Vitamin C Protects Human Vascular Smooth Muscle Cells Against Apoptosis Induced by Moderately Oxidized LDL Containing High Levels of Lipid Hydroperoxides

Richard C. M. Siow, Justin P. Richards, Kevin C. Pedley, David S. Leake, Giovanni E. Mann

Abstract—Vascular cell death is a key feature of atherosclerotic lesions and may contribute to the plaque “necrotic” core, cap rupture, and thrombosis. Oxidatively modified low-density lipoproteins (LDLs) are implicated in the pathogenesis of atherosclerosis, and dietary antioxidants are thought to protect the vasculature against LDL-induced cytotoxicity. Because LDL oxidative modification may vary within atherosclerotic lesions, we examined the effects of defined, oxidatively modified LDL species on human arterial smooth muscle cell apoptosis and the cytoprotective effects of vitamin C. Moderately oxidized LDL (0 to 300 μg protein/mL), which has the highest content of lipid hydroperoxides, induced smooth muscle cell apoptosis within 6 hours, whereas native LDL and mildly and highly oxidized LDL had no effect. Moderately oxidized LDL increased cellular DNA fragmentation, release of fragmented DNA into the culture medium, and annexin V binding and decreased mitochondrial dehydrogenase activity and expression of the antiapoptotic mediator Bcl-xL. Treatment of cells with native LDL together with the lipid hydroperoxide 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (HPODE, 200 μmol/L, 6 to 24 hours) also induced apoptotic cell death. Pretreatment of smooth muscle cells with vitamin C (0 to 100 μmol/L, 24 hours) attenuated the cytotoxicity and apoptosis induced by both moderately oxidized LDL and HPODE. Our findings suggest that moderately oxidized LDL, with its high lipid hydroperoxide content, rather than mildly or highly oxidized LDL, causes apoptosis of human smooth muscle cells and that vitamin C supplementation may provide protection against plaque instability in advanced atherosclerosis.

Key Words: smooth muscle, vascular low-density lipoprotein, oxidized apoptosis ascorbic acid Bcl-xL atherosclerosis

Oxidatively modified LDLs are involved in the initiation and progression of atherosclerosis and have been localized within atherosclerotic lesions. Evidence that oxidized LDL induces vascular cell death by apoptosis and the presence of apoptotic cells in atherosclerotic and restenotic lesions has potential clinical implications for atherogenesis and thrombosis. Although smooth muscle cell death may enhance lesion regression, it can also result in the accumulation of necrotic debris within the lesion core and instability of the fibrous plaque cap, leading to its rupture and thrombosis. The extent to which LDL becomes oxidized within atherosclerotic lesions has not been well defined, and levels of oxidation may differ within lesions. Because the toxicity of oxidized LDL appears to increase with the degree of oxidative modification, this may contribute to the impaired vascular reactivity in patients with coronary artery disease.

Oxidized LDL is cytotoxic to smooth muscle cells, endothelial cells, and macrophages and has been shown to induce apoptosis in several vascular cell types. Although a mildly oxidized species of LDL (moxLDL) has been reported to induce endothelial cell toxicity and apoptosis, only limited information is available on the effects of moderately oxidized LDL (modLDL), which contains the highest lipid hydroperoxide levels. Dietary antioxidants attenuate the cytotoxic effects of oxidatively modified LDL and restore endothelial function in patients with coronary artery disease. Moreover, a low plasma vitamin C concentration is implicated as an independent predictor of unstable coronary artery disease. In this context, we previously reported that vitamin C protects human vascular smooth muscle cells against oxidized LDL–induced adaptive increases in the synthesis of the key intracellular antioxidant glutathione. These results provided the first evidence that vitamin C spares endogenous antioxidant responses in human vascular smooth muscle cells exposed to atherogenic lipoproteins.

In this study, we investigated further the cytoprotective effects of vitamin C by examining LDL-induced apoptosis of human smooth muscle cells. modLDL, which contains the peak level of lipid hydroperoxides, but not highly oxidized...
Lipid Hydroperoxide Content and Electrophoretic Mobility of LDL

<table>
<thead>
<tr>
<th>Degree of LDL Oxidation</th>
<th>Lipid Hydroperoxide Content (nmol/mg protein)</th>
<th>Electrophoretic Mobility (% Increase Relative to nLDL)</th>
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<tbody>
<tr>
<td>nLDL</td>
<td>43±24</td>
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<tr>
<td>moxLDL</td>
<td>49±21</td>
<td>19±8*</td>
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<tr>
<td>modLDL</td>
<td>615±122*</td>
<td>142±40*</td>
</tr>
<tr>
<td>oxLDL</td>
<td>110±47*</td>
<td>347±80*</td>
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Human nLDL was oxidized with CuSO4 as described in Methods to obtain moxLDL, modLDL, and oxLDL. Values are mean±SD, n=30.

LDL (oxLDL), caused smooth muscle cell apoptosis within 6 hours; this apoptosis was attenuated markedly by pretreatment with vitamin C. In addition, cells treated with native LDL (nLDL) and the lipid hydroperoxide 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (HPODE) also underwent apoptosis, which was attenuated by vitamin C. Our findings may have important clinical implications for therapies designed to arrest LDL oxidation, because modLDL appears to be potentially more atherogenic than oxLDL.

Methods

Reagents
Smooth muscle cell culture medium (MCDB 131) was obtained from Clonetics; cellular DNA fragmentation ELISA kit, from Boehringer Mannheim; ApoAlert annexin V apoptosis kit, from Clontech; HPODE, from Biomol Research Laboratories; Bcl-xL polyclonal antibody, from Transduction Laboratories; and the enhanced chemiluminescence kit, from Amersham. All other reagents were of the highest analytical grade and were obtained from Sigma Chemical Co.

Isolation and Oxidation of LDL
LDL was isolated from the blood of healthy volunteers by sequential density ultracentrifugation (1.019 to 1.063 g/mL) in the presence of 1 mmol/L EDTA. nLDL was diluted to 100 μg protein/mL in sterile PBS. nLDL was oxidized with CuSO4 (5 μmol/L net above the EDTA added to carry over from the LDL) at 37°C, and the conjugated diene content was determined every 15 minutes by removing an aliquot, measuring its absorbance at 234 nm (A234), and then returning it to the LDL sample. When A234 increased by 0.2 above the initial value after 80 to 100 minutes, moxLDL was removed, 1 mmol/L EDTA was added to stop oxidation, and the sample was stored at 4°C. The remaining LDL was further oxidized for ~3 hours until A234 reached a peak (an increase of 1.5 to 1.9). We defined this as modLDL, which contains the peak levels of lipid hydroperoxides. The level of conjugated dienes closely follows the level of lipid hydroperoxides during the early stages of LDL oxidation. A portion was removed, 1 mmol/L EDTA was added, and the sample was stored at 4°C. The remaining LDL was oxidized at 37°C for 24 hours before EDTA (1 mmol/L) was added to obtain what we define as oxLDL. During this time, the lipid hydroperoxides fall, but the later secondary products of lipid and protein oxidation increase. Solid KBr was removed by centrifugation at 250g for 10 minutes at 4°C. The oxidized LDL species were then centrifuged at 149,000g for 18 hours at 4°C to concentrate them. The oxidized LDL layer was removed and dialyzed overnight at 4°C in the dark against four 1-L volumes of phosphate buffer (140 mmol/L NaCl, 1.9 mmol/L Na2HPO4, 8.1 mmol/L NaH2PO4, and 100 μmol/L EDTA, pH 7.4). The oxidized LDL species were then filter-sterilized and stored at 4°C. LDL protein content was determined by a modified Lowry assay; lipid hydroperoxide content was determined by an iodometric method; and relative electrophoretic mobility was compared with nLDL, as an index of protein modification, using Beckman Paragon Lipo gels. The mobility of the LDL increased with the degree of oxidative modification, whereas the lipid hydroperoxide content was maximal in modLDL and significantly lower in moxLDL and oxLDL (see Table).

Culture of Vascular Smooth Muscle Cells
Human arterial smooth muscle cells (HUASMCs) were cultured from umbilical artery explants in MCDB131 medium supplemented with 10% (vol/vol) FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO2/95% air atmosphere. Cells were confirmed as smooth muscle by their typical “hill-and-valley” morphological features and by immunofluorescent staining for smooth muscle α-actin. HUASMCs between passages 3 and 7 were used in all experiments. Confluent, randomly cycling cells were incubated in the absence or presence of up to 300 μg protein/mL nLDL, moxLDL, modLDL, or oxLDL for 3 to 24 hours or nLDL in combination with HPODE (200 μmol/L) for 6 or 24 hours. Exposure to LDL was terminated by gently washing the cells twice with warmed PBS.

In experiments examining the effects of vitamin C, cells were pretreated with vitamin C (0 to 100 μmol/L) for 24 hours in serum-containing medium, monolayers were washed with warmed PBS, and cells were reincubated for an additional 3 to 24 hours in complete medium containing either defined LDL species or nLDL in combination with HPODE but in the absence of vitamin C.

Measurements of Cytotoxicity
Mitochondrial dehydrogenase activity was used as an index of cell viability and was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Confluent HUA-SCMCs were pretreated in the absence or presence of vitamin C (100 μmol/L, 24 hours), washed with PBS, and then incubated with defined LDL species (3 to 24 hours) in the absence of vitamin C. Culture medium was then replaced with fresh medium containing MTT (50 μg/mL), and cells were incubated at 37°C and 5% CO2 for an additional 1 to 2 hours. DMSO was then added to each well, and the microtiter plates were incubated at room temperature for another 20 minutes before absorbance was measured at 540 nm.

Plasma membrane damage in HUA-SCMCs was also assessed by monitoring the leakage of DNA fragments into the culture medium after treatment of confluent HUA-SCMC monolayers with oxidized LDL for defined time periods. Proliferating cells in 96-well microtiter plates were labeled with 10 μmol/L 5-bromo-2′-deoxyuridine (BrdU) for 24 hours, washed with PBS, and treated with LDL in the absence of BrdU for defined time periods. The culture medium was then collected, and labeled DNA fragments released by the cells were assayed using a DNA fragmentation ELISA (see below). Levels of fragmented DNA were expressed as absorbance at 450 nm.

Measurements of Apoptosis

DNA Fragmentation
As described previously, cellular DNA fragmentation was measured using a commercially available cellular DNA fragmentation ELISA kit. Proliferating cells in 96-well microtiter plates were incubated with 10 μmol/L BrdU for 24 hours, washed with PBS, and treated in the absence of BrdU with nLDL or oxidized LDL for defined time periods. Cells were then washed with PBS and incubated with the kit lysis buffer (BSA, EDTA, and Tween 20) for 30 minutes at room temperature, and soluble BrdU-labeled DNA fragments present in the buffer were quantified using the ELISA. Briefly, an anti-DNA antibody was adsorbed onto the wells of a microtiter plate, and lysis buffer supernatants containing BrdU-labeled DNA fragments were allowed to bind the immobilized anti-DNA antibody. After washing, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed to the surface of the microtiter plate by microwave irradiation. Labeled fragments...
were detected using an anti-BrdU antibody conjugated to horseradish peroxidase. The amount of peroxidase bound to the immune complex was determined by addition of tetramethyl benzidine substrate, and DNA fragmentation was expressed as absorbance at 450 nm.

**Annexin V Binding**

Active externalization of phosphatidylserine to the cell surface is an early event in apoptotic cell death, and annexin V binding to phosphatidylserine can be used as a marker of early apoptotic events that precede nuclear changes.31 In this study, binding of FITC-conjugated annexin V to HUASMCs treated with nLDL, oxidized LDL, or nLDL combined with HPODE was assessed using a commercially available kit (ApoAlert annexin V, Clontech). Cells were also costained with propidium iodide as a marker of cell membrane permeability, which occurs during the later stages of apoptosis and in necrosis. Cells were washed with PBS and incubated with FITC-annexin V (0.5 μg/mL) or propidium iodide (0.125 μg/mL). Staining was determined by fluorescence microscopy using dual-filter sets for FITC and rhodamine to visualize annexin V binding and nuclear propidium iodide staining, respectively.

**Bcl-xL Protein Expression**

Changes in protein expression of the antiapoptotic mediator Bcl-xL32 were determined by Western blot analysis in cells treated with LDL. In brief, confluent HUASMCs were treated with oxidized LDL for defined periods, and incubations were terminated by washing cells with ice-cold PBS. Cells were lysed in buffer (2% wt/vol SDS, 10% vol/vol glycerol, 50 mmol/L Tris-HCl, and 1 mmol/L PMSF; pH 6.8), and the lysate was heated at 100°C for 5 minutes. Sample protein content was determined, and samples were heated at 100°C in a mixture of 1% 2-mercaptoethanol and 0.05% bromophenol blue for an additional 3 minutes. Equal protein amounts were separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with a polyclonal rabbit anti-rat Bcl-xL antibody. A horseradish peroxidase–conjugated goat anti-rabbit secondary antibody was used in conjunction with enhanced chemiluminescence to visualize the 26-kDa Bcl-xL bands on autoradiographic film, and the level of expression was quantified using image analysis software.

**Statistical Analysis**

Statistical variance from the mean was determined using the normal distribution and represented by mean±SE (n=number of different cell cultures). When 2 values were compared, confidence limits were
established using the unpaired Student’s *t* test. ANOVA was used to compare multiple groups. Statistical significance between data sets was established at *P* < 0.05.

**Results**

**LDL-Mediated Cytotoxicity**

The activity of mitochondrial dehydrogenases (MTT assay) was significantly decreased after incubation of HUASMCs with modLDL (300 µg protein/mL) for 24 hours, whereas nLDL and moxLDL had negligible effects and oxLDL increased mitochondrial dehydrogenase activity above basal levels (Figure 1). The decrease in MTT activity induced by modLDL was time-dependent (0 to 24 hours) and concentration-dependent (0 to 300 µg protein/mL) (Figure 2A and 2B). Pretreatment of cells with vitamin C (100 µmol/L, 24 hours) before LDL exposure abolished the changes in MTT activity induced by modLDL and oxLDL (Figure 1), with maximal effects observed at vitamin C concentrations >50 µmol/L (Figure 2C). Plasma membrane permeability, assessed by release of fragmented DNA into the culture medium, was increased in HUASMCs treated with

![Figure 3](image_url) **Figure 3.** Effects of nLDL and oxidatively modified LDL species on release of fragmented DNA and intracellular DNA fragmentation in human arterial smooth muscle cells. After pretreatment in the absence (■) or presence (□) of vitamin C (100 µmol/L, 24 hours), cells were exposed to either nLDL, moxLDL, modLDL, or oxLDL (300 µg protein/mL) for an additional 24 hours, and release of fragmented DNA into the culture medium (A) or intracellular DNA fragmentation (B) was determined by ELISA. Values are mean ± SE (n = 3 to 6 cell cultures). *P* < 0.05 relative to control cells incubated in the absence of vitamin C; +*P* < 0.05 relative to cells treated with modLDL in the absence of vitamin C.

![Figure 4](image_url) **Figure 4.** Vitamin C inhibits DNA fragmentation induced by modLDL. After pretreatment in the absence or presence of vitamin C (0 to 100 µmol/L, 24 hours), cells were exposed to either nLDL or modLDL for an additional 24 hours, and cellular DNA fragmentation was determined by ELISA. A and B, Time-dependent (0 to 24 hours, 300 µg protein/mL) and concentration-dependent (0 to 300 µg protein/mL, 24 hours) effects of nLDL (■) or modLDL (○). C, Effects of vitamin C (0 to 100 µmol/L, 24 hours) pretreatment on nLDL-induced (■) or modLDL-induced (○) DNA fragmentation. Values are mean ± SE (n = 3 to 6 cell cultures). *P* < 0.05 relative to respective values in cells treated with native LDL; +*P* < 0.05 relative to cells treated with modLDL in the absence of vitamin C.
modLDL (300 μg protein/mL, 24 hours) but not in those treated with nLDL, moxLDL, or oxLDL (Figure 3A). This release of DNA induced by modLDL was abolished after preincubation of cells with vitamin C (100 μmol/L, 24 hours) (Figure 3A).

**LDL- and HPODE-Mediated Apoptosis**

modLDL (300 μg protein/mL, 24 hours) caused an increase in cellular DNA fragmentation (Figure 3B) that was both time-dependent (Figure 4A) and concentration-dependent (Figure 4B). nLDL, moxLDL, and oxLDL had negligible effects on cellular DNA fragmentation (Figure 3B). Pretreatment of cells with vitamin C for 24 hours attenuated DNA fragmentation induced by modLDL (Figure 3B), with vitamin C providing protection at concentrations >50 μmol/L (Figure 4C). Binding of annexin V, a marker of early apoptosis, was observed in cells treated for 6 or 24 hours with modLDL (300 μg protein/mL) (Figure 5A and 5C), whereas no binding was detected in HUASMCs treated with nLDL, moxLDL, or oxLDL (300 μg protein/mL) (data not shown). Moreover, cells treated with modLDL for 24 hours, but not for 6 hours, were permeable to propidium iodide (Figure 5C), suggesting that cells progressed to a late stage of apoptosis after prolonged exposure to modLDL. Vitamin C pretreatment (100 μmol/L, 24 hours) abolished the early (6 hours) annexin V binding (Figure 5B) and attenuated propidium iodide staining (Figure 5D) in cells exposed to modLDL for 24 hours.

When smooth muscle cells were treated for 6 or 24 hours with nLDL (300 μg protein/mL) together with 200 μmol/L HPODE (Figure 6A and 6C), a level comparable to the lipid hydroperoxide content in 300 μg protein/mL modLDL. In addition, cells treated with nLDL and HPODE for 24 hours, but not for 6 hours, were also permeable to propidium iodide (Figure 6C), indicating late-stage apoptosis. Pretreatment with vitamin C (100 μmol/L, 24 hours) abolished the early (6 hours) annexin V binding (Figure 6B) and propidium iodide staining (Figure 6D) in cells exposed to HPODE for 24 hours.

**Bcl-x<sub>L</sub> Expression**

Basal expression of the antiapoptotic mediator Bcl-x<sub>L</sub> was unaltered in HUASMCs treated for up to 24 hours with nLDL, moxLDL, or oxLDL (300 μg protein/mL, data not shown), whereas treatment with modLDL for 24 hours significantly decreased Bcl-x<sub>L</sub> expression (Figure 7A and 7B). Pretreatment of cells with vitamin C (100 μmol/L, 24 hours) prevented the decrease in Bcl-x<sub>L</sub> expression induced by modLDL and appeared to slightly elevate Bcl-x<sub>L</sub> expression, although this increase was not significant. The lower-molecular-weight band observed may reflect nonspecific cross-reactivity of the antibody with the closely related Bcl-x<sub>S</sub> protein, but this was not investigated further.

**Discussion**

This study established that modLDL and the lipid hydroperoxide HPODE, but neither nLDL, moxLDL, nor oxLDL,
induced apoptosis of human vascular smooth muscle cells. Our findings suggest that the high lipid hydroperoxide content of modLDL is more cytotoxic to smooth muscle cells than secondary lipid oxidation products, such as aldehydes, ketones, and lipid alcohols, generated during extended copper oxidation of LDL. Moreover, pretreatment of cells with physiologically relevant vitamin C concentrations attenuated the cytotoxicity induced by modLDL and HPODE. The protection against smooth muscle cell apoptosis provided by vitamin C may reflect that this dietary antioxidant maintained cellular levels of the antiapoptotic mediator Bcl-xL.

Increased apoptotic cell death within atherosclerotic lesions and its contribution to plaque rupture and accumulation of the necrotic lipid core has been established. LDL is oxidized within lesions and contributes to the impaired vascular cell function observed in atherogenesis. Although the toxicity of LDL increases with the extent of LDL oxidation, the methods used to oxidize LDL vary considerably between different laboratories and are often not well defined. In this study, we used a well-defined protocol for LDL oxidation to obtain mildly, moderately, and highly oxidized LDL species. The method relies on oxidizing a low concentration of LDL (100 μg protein/mL) with copper; the kinetics of LDL oxidation under these conditions have been characterized in detail elsewhere (see Table). In agreement with our observations in human smooth muscle cells, Kuzuya et al reported that the toxicity of LDL to endothelial cells initially increases and then decreases with time of LDL oxidation. The enhanced mitochondrial enzyme activity observed in human smooth muscle cells treated with oxLDL may have arisen from increased mitochondrial metabolism of oxysterols present in higher amounts in oxLDL. Although some studies have reported that oxidized LDL enhances smooth muscle cell proliferation, this seems an unlikely explanation for our findings because protein concentrations were similar in cell monolayers treated with the different LDL species.

Recent studies have identified the presence of epitopes of LDL, oxidized by copper to increasing extents, within animal and human atherosclerotic lesions and in circulating plasma LDL. Chisholm et al proposed that the cytotoxicity of copper-oxidized LDL to cells is mainly due to cholesterol hydroperoxides, which may be more cytotoxic than hydroxycholesterols or ketocholesterols. We showed that oxLDL, which contains a higher level of hydroxycholesterols and ketocholesterols than modLDL, does not induce human smooth muscle cell apoptosis. Our findings contrast with those of Nishio and Watanabe and Harada-Shiba et al, who reported that oxidized LDL species induced smooth muscle and endothelial cell apoptosis. This discrepancy may reflect the differences in the degree of LDL oxidation and the criteria used to define oxidized LDL. Nevertheless, it is interesting that 7-ketocholesterol and 25-hydroxycholesterol have been shown to induce endothelial and smooth muscle cell apo-
Although studies in rat pheochromocytoma cells and human muscle cells than those with a hydroxyl or ketone group. Droperoxyl group may be more cytotoxic to human smooth cholesterol, cholesteryl esters, or phospholipids with a hydroperoxyl group.44 We here report the first evidence that modLDL, an endogenous antioxidant, inhibiting lipid peroxidation in apoptosis.47 We have previously reported that a combination of vitamins C and E, vitamins C and E prevented the endothelial cell apoptosis induced by oxidized LDL by inhibiting CPP32 activation. Moreover, these vitamins also prevent endothelial cell apoptosis induced by lipopolysaccharide by preserving Bcl-2 protein levels.48 Thus, vitamin C could protect against apoptosis induced by oxidative stress and lipid hydroperoxides by modulating mediators of apoptosis. It was recently shown that enhanced expression of Bcl-xL protects against atherosclerosis and endothelial cell activation,49 so maintained or enhanced expression of antiapoptotic genes by vitamin C supplementation may be protective in atherogenesis.

The extent of LDL oxidation within atherosclerotic lesions remains a controversial issue, yet our findings suggest that protein modifications associated with oxLDL are not necessarily required for LDL-induced cytotoxicity. Although antioxidant dietary vitamins protect against vascular dysfunction and plaque instability,22,23 and reduce the oxidative modification of LDL,26 it is also possible that LDL modification may be arrested at a more atherogenic “moderately oxidized” level by vitamin supplementation. Thus, modLDL could induce more smooth muscle cells within the lesion to undergo apoptosis than oxLDL. Prolonged dietary antioxidant supplementation may possibly have different effects on advanced atherosclerotic lesions, because initially antioxidants would inhibit oxidative modification of LDL in the arterial wall and limit the formation of lesions. It is beyond the scope of this study to determine whether antioxidants such as vitamin C are proatherogenic through the arrest of LDL oxidation and inhibition of neointimal smooth muscle cell apoptosis. Our study supports the current consensus that antioxidant consumption reduces coronary heart disease,50 although additional clinical trials seem warranted to establish the efficacy of vitamin C against accumulation of necrotic debris within the lesion core and instability of the fibrous plaque in atherosclerosis.

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