis and the pathophysiological role of LDL in this process.

Recently, there has been increasing evidence that oxidative modification of LDL may increase its atherogenic potential.\(^5\) The earliest recognized lesion in atherogenesis is the fatty streak, which consists mostly of an accumulation of macrophages loaded with cholesterol esters (foam cells) just beneath the endothelium. Ten years ago, Henriksen et al\(^6,7\) demonstrated that oxidized LDL (Ox-LDL) was taken up three to 10 times more rapidly by cultured macrophages than was native LDL (N-LDL). Furthermore, the cytotoxicity of LDL during atherogenesis seems to depend on oxidative modification.\(^8,9\) During atherogenesis, smooth muscle cells accumulate in the arterial intima by cell proliferation and migration. Ox-LDL has been shown to be chemotactic for arterial smooth muscle cells in culture, whereas N-LDL is without significant activity.\(^10\) It has been demonstrated that peroxidation of LDL is dependent on low concentrations of copper or iron and, in consequence, is inhibited by EDTA or antioxidants such as butylated hydroxytoluene and vitamin E.\(^11-13\) To date, the mechanisms for the increased atherogenic potential induced by oxidation are unclear.

Because two key mechanisms of atherogenesis, proliferation and cell contractility of vascular smooth muscle cells (VSMCs),\(^14\) are probably triggered by changes in intracellular calcium \([\text{Ca}^{2+}]\), and other mechanisms, we studied \([\text{Ca}^{2+}]\), of VSMCs exposed to Ox-LDL. This was compared with that of N-LDL, which in previous studies from our laboratory had been shown to activate VSMCs and to elevate \([\text{Ca}^{2+}]\),.\(^15-17\) The determination of this parameter...
might further elucidate the role of LDL and the mechanisms of action of its oxidized forms in cardio-vascular disease.

Methods

Materials

Fura 2→penta-acetoxyethyl ester (fura 2-AM) was purchased from Calbiochem (Zürich, Switzerland). Dulbecco’s modified Eagle’s medium (DMEM) and Dulbecco’s phosphate-buffered saline (PBS) were obtained from Amimed (Zürich, Switzerland). Na₂EDTA was obtained from Merck-Schuchardt (Zürich, Switzerland). Anti-desmin antibody and anti-mouse immunoglobulin G-fluorescein isothiocyanate antibody were purchased from Boehringer Mannheim (Rotkreuz, Switzerland). All other chemicals were obtained from Sigma Chemical (Zürich, Switzerland).

Preparation of Vascular Smooth Muscle Cells

VSMCs were cultured from rat aortas (female animals of the Wistar-Kyoto strain, 6–8 weeks old) and grown for several passages according to Ross. VSMCs were identified as such according to morphological criteria and immunologically with an anti-desmin antibody plus a second fluorescent anti-immunoglobulin G antibody according to Debus et al. The cells were allowed to grow for 4–5 days in 5% CO₂ and 95% air at 37°C. Cells were cultured in DMEM supplemented with 2 mM L-glutamine, 1% nonessential amino acids, and 10% fetal calf serum. VSMCs were used after five to 10 passages. Confluent cells were detached by trypsinization (0.05% trypsin) and were subsequently suspended in PBS supplemented with 1% bovine serum albumin. After detachment, cells were washed twice and suspended in PBS (approximately 2×10⁶ cells/ml) and incubated with 2 μM fura 2-AM at 37°C for 20 minutes.

Measurement of Intracellular Free Calcium Concentration

After loading the cells with 2 μM fura 2-AM, 1-ml aliquots of the cell suspensions were spun down at 100g and the supernatant was removed. Cells were resuspended in PBS buffer immediately before use. Fluorescence of cells was measured at 37°C, while stirring, in a cuvette placed in an SLM-Aminco SPF-500C spectrofluorometer (SLM Instruments, Urbana, Ill.). This fluorometer allows rapid, computer-sustained alterations of excitation wavelength. For determination of [Ca²⁺]ᵢ, excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm were chosen. Excitation bandwidth was set at 7.5 nm and the emission bandwidth at 5 nm. Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated against 0.5% Triton X-100 for the maximum, followed by addition of 1.0 M tris(hydroxymethyl)aminomethane (Tris) buffer and 300 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH <8.8, for minimum fluorescence according to Grynkiewicz et al. Computer-assisted calculation of the ratio of the emitted fluorescence signals permits calculation of [Ca²⁺].

Preparation of Low Density Lipoprotein

LDL was isolated from freshly drawn plasma samples (50 ml) from 17 different human donors. N-LDL was prepared in the presence of 1 mM EDTA by ultracentrifugation according to Redgrave et al. The density of LDL was 1.019–1.063 g/ml. LDL was then dialyzed at 4°C for 24 hours against three changes of 1.5 l of 0.15 M NaCl and 1 mM EDTA at pH 7.4 and used within 3 weeks. LDL was sterilized by the use of 0.45-μM Millipore filters (Millipore, Molsheim, France) and stored in the dark at 4°C. Because oxidation of LDL is dependent on low concentrations of copper and iron, substantial further oxidation of LDL cannot occur after dialysis against EDTA and without addition of small amounts of copper or iron.

Oxidation of Low Density Lipoprotein

The oxidative modification of LDL was performed by incubation of freshly prepared LDL with 5 μM CuSO₄ in the absence of EDTA for 24 hours at 37°C. To stop further oxidation, LDL was then refrigerated at 4°C, and 1 mM EDTA was added, followed by extensive dialysis against three changes of 1.5 l of 0.15 M NaCl and 1 mM EDTA at pH 7.4 and sterilization by the use of 0.45-μM Millipore filters. Oxidation of LDL in comparison to N-LDL was controlled with absorption at wavelength 234 nm. Absorption increases if LDL is oxidized, indicating an increase in diene formation of fatty acids.

Furthermore, LDL oxidation was measured by a slight modification of Yagi's fluorometric method. The extent of oxidation was assessed by determination of thiobarbituric acid–reactive substances (TBARS). LDL (20 μl) was mixed with 1 ml 12N sulfuric acid, and 0.5 ml 10% phosphotungstic acid was then added. After centrifugation at 2,000g for 5 minutes, the sediment was mixed with 1 ml 12N sulfuric acid before the centrifugation was repeated. The sediment was then suspended in fresh thiobarbituric acid reagent (1 ml of a solution of 0.67% aqueous thiobarbituric acid in 0.5 M Tris buffer, adjusted to pH 3.2–3.8 with glacial acetic acid). The suspension was then heated at 95°C for 1 hour. After cooling at room temperature and centrifugation, the supernatant was removed and fluorometric measurement (515 nm excitation, 553 nm emission) was performed in an SLM-Aminco SPF 500 spectrofluorometer (SLM Instruments). The standard was obtained by reacting 0.5 nmol of 98% malonaldehyde bis(dimethylacetal) with thiobarbituric acid reagent. The intensity of fluorescence was linearly related to the malondialdehyde concentration. The concentration of Ox-LDL was calculated according to the relation of the fluorometric signal obtained with LDL and the standard. All LDL samples were measured in duplicate.
It has been shown that in vitro, auto-oxidized LDL has very similar properties to LDL isolated from macrophages and to LDL incubated with cultured endothelial cells.\(^5\) Determination of protein was performed by Lowry's method.\(^2^5\) Concentrations of LDL are reported as micrograms protein per milliliter throughout this study. Statistical analyses were performed with the paired t test for comparison between N-LDL and Ox-LDL. Data are given as mean±SEM.

**Results**

**Oxidation of Low Density Lipoprotein**

As shown in previous studies,\(^2^3\) oxidation of LDL was accompanied by a decrease in the yellow carotenoid color that is characteristic of freshly prepared LDL. Oxidation parameters were significantly (\(p<0.001\)) higher for Ox-LDL, indicating that auto-oxidation had occurred in the presence of small amounts of copper and iron.

With both methods, absorption at wavelength 234 nm and measurement of TBARS, increased oxidation could be demonstrated. The mean values for absorption and TBARS obtained with N-LDL and Ox-LDL are shown in Table 1. Mean concentration of TBARS in Ox-LDL was increased more than twofold compared with N-LDL.

**Intracellular Calcium Concentration**

Both N-LDL and Ox-LDL significantly (\(p<0.01\)) increased \([\text{Ca}^{2+}]\) in VSMCs. In Figure 1, a representative original tracing of \([\text{Ca}^{2+}]\), as assessed by the fura-2 method is shown. Peak calcium levels were reached about 30 seconds after LDL was added to the muscle cell suspensions. In this figure, \([\text{Ca}^{2+}]\), induced by N-LDL and Ox-LDL (20 \(\mu\)g protein/ml) are compared with calcium transients induced by angiotensin II (100 nmol/1). In this particular experiment, angiotensin II induced an increase in \([\text{Ca}^{2+}]\), from 98 to 290 nmol/l as opposed to smaller increases seen after addition of Ox-LDL (95–180 nmol/l) or N-LDL (92–141 nmol/l). The peaks in calcium elevation seen after addition of angiotensin II tended to be reached earlier than those after LDL stimulation. In the experiment shown in Figure 1, peak \([\text{Ca}^{2+}]\), was reached about 10 seconds after addition of angiotensin II and 20–30 seconds after LDL was added to the VSMC suspensions.

<table>
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<tr>
<th>TABLE 1. Thiobarbituric Acid–Reactive Substances Content and Absorption at 234 nm of Native and Oxidized Low Density Lipoprotein</th>
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<tr>
<td><strong>Ox-LDL</strong></td>
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<td>((n=17))</td>
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<tr>
<td>Absorption (234 nm)</td>
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<td>TBARS (nmol/mg protein)</td>
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<td><strong>Values are mean±SEM.</strong></td>
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Oxidized low density lipoprotein (Ox-LDL) was obtained by oxidation of native low density lipoprotein (N-LDL) with 5 \(\mu\)M CuSO\(_4\) for 24 hours at 37°C. TBARS, thiobarbituric acid–reactive substances.

**FIGURE 1.** Original tracing of a representative experiment showing the effect of angiotensin II (A II), oxidized low density lipoprotein (ox-LDL), and native LDL (n-LDL) on intracellular free calcium (nmol/l). Arrows indicate the addition of LDL (20 \(\mu\)g protein/ml) or angiotensin II to the muscle cell suspensions. Time course of the elevation of intracellular free calcium is shown.

Mean \([\text{Ca}^{2+}]\), at baseline and after stimulation with 20 \(\mu\)g LDL protein/ml are shown in Table 2. In Figure 2, mean increases in \([\text{Ca}^{2+}]\), (percent) are compared with increases obtained by stimulation with 100 nmol/l angiotensin II. In 15 of the 17 LDL preparations from different donors, Ox-LDL–induced elevations in \([\text{Ca}^{2+}]\) were more pronounced than in those seen with N-LDL.

**Dose–Response Relation**

Increasing doses of LDL were added to the VSMCs. The starting dose was 1 \(\mu\)g protein/ml. Thereafter, doses of 2.5, 5, 10, and 20 \(\mu\)g/ml were used. A significantly (\(p<0.01\)) more pronounced elevation in \([\text{Ca}^{2+}]\) was induced by all doses of Ox-LDL compared with N-LDL. The dose–response curves are shown in Figure 3. Data are given as change (percent) in \([\text{Ca}^{2+}]\). The configuration of both curves is similar. After an initial sharp, dose-dependent increase, higher concentrations of LDL did not produce substantial further calcium elevations. Both N-LDL and Ox-LDL dose–response curves seem to display a saturation characteristic. Even with higher doses of N-LDL, the increase in \([\text{Ca}^{2+}]\), did not reach the levels obtained with Ox-LDL.

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<th>TABLE 2. Intracellular Free Calcium Concentration in Vascular Smooth Muscle Cells Before and After Stimulation With Oxidized and Native Low Density Lipoprotein</th>
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<tr>
<td><strong>Ox-LDL</strong></td>
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<td>((n=17))</td>
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<tr>
<td>Baseline ([\text{Ca}^{2+}]) (nM)</td>
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<tr>
<td>Stimulated ([\text{Ca}^{2+}]) (nM)</td>
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Vascular smooth muscle cells were loaded with fura-2. Cells were stimulated at 37°C with 20 \(\mu\)g protein/ml of native (N) or oxidized (Ox) low density lipoprotein (LDL). Values are mean maximum increases±SEM. Time course of the increase in intracellular free calcium (\([\text{Ca}^{2+}]\)) is shown in Figure 1.
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FIGURE 2. Bar graph showing mean increases in intracellular free calcium concentration as a percentage of baseline values after exposure of vascular smooth muscle cell suspensions to native low density lipoprotein (n-LDL), oxidized LDL (ox-LDL), and angiotensin II (A II). Concentrations were 20 μg protein/ml for n-LDL and ox-LDL and 100 nM for angiotensin II. Oxidized LDL caused a significantly (p<0.01) more pronounced increase than did native LDL. Both increases were significantly (p<0.01) lower than that after angiotensin II. Mean±SEM of 17 experiments is given.

Discussion

The data presented in this study indicate that oxidation of LDL increases its potential to raise [Ca²⁺], compared with N-LDL. What are the implications of these results for the influence of Ox-LDL on atherogenesis?

It is not known which concentration of Ox-LDL is likely to exist in the neighborhood of the smooth muscle cells in the arterial wall, but our results were obtained with Ox-LDL concentrations substantially lower than those that occur normally in serum (85–130 mg/100 ml). The possibility cannot be excluded that aggregation of Ox-LDL, not only oxidation per se, also contributes to the results of our study. Furthermore, it could be argued that in vitro oxidation produces an Ox-LDL that might be oxidized more extensively than that in vivo. However, in a recent study, Ox-LDL was shown to be present in vivo. In that study, the concentration of Ox-LDL measured in TBARS (as in our investigation) approximately doubled in healthy volunteers after they smoked five to seven cigarettes. Although our absolute TBARS values for both N-LDL and Ox-LDL are higher than theirs, the range of oxidation expressed as an increase of TBARS relative to N-LDL is comparable. In addition, Ox-LDL has been shown by immunological methods to be present in serum and in the arterial wall of atherosclerotic animals.

Little is known about the action of Ox-LDL on VSMCs. However, there has been increasing evidence that oxidative modification of LDL increases its atherogenic effects on the arterial wall. In experimental models of atherosclerosis, many steps that lead to the development of lesions of the arterial wall, such as the fatty streak, have been shown to be promoted by the oxidation of LDL. The earliest steps in atherogenesis, including the recruitment of circulating monocytes, the subsequent migration of monocytes into the arterial wall, and the uptake of LDL by monocytes/macrophages, have all been shown to be increased by oxidative modification of LDL, and at least some of the Ox-LDL is taken up by the scavenger receptor. Once LDL is incorporated into foam cells, cytotoxic action of LDL may produce endothelial damage. Again, the cytotoxic action of LDL seems to be enhanced by oxidation of LDL.

Endothelial cells have been shown to oxidize LDL in vitro, and LDL may be oxidized when it is taken up by endothelial cells. Our data might indicate that LDL could have increased biologic action on the VSMCs in the arterial wall after a possible oxidative modification during its passage through the endothelial layer. Furthermore, platelets have been shown to render LDL more atherogenic, and this mechanism appears to be dependent on an oxidative modification of LDL by the platelets. Recent clinical data could be added to the experimental results indicating increased atherogenicity of oxidized lipids. Plasma lipid oxidation was found to be significantly higher in patients with severe atherosclerosis compared with normal controls. Furthermore, probucol, an antioxidant drug, has been shown to prevent progression of atherosclerosis in familial hypercholesterolemic rabbits even without a lowering of plasma cholesterol.
levels. Our findings might explain, at least in part, the increased atherogenic potential of Ox-LDL, as atherosclerosis and atherosclerosis have been linked to changes in [Ca^{2+}]. Several processes involved in atherosclerosis, such as membrane permeability, secretion of extracellular matrix proteins, cell migration, and cellular damage, are, among others, regulated by changes in [Ca^{2+}]. Thus, not only end-stage atherosclerotic disease, such as calcification of plaques and necrosis, but also the initial steps leading to vascular damage are associated with alterations of intracellular calcium metabolism.

Furthermore, calcium-chelating agents and calcium antagonists have been shown to induce regression of atherosclerotic lesions caused by dietary cholesterol in animals. Clinical evidence for the beneficial effects of calcium channel blockers is provided by a placebo-controlled, double-blinded study showing that nifedipine retarded the angiographically demonstrated progression of lesions of the coronary arteries in patients with coronary artery disease.

These and other findings demonstrate the possible involvement of alterations in intracellular calcium metabolism in the process of atherosclerosis. Although many risk factors for cardiovascular disease are known, atherosclerosis represents a relatively uniform response to different stimuli, and there is the hypothesis that many stimuli of atherosclerosis may act through changes in calcium metabolism.

In conclusion, our results may indicate a link between alterations in intracellular calcium metabolism and the importance of oxidation of LDL for its atherogenic potential. Many of the cellular processes leading to atherosclerosis and cardiovascular disease are, among others, regulated by changes in [Ca^{2+}]. We propose that at least some of the mechanisms leading to an increased atherogenic potential of Ox-LDL may be regulated by alterations in intracellular calcium metabolism, which is an important second-messenger system.

**KEY WORDS**  oxidized low density lipoproteins  vascular smooth muscle cells  cytosolic calcium  atherogenesis
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