Oxidized LDL Modulates Bax/Bcl-2 Through the Lectinlike Ox-LDL Receptor-1 in Vascular Smooth Muscle Cells

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Abstract—Oxidized low density lipoprotein (Ox-LDL) induces apoptosis in vascular smooth muscle cells (VSMCs), which may increase atherosclerotic plaque instability. In this study, we examined the molecular mechanisms causing the Ox-LDL–induced apoptosis in VSMCs, especially focusing on the involvement of Bax/Bcl-2 and the lectinlike Ox-LDL receptor-1 (LOX-1). In cultured bovine aortic smooth muscle cells (BASMCs), Ox-LDL at high concentrations (>60 μg/mL) induced cell death as demonstrated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. DNA fragmentation was increased in BASMCs treated with high concentrations of Ox-LDL, indicating that the Ox-LDL–induced cell death in VSMCs was apoptosis. Ox-LDL upregulated LOX-1 expression through phosphorylation of extracellular signal–regulated kinase in BASMCs, and a neutralizing anti–LOX-1 monoclonal antibody, which can block LOX-1–mediated cellular uptake of Ox-LDL, prevented the Ox-LDL–induced apoptosis in BASMCs. This antibody also suppressed the increase in the Bax to Bcl-2 ratio induced by Ox-LDL in BASMCs. Furthermore, LOX-1 expression was well colocalized with Bax expression in the rupture-prone shoulder areas of human atherosclerotic plaques in vivo. LOX-1 may play an important role in Ox-LDL–induced apoptosis in VSMCs by modulating the Bax to Bcl-2 ratio. These molecular mechanisms may be involved in destabilization and rupture of atherosclerotic plaques.

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everal lines of evidence have suggested that oxidized LDL (Ox-LDL) may play crucial roles in the pathogenesis of atherosclerosis and destabilization of the atherosclerotic plaque.1 Ox-LDL can bind to scavenger receptors and subsequently be internalized by macrophages, resulting in foam cell formation.2 Ox-LDL and its lipid constituents can be major factors that cause endothelial dysfunction by transcriptionally inducing proatherogenic genes.3–5 In addition to its role in foam cell transformation of macrophages and endothelial dysfunction, Ox-LDL is known to induce apoptosis of vascular smooth muscle cells (VSMCs).6,7 Because areas of the fibrous cap in vulnerable plaques are typically thin and contain fewer VSMCs, the Ox-LDL–induced apoptosis in intimal VSMCs may increase plaque instability, leading to plaque rupture in acute coronary syndromes. However, the molecular mechanisms involved in Ox-LDL–induced apoptosis in VSMCs remain to be elucidated.

The lectinlike Ox-LDL receptor-1 (LOX-1) is a receptor for Ox-LDL that was initially cloned from cultured vascular endothelial cells.8 LOX-1 can support binding, internalization, and degradation of Ox-LDL.9 Subsequent studies have revealed that LOX-1 is also expressed by VSMCs in vitro10,11 and in vivo,12 suggesting that LOX-1 may be involved in some of the functional modulations caused by Ox-LDL in VSMCs.

In contrast to necrosis, apoptosis is regulated by various apoptosis-related proteins. Bcl-2 acts as an anti-death factor, preventing the release of cytochrome c and other apoptogenic factors from mitochondria.13 In contrast, Bax reduces mitochondrial membrane potential and thereby causes cytochrome c release and caspase activation, which leads to apoptosis.14 Bcl-215 and Bax16 are expressed in human atheromas, suggesting that these factors may in fact regulate apoptotic cell death in atherosclerotic plaques.

Therefore, in the current study, we examined whether Bax/Bcl-2 can be modulated by Ox-LDL and whether LOX-1 is involved in this process in VSMCs by use of a neutralizing anti–LOX-1 monoclonal antibody. Here we provide evidence for the first time that LOX-1 mediates, at least in part, the Ox-LDL–induced Bax/Bcl-2 modulation and apoptosis in VSMCs.

Key Words: atherosclerosis ■ lipoproteins ■ receptors ■ apoptosis

Methods

Cells and Reagents

Bovine aortic smooth muscle cells (BASMCs) were isolated and cultured as previously described.10 Cells used for experiments were

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between passage levels 2 and 5. Isolation of human LDL, oxidative modification of LDL with cupric ion, and labeling of LDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes) were performed as previously described.17 Values for thiobarbituric acid–reactive substances in Ox-LDL and native LDL were 9.86±1.32 and 1.15±0.21 nmol malondialdehyde per milligram protein, respectively. Anti–LOX-1 monoclonal antibodies (5-14418 and 2-3212) were prepared as previously described. Monoclonal antibodies for Bax (B-9) and Bcl-2 (C-2) were purchased from Santa Cruz. The mitogen-activated protein kinase (MAPK) inhibitors SB203580 and PD098059 were obtained from Calbiochem.

Measurements of Cytotoxicity
Mitochondrial dehydrogenase activity was used as an index of cell viability and was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.19 BASMCs seeded onto 96-well plates at 10⁴ cells per well were incubated in Dulbecco’s modified Eagle’s medium/1% fetal calf serum with various reagents for the indicated times. Then MTT (final concentration, 0.5 mg/mL; Roche) was added to each well, and the wells were incubated at 37°C for 4 hours. DNA fragmentation was visualized in situ on fixed cells by using the DNA fragmentation ELISA kit (Roche), followed by nuclear staining with 500 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature. DNA fragmentation was also quantified by using a cellular DNA fragmentation ELISA kit (Roche) as previously described.21 Data are expressed as mean±SD of quadruplicate wells.

Determination of DNA Fragmentation
DNA fragmentation was visualized in situ on fixed cells by using the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) procedure by Gavrielli et al20 by use of an in situ cell death detection kit (Roche), followed by nuclear staining with 500 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature. DNA fragmentation was also quantified by using a cellular DNA fragmentation ELISA kit (Roche) as previously described.21 Data are expressed as mean±SD of quadruplicate wells.

Western Blot Analysis
After BASMCs were treated with various reagents in Dulbecco’s modified Eagle’s medium/1% fetal calf serum for the indicated times, Western blot analyses for detection of LOX-1, Bax, and Bcl-2 were performed as previously described.17

Immunohistochemistry
A total of 21 human carotid endarterectomy specimens were prepared as previously described.12 After fixation with cold acetone and blocking, frozen sections were first incubated with an anti–LOX-1 monoclonal antibody (2-32), which was followed by incubation with a fluorescence dye, Alexa 488–conjugated secondary antibody for rat IgG (Molecular Probes). They were subsequently incubated with an anti-Bax monoclonal antibody, which was followed by incubation with a carbocyanine 3 (Cy3)-labeled anti-mouse IgG antibody (Chemicon International, Inc). Sections were observed under the fluorescence microscope.

Statistical Analysis
Statistical significance in the difference between 2 groups was determined by unpaired Student’s t test. ANOVA was used for comparisons among multiple groups. Statistical significance was established at P<0.01.

Results
High Concentrations of Ox-LDL Induce Apoptosis in BASMCs
To examine the effect of fully Ox-LDL on the viability of BASMCs, they were treated with various concentrations of Ox-LDL or native LDL and then subjected to the MTT assay. As shown in Figure 1A, Ox-LDL at high concentrations (>60 μg/mL) induced cell death, although it caused cellular proliferation at low concentrations (10 to 20 μg/mL). In contrast, native LDL did not affect cell viability. To determine whether

Ox-LDL Upregulates LOX-1 Expression in BASMCs Through Phosphorylation of ERK
To explore the expression of LOX-1 in BASMCs, we performed Western blot analysis in both untreated and Ox-LDL–treated BASMCs. Although expression levels of LOX-1 protein were very low under basal condition in BASMCs, they were markedly upregulated by 40 to 80 μg/mL Ox-LDL, but not by native LDL (Figure 1A; please see http://atvb.ahajournals.org/). Expression of LOX-1 protein was increased in a dose-dependent manner and peaked at 60 μg/mL Ox-LDL.
Ox-LDL has been known to activate MAPK in VSMCs. To explore whether the MAPK kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway was involved in Ox-LDL–induced LOX-1 expression, the effects of PD98059, a specific inhibitor of MEK1, were examined. As shown in Figure IB, PD98059, but not the p38 MAPK inhibitor SB202190, dose-dependently inhibited Ox-LDL–induced LOX-1 expression. These results thus indicate that MEK-ERK, but not p38 MAPK, is involved in the Ox-LDL–induced expression of LOX-1 in BASMCs.

**Ox-LDL–Induced Upregulation of LOX-1 Protein Is Followed by Ox-LDL–Induced Apoptosis in BASMCs**

To examine whether LOX-1 is involved in Ox-LDL–induced apoptosis, time-course experiments were carried out in BASMCs. LOX-1 protein levels were increased as early as 4 hours and were sustained for 24 hours (Figure 2A). In contrast, Ox-LDL–induced cell death was detectable after 8 hours and became statistically significant after 12 hours in BASMCs (Figure 2B). These data indicate that the Ox-LDL–induced upregulation of LOX-1 precedes apoptotic cell death in BASMCs.

**A Neutralizing Anti–LOX-1 Antibody Prevents Ox-LDL–Induced Apoptosis in BASMCs**

To determine whether LOX-1 was involved in the Ox-LDL–induced apoptosis in VSMCs, BASMCs were incubated with Ox-LDL (60 µg/mL) or tumor necrosis factor-α (TNF-α; 500 IU/mL) in combination with 300 µg/mL anti–LOX-1 monoclonal antibody (5-144) or an isotype-matched control IgG for 24 hours. Cell viability was assessed by MTT assay. Results are expressed as percent of initial level. Values are mean ± SD of triplicate wells. (B) BASMCs were treated with Ox-LDL (60 µg/mL) in the presence or absence (right) of 300 µg/mL anti–LOX-1 monoclonal antibody (5-144) (center) or isotype-matched control IgG (left) for 24 hours. Upper panels show DNA fragments labeled in situ by TUNEL procedure. Lower panels show nuclear staining by DAPI of the same field of view. Representative data of 3 independent experiments are shown.

**A Neutralizing Antibody for LOX-1 Inhibits Ox-LDL–Induced Modulation of Bax and Bcl-2 Expression in BASMCs**

In BASMCs, Ox-LDL increased Bax expression and decreased Bcl-2 expression in a dose-dependent manner (data not shown). In the MTT assay, this neutralizing antibody for LOX-1 dramatically reduced Ox-LDL–induced cell death in BASMCs (Figure 3A). DNA fragmentation induced by Ox-LDL was significantly decreased by incubation with this neutralizing antibody for LOX-1 (Figures 3B and 3C). In contrast, an isotype-matched control IgG did not show any significant effects on either cell viability or DNA fragmentation in Ox-LDL–treated BASMCs. This neutralizing antibody did not block either cell death (Figure 3A) or DNA fragmentation (Figure 3B) induced by tumor necrosis factor-α, suggesting the specificity of LOX-1 for the Ox-LDL–induced apoptosis. These data indicate that LOX-1 mediates the Ox-LDL–induced apoptosis in BASMCs.
Figure 4. Effects of the anti-LOX-1 neutralizing antibody on the Ox-LDL–induced increase in the Bax to Bcl-2 ratio in BASMCs. Confluent BASMCs were treated with 80 μg/mL Ox-LDL in the presence or absence of 300 μg/mL of an anti–LOX-1 neutralizing antibody (5-144) or an isotype-matched control IgG for 12 hours, and Western blot analyses were performed to evaluate the amounts of Bax (A) or Bcl-2 (B) protein. A representative figure from 3 independent experiments is shown.

Figure 5. Colocalization of LOX-1 and Bax by double-label immunohistochemistry in 2 different sections (A through C, shoulder region; D through F, a fibrous cap) from human carotid endarterectomy specimens. LOX-1 was well colocalized with Bax in both the fibrous cap and shoulder regions of human atherosclerotic plaques. The expression of LOX-1 and Bax was more abundant in the shoulder regions than in the fibrous cap regions. These data suggest that Bax expression may be upregulated through a LOX-1–mediated pathway in vivo as well as in vitro.

Discussion

Apoptosis of VSMCs may play important roles in the destabilization of atherosclerotic plaques. The present study has established that LOX-1 mediates the Ox-LDL–induced apoptosis in VSMCs through a Bax/Bcl-2–dependent pathway. Nishio and Watanabe24 revealed that the Ox-LDL–induced apoptosis of VSMCs involved downregulation of Bcl-2 protein and activation of caspase 3. We have further demonstrated in this study that Ox-LDL upregulates Bax expression in VSMCs and that both the increase in Bax and the decrease in Bcl-2 were dependent on LOX-1–mediated Ox-LDL uptake or binding. The ratio of Bax to Bcl-2 appears to determine the susceptibility of cell death after application of apoptotic stimuli in various cell types.25 Recent studies have revealed that VSMCs in human atherosclerotic lesions express Bax, which increases the susceptibility of these cells to undergo apoptosis.18 Taken together with our immunohistochemistry data (Figure 5) showing the colocalization of LOX-1 and Bax in human atherosclerotic plaques, LOX-1 may mediate the Ox-LDL–induced apoptosis in VSMCs through the increase in the Bax to Bcl-2 ratio in vivo.

Ox-LDL can also induce cell proliferation in VSMCs at low concentrations,26,27 which was confirmed by our data for the MTT assay (Figure 1A). Ox-LDL–induced proliferation of VSMCs may not depend on LOX-1 because LOX-1 expression levels were low at low concentrations of Ox-LDL. High concentrations of Ox-LDL were required to induce high levels of LOX-1 expression and apoptosis in VSMCs, thus supporting the conclusion that Ox-LDL–induced apoptosis of VSMCs is LOX-1 dependent.

LOX-1 is a receptor for Ox-LDL expressed in vascular endothelial cells,17,28 macrophages,29,30 and VSMCs10,11 in vitro and in vivo.12,31 These results suggest that LOX-1 may mediate some of the pathophysiological consequences induced by Ox-LDL in various cell types. In endothelial cells, LOX-1 is known to mediate upregulated expression of monocyte chemotactic protein-1,32 apoptotic cell death,33 and activation of nuclear factor-κB.34 However, the consequences of Ox-LDL binding or uptake through LOX-1 in VSMCs remain to be clarified. In this study, we demonstrated for the first time the functional alterations of VSMCs caused by Ox-LDL/LOX-1 interactions, which may play important roles in plaque rupture.

During atherogenesis, medial VSMCs dedifferentiate, proliferate, and transmigrate into the intima, resulting in the formation of fibrous caps together with extracellular matrixes.1 VSMCs produce the extracellular matrixes, which can be degraded by metalloproteinases that are produced mainly by macrophages.35 Ox-LDL has been known to promote plaque instability by regulating the expression of metalloproteinases in macrophages.36,37 In addition, Ox-LDL is likely to destabilize plaques by inducing apoptosis in intimal VSMCs. As demonstrated in this study, LOX-1 may be a key molecule in...
mediating the Ox-LDL–induced apoptosis in VSMCs leading to plaque rupture. Because LOX-1 expression in VSMCs can be upregulated by cytokines,10 lysophosphatidylcholine,11 and Ox-LDL (please see online Figure IA), inflammatory responses in atheromas may enhance plaque vulnerability by inducing LOX-1 expression in intimal VSMCs. Our data from the double immunohistochemistry study showed that LOX-1 and Bax were colocalized, especially in the rupture-prone shoulder regions, thus supporting the notion that increased LOX-1 expression by inflammatory stimuli mediates the modulation of Bax and apoptosis in VSMCs, leading to plaque rupture. Moreover, we have recently identified soluble forms of LOX-1 that are proteolytically cleaved from the cell surface.12 Serum levels of soluble LOX-1 may reflect the cellular expression levels of LOX-1 and thus, can be a predictor of acute coronary syndromes.

In conclusion, the present study has revealed 1 of the molecular mechanisms involved in the Ox-LDL–induced apoptosis in VSMCs. LOX-1 mediates the Ox-LDL–induced apoptosis in VSMCs, at least in part, through an increase in the Bax to Bcl-2 ratio. To our knowledge, this is the first evidence that demonstrates a functional consequence mediated by LOX-1 in VSMCs. Ox-LDL upregulates LOX-1 expression, which in turn may amplify the Ox-LDL–induced apoptosis in intimal VSMCs and thus finally elicit plaque rupture in coronary heart disease. Further studies related to the molecular mechanisms linking the Ox-LDL/LOX-1 interplay to the altered expression of Bax and Bcl-2 may provide new insights into the pathogenesis of atherosclerosis and plaque rupture.

Acknowledgments

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References


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**LOX-1**

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**Ox-LDL**

**LOX-1**