Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas

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Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas.

Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H568–H576, 1998.—Oxidized low-density lipoprotein (ox-LDL) plays a critical role in the development of atherosclerosis. Recent studies show that ox-LDL may induce apoptosis of cultured rabbit smooth muscle cells and human macrophages. This study was designed to determine the modulation by ox-LDL of apoptosis in cultured human coronary arterial endothelial cells (HCAEC) during hypoxia-reoxygenation and to determine underlying mechanisms. When HCAEC were ~85% confluent, the cells were exposed to hypoxia (24 h)-reoxygenation (3 h), native LDL, or ox-LDL. Fragmented DNA end-labeling, DNA ladder formation, and electron microscopy were used to determine changes characteristic of apoptosis. Ox-LDL (20 µg/ml) increased apoptosis during hypoxia-reoxygenation compared with hypoxia-reoxygenation alone (P < 0.05). Low concentrations of ox-LDL (5 µg/ml) and native LDL (20 µg/ml) under identical conditions had no effect on the degree of apoptosis. Ox-LDL markedly decreased endogenous superoxide dismutase activity and increased lipid peroxidation in HCAEC. The presence of ox-LDL, but not native LDL, in cultured HCAEC resulted in the activation of protein kinase C (PKC) and protein tyrosine kinase (PTK). The specific PKC and PTK inhibitors significantly reduced ox-LDL-mediated apoptosis of HCAEC (P < 0.05). Hypoxia-reoxygenation significantly increased Fas expression and decreased bcl-2 expression in HCAEC lysate as determined by Western analysis. Ox-LDL further increased Fas expression and decreased bcl-2 expression. These data indicate that ox-LDL enhances hypoxia-reoxygenation-mediated apoptosis in HCAEC. Ox-LDL-mediated apoptosis of HCAEC appears to involve activation of PKC and PTK. In addition, ox-LDL modulates Fas and bcl-2 protein expression in HCAEC. This study also suggests that ox-LDL is more important than native LDL in hypoxia-reoxygenation-induced apoptosis.

In a reduction in cardiac events such as myocardial infarction and the need for revascularization in patients with hyperlipidemia (43).

Apoptosis is a mechanism of cell death and is an important process for normal development as well as genesis of many pathological conditions (27, 45). Its hallmark is the cleavage of genomic DNA into nucleosomal fragments of 180 bp (8). Recent studies indicate that ox-LDL can induce apoptosis in a variety of tissues, including cultured smooth muscle cells (32, 33), macrophages (13, 46), cultured rat dorsal root ganglia (35), and lymphoblastoid (5) cell lines.

The most effective therapy for myocardial ischemia is restoration of coronary blood flow. However, reperfusion itself may lead to additional tissue injury by induction of apoptosis and necrosis (9). Ischemia-reperfusion leads to the release of large amounts of free radicals. Free radicals have been implicated in the oxidative modification of LDL (19). Ox-LDL results in coronary endothelial dysfunction, loss of myocardial contractile function, and damage to the cardiac ultrastructure (14). We speculated that ox-LDL may enhance the injury to endothelial cells caused by ischemia-reperfusion.

In the present study, we investigated ox-LDL-enhanced apoptosis of cultured human coronary arterial endothelial cells (HCAEC) during hypoxia-reoxygenation and the underlying signal transduction mechanisms.

MATERIALS AND METHODS

Cell Culture

HCAEC were purchased from Clonetics (lot no. 6F0756). Microvascular endothelium growth medium consisted of 500 ml endothelial cell basal medium, 0.5 ml of 10 ng/ml human recombinant epidermal growth factor, 0.5 ml of 1.0 mg/ml hydrocortisone, 0.5 ml of 50 mg/ml gentamicin, 50 µg/ml amphotericin B, 2 ml of 3.0 mg/ml bovine brain extract, and 25 ml fetal bovine serum. HCAEC (4,000 cells/cm²) were seeded in 5 ml growth medium in a 25-cm² flask and incubated at 37°C in 95% air-5% CO₂. HCAEC (1 × 10⁶) were used at the fourth and fifth subcultivations. Cells were examined under phase-contrast microscopy, and when cells were ~85% confluent, culture medium was changed and the cells were divided into several groups: a control group, in which cells were incubated in 95% air-5% CO₂; a hypoxia-reoxygenation group, in which cells were exposed to hypoxia for 24 h followed by reoxygenation for 3 h; an ox-LDL or native LDL group, in which cells were incubated for 27 h with ox-LDL (5–20 µg/ml) or native LDL (20 µg/ml); an ox-LDL + hypoxia-reoxygenation group, in which cells were exposed to hypoxia-reoxygenation in the presence of ox-LDL (5–20 µg/ml); a native LDL + hypoxia-reoxygenation group, in which cells were exposed to hypoxia-reoxygenation in the presence...
of native LDL (20 μg/ml); and a protein kinase C (PKC) inhibitor or protein tyrosine kinase (PTK) inhibitor + ox-LDL group, in which cells were treated with PKC inhibitor (100 μM) or PTK inhibitor (24 μM) along with ox-LDL (20 μg/ml) for 27 h.

Cells were made hypoxic by exposure to 95% N2-5% CO2 in a specially designed chamber. The amount of dissolved oxygen in medium (PO2) declined from 150 mmHg at baseline to 30–40 mmHg within 1 h of hypoxia. This decrease in PO2 remained stable over the course of the hypoxic period. Reoxygenation of cells was performed by transferring cells into an incubator maintained at normal atmospheric O2 and 5% CO2.

Preparation of Ox-LDL

Native LDL was purchased from Sigma. Native LDL (200 μg protein/ml) was oxidized by exposure to CuSO4 (5 μmol/l free Cu2+) in phosphate-buffered saline at 37°C for 18–20 h. Control incubations were done in the presence of 200 μmol/l EDTA without CuSO4. Oxidation was terminated by refrigeration. Oxidation of LDL was confirmed by the measurement of thiobarbituric acid-reactive substances (TBARS) with malonaldehyde bis(dimethyl acetal) (MDA) as the standard. The TBARS content of ox-LDL was 1.12 ± 0.08 versus 0.24 ± 0.06 nmol/100 μg protein in the native LDL preparation (P < 0.01). Protein content was determined by bicinchoninic acid (BCA) protein assay kit (Pierce) with the use of bovine serum albumin as the standard (2).

Phase-Contrast Microscopy

Phase-contrast microscopy was performed to examine the morphological features of apoptosis in cultured HCAEC in different experimental conditions.

Transmission Electron Microscopy

HCAEC exposed to hypoxia-reoxygenation with or without ox-LDL were fixed in cacodylate buffer containing 2.0% glutaraldehyde (pH 7.4) for 1 h. After three buffer washes, cells were postfixed in OsO4 in cacodylate buffer for 1 h. After three buffer washes, cells were pelleted by dehydration in ethanol and embedded in epoxy resin (Agar 100). Thin sections were stained in uranyl acetate and Reynolds lead citrate and were viewed at 75 kV in a Hitachi electron microscope (model H 7000).

Quantification of Apoptosis by End-Labeling In Situ

A FragEL (Klenow) DNA fragmentation detection kit was purchased from Calbiochem. This method of end-labeling has been used by other investigators (10). In brief, the cells were pelleted by gentle centrifugation (800 g) for 5 min at 4°C, resuspended in 4% buffered formaldehyde at a cell density of 1 × 10⁶ cells/ml, and incubated at room temperature for 10 min. Cells were then centrifuged and resuspended at the same concentration, in 80% ethanol. The fixed cells (100 μl) were immobilized onto glass slides using a Cytospin and incubated at room temperature for 20 min with 100 μl of 20 μg/ml proteinase K to increase cell permeability. The cells were then incubated at room temperature for 5 min with 100 μl of 3% H2O2 to inactivate endogenous peroxidases. The cells were then incubated with 60 μl Klenow labeling reaction mixture [58.4 μl Klenow labeling reaction mixture (biotin-dNTP) plus 1.6 μl Klenow enzyme] in a humidified chamber at 37°C for 1.5 h. After cells were rinsed with Tris-buffered saline (TBS), the cells were incubated with 100 μl conjugate (streptavidin-horseradish peroxidase) at room temperature for 30 min and then with 100 μl dianaminobenzidine at room temperature for 10 min. Methyl green was used for counterstain. A negative control sample was generated by substituting distilled water for the Klenow in the reaction mixture during the labeling step. The positive control sample was generated by covering the entire sample with 1 μg/ml DNase I in 1 x TBS-1 mM MgSO4 at room temperature for 20 min after proteinase K treatment. The negative control sample contained predominantly rounded cells that appeared counterstained with methyl green. The distinctive changes of apoptosis include nuclear chromatin condensation and aggregation on the inner surface of the nuclear membrane. A dark brown signal under microscopy generally indicated an apoptotic cell. At least 500 cells from randomly selected fields were counted to determine the percentage of apoptotic cells.

Extraction and End-Labeling of DNA Fragmentation (DNA Laddering)

Cultured HCAEC (1 × 10⁶) were removed from culture dishes, washed twice in PBS, resuspended in 1 ml of lysis buffer (25 mM EDTA, 10 mM Tris, 100 mM NaCl, 0.5% SDS, and 0.1 mg/ml proteinase K), and incubated at 50°C overnight. Samples were extracted twice with equal volumes of phenol-chloroform-isooamyl alcohol (25:24:1 vol:vol:vol) and incubated with RNase A (0.5 μg/ml) for 1 h at 37°C. DNA was precipitated in 2 vol of ethanol, 0.1 vol of 2 M NaCl, and 0.02 vol of 0.5 M MgCl2 overnight and were then centrifuged at 13,000 g for 10 min. The resultant pellet was resuspended in 30 μl Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA). End-labeling of DNA was performed in a 0.5-ml microcentrifuge tube by adding 3 μg of cellular DNA, 6 units of Klenow polymerase, 6 μCi of [3H]dCTP, and buffer [10 mM Tris-HCl (pH 7.5), 5 mM MgCl2]. The reaction was terminated with the addition of 10 mM EDTA. The unincorporated nucleotides were removed by Bio-Spin 6 columns (Bio-Rad). Radiolabeled DNA (3,000–5,000 counts) was electrophoresed on a 1.8% agarose gel for 2–3 h at 100 V. After the gel dried on 3-mm Whatman paper, it was used for autoradiography (36).

Western Blot Analysis for bcl-2 and Fas Protein Expression in Cultured HCAEC

Cells were solubilized directly in boiling 2 x concentrated electrophoresis sample buffer [1 x = 125 mM Tris-HCl (pH 6.8), 2% SDS, 12.5 μg/ml aprotinin, 5 μM leupeptin, 5% glycerol, 0.003% bromphenol blue, and 1% β-mercaptoethanol] and centrifuged at 12,500 g for 15 min at 4°C. The cytosolic protein from aliquots of different groups of cells (10 μg/lane) was separated by 8% SDS-PAGE with the use of a Bio-Rad Mini-Protein Cell, transferred to nitrocellulose filters (Amersham), and then immunoblotted with a rabbit monoclonal antibody against human bcl-2 (Transduction Laboratories) at 1:800 dilution. Anti-human alkaline phosphatase-conjugated antibody was used as a secondary antibody at 1:4,000 dilution. The blot was directly detected for color development on membrane. Sites of antigen localization turned a dark purple color as a result of alkaline phosphatase activity. Total protein content of different samples was quantified by BCA protein assay kit (Pierce). Relative intensities of bands of interest were analyzed with the use of an MSF-300G scanner (Microtek Lab). The process of determination of Fas protein was similar to the procedure used for bcl-2 analysis, except for the use of rabbit monoclonal antibody against human Fas (Transduction Laboratories) (2, 30).

PTK Activity

Cells (5–10 × 10⁶ cells/100-mm dish) were divided into different groups: control cells treated with 20 μg/ml of ox-
LDL; cells treated with the PTK inhibitor genistein (Sigma) + ox-LDL; cells exposed to hypoxia (24 h)-reoxygenation (3 h); cells treated with ox-LDL and then exposed to hypoxia-reoxygenation; and cells treated with genistein + ox-LDL and then exposed to hypoxia-reoxygenation. Cells were washed twice with PBS and scraped into 1 ml of membrane-bound PTK extraction buffer containing (in mM) 20 HEPES (pH 7.4), 0.5 EGTA, 10 β-mercaptoethanol, 100 phenylmethylsulfonyl fluoride, 0.05% Triton X-100, 1 µg/ml leupeptin, and 1 µg/ml aprotnin. The lysate was homogenized and centrifuged at 14,000 g for 30 min, and the supernatant was saved for determining PTK activity. An assay system (Promega) was used to determine PTK activity (44). Results were expressed as picomoles of phosphate per minute per microgram of protein.

PKC Activity

The groups of cells used were the same as for the PTK assay, except that myristoylated PKC peptide inhibitor (Promega) instead of genistein was used. Cells were washed twice with PBS and scraped into 0.5 ml of cold extraction buffer containing (in mM) 25 mM Tris (pH 7.4), 0.5 EDTA, 0.5 EGTA, 10 β-mercaptoethanol, 100 phenylmethylsulfonyl fluoride, 0.05% Triton X-100, 1 µg/ml leupeptin, and 1 µg/ml aprotnin. The lysate was homogenized and centrifuged at 14,000 g for 30 min, and the supernatant was saved for PKC assay. A specific assay system (Promega) was used for determination of PKC activity (1). Results were expressed as picomoles of ATP per minute per microgram of protein.

MDA in Cultured HCAEC Medium

MDA was measured in duplicate in the media bathing different groups of cells by a modification of the method of Ohkawa et al. (34). The assay mixture consisted of 0.1 ml of the medium, 0.4 ml of 0.9% NaCl, 0.5 ml of 3% SDS, and 3 ml thiobarbituric acid reagent (containing equal parts of 0.8% aqueous thiobarbituric acid and acetic acid) and was heated for 75 min at 95°C. Thereafter, 1 ml cold 0.9% NaCl was added to the mixture, which was cooled and extracted with 5 ml n-butanol. After centrifugation at 3,000 rpm for 15 min, the butanol phase was assayed spectrophotometrically at 532 nm. MDA (in amounts of 0, 0.1, 0.2, 0.4, 0.8, and 1.0 nmol) was used as an external standard. MDA content in the medium was expressed as nanomoles per milliliter.

Superoxide Dismutase Activity in Cultured HCAEC Medium

Total superoxide dismutase (SOD) activity was measured spectrophotometrically in duplicate in the media of different groups of cells by monitoring the SOD-inhibitable autoxidation of pyrogallol as described by Marklund and Marklund (26). The reaction mixture (4.5 ml) consisted of 0.2 mM pyrogallol, 1 mM diethylenetriamine pentaacetic acid, 50 mM Tris-cacodylic acid buffer (pH 8.2), and 4 µg catalase. The reaction was carried out at 25°C. The rate of increase in absorbance at 420 nm was recorded. One unit of enzyme activity is defined as 50% inhibition of pyrogallol autoxidation under the assay condition. The SOD activity in plasma was expressed as units per milliliter.

Data Analysis

All data represent means of duplicate samples from at least four independently performed experiments. Data are presented as means ± SD. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the F-test indicated the presence of significant differences. A P value ≤ 0.05 was considered significant.

RESULTS

Demonstration of Apoptosis

Phase-contrast microscopy. Adherent HCAEC showed typical cobblestone morphology under control conditions. After exposure to hypoxia-reoxygenation, parts of the cells became rounded and partially detached, indicating apoptotic cells. The presence of ox-LDL further increased these effects of hypoxia-reoxygenation (Fig. 1).

Electron microscopy. Transmission electron microscopy was performed to further document that the phase-contrast microscopic features of apoptosis were accompanied by appropriate ultrastructural morphology. Under control conditions, normal cellular structure was identified. In contrast, cells exposed to ox-LDL and hypoxia-reoxygenation showed typical features of apoptosis, including DNA fragmentation, condensation of chromatin at the periphery of the nucleus, and extensive vacuolation, condensation of cytoplasm, and shrinkage of cell (Fig. 2).

Fragmented DNA end-labeling in situ. Because a small number of cells die of apoptosis during culture or are damaged during processing, 1–6% of control cells stained positive. Low concentrations of ox-LDL (5 µg/ml) and native LDL (20 µg/ml) did not induce apoptosis. In contrast, ox-LDL (20 µg/ml) alone caused a marked increase in apoptosis of HCAEC (number of apoptotic cells was 21.0 ± 6.0% of all cells). Three hours of reoxygenation following 24 h of hypoxia caused the number of apoptotic cells in cultured HCAEC to increase to 27.1 ± 7.3%. Low concentrations of ox-LDL (5 µg/ml) and native LDL (20 µg/ml) did not significantly increase apoptosis of cultured HCAEC during hypoxia-

![Fig. 1](https://example.com/fig1.jpg)
reoxygenation. High concentrations of ox-LDL (20 µg/ml), on the other hand, significantly enhanced the number of apoptotic cells during hypoxia-reoxygenation (40.1 ± 8.8%, P < 0.01 vs. hypoxia-reoxygenation group) (Fig. 3).

DNA laddering. HCAEC cultured under normoxic conditions showed no DNA laddering. Under hypoxia-reoxygenation conditions, cultured HCAEC contained fragmented DNA that produced a ladder of DNA bands representing integer multiples of the internucleosomal DNA length (~180 bp), indicating apoptotic cell death during hypoxia-reoxygenation. The proportion of the fragmented DNA was increased by ~50% in HCAEC treated with ox-LDL (20 µg/ml). The proportion of the fragmented DNA did not significantly increase in the presence of low concentrations of ox-LDL and 20 µg/ml of native LDL (alone or with hypoxia-reoxygenation) (Fig. 4).

Signal Conduction Pathways

Ox-LDL-induced PKC Activity. Ox-LDL (20 µg/ml) alone increased PKC activity in cultured HCAEC. Hypoxia-reoxygenation also increased PKC activity in HCAEC. The presence of ox-LDL further increased PKC activity in cells exposed to hypoxia-reoxygenation (P < 0.01 vs. hypoxia-reoxygenation alone). The effect of ox-LDL (alone or with hypoxia-reoxygenation) was abolished by the PKC inhibitor (Fig. 5).

Ox-LDL-induced PTK Activity. Ox-LDL alone increased PTK activity in cultured HCAEC. Hypoxia-reoxygenation alone did not affect PTK activity. However, ox-LDL significantly increased PTK activity exposed to hypoxia-reoxygenation (P < 0.01). This effect was markedly diminished by the PTK inhibitor (Fig. 6).

Critical role of PKC and PTK in apoptosis. To determine the role of PKC and PTK activation in apoptosis,

Fig. 2. Transmission electron microscopy of cultured HCAEC exposed to H-R alone (middle) and to ox-LDL + H-R (right). Whereas control cells (left) show normal architecture, cells exposed to ox-LDL + H-R show condensation of chromatin at periphery, fragmentation of nucleus, and vacuolation, shrinkage, and fragmentation of cytoplasm. Original magnifications: ×8,750, control cell; ×10,000, H-R; ×12,500, Ox-LDL + H-R.

Fig. 3. Summary of data on the number of apoptotic HCAEC as a percentage of all cells measured by end-labeling (see text for detailed methodology). Control cells show only modest apoptosis. Ox-LDL (5 µg/ml) or native LDL (n-LDL; 20 µg/ml) did not affect the number of apoptotic cells. H-R causes a marked increase in the number of apoptotic cells. Presence of a small amount of ox-LDL or 20 µg/ml of native LDL during H-R causes a small, insignificant increase in the number of apoptotic cells. However, presence of 20 µg/ml of ox-LDL during H-R causes a significant increase in the number of apoptotic cells. Data are means ± SD from 4 separate experiments. *P < 0.01 vs. control; †P < 0.05 vs. ox-LDL alone or H-R alone.
HCAEC were treated with specific inhibitors and then exposed to hypoxia-reoxygenation or ox-LDL (20 µg/ml). Inhibitors of PKC as well as PTK significantly decreased ox-LDL-mediated apoptosis in HCAEC. PKC inhibitor treatment also reduced apoptosis in HCAEC exposed to hypoxia-reoxygenation alone (Fig. 7). The effect of PTK inhibitor on apoptosis induced by hypoxia-reoxygenation alone was not examined because hypoxia-reoxygenation did not activate PTK (Fig. 6).

Modulation of Fas and bcl-2 Protein Expression by Ox-LDL

Hypoxia-reoxygenation alone increased Fas protein expression (Fas band density 2–3× that of control group, P < 0.05, n = 4). The presence of ox-LDL further increased Fas protein expression (Fas band density 2–3× that of hypoxia-reoxygenation group alone, P < 0.05, n = 4) (Fig. 8).

In contrast to the effect on Fas protein expression, hypoxia-reoxygenation decreased bcl-2 protein expression (bcl-2 band density 0.3–0.6× that of control group, P < 0.05, n = 4). The presence of ox-LDL further decreased bcl-2 protein expression (bcl-2 band density 0.5× that of hypoxia-reoxygenation group, P < 0.05, n = 4) (Fig. 8).

Modulation of Lipid Peroxidation and SOD Activity in HCAEC by Ox-LDL

MDA and SOD activities were measured in the supernatants of HCAEC treated under different conditions. MDA, an index of lipid peroxidation, was increased in the group exposed to hypoxia-reoxygenation alone (P < 0.05 vs. control group). A low concentration of
of ox-LDL (5 µg/ml) or native LDL (20 µg/ml) had no significant effect. On the other hand, the presence of 20 µg/ml of ox-LDL significantly enhanced the MDA level during hypoxia-reoxygenation (P < 0.01 vs. hypoxia-reoxygenation alone) (Fig. 9).

SOD activity in HCAEC fell during hypoxia-reoxygenation (P < 0.01 vs. control group). The presence of both concentrations of ox-LDL (5 and 20 µg/ml) during hypoxia-reoxygenation further decreased SOD activity (P < 0.01 vs. hypoxia-reoxygenation group), whereas native LDL had no effect (Fig. 9).

**DISCUSSION**

This study shows that ox-LDL (20 µg/ml) causes apoptosis and enhances hypoxia-reoxygenation-mediated apoptosis in cultured HCAEC. Native LDL (20 µg/ml) and a low concentration of ox-LDL (5 µg/ml) does not have a significant proapoptotic effect. These obser-

**Fig. 8.** Fas and bcl-2 protein expression in HCAEC exposed to H-R or ox-LDL + H-R. H-R increases Fas protein expression by ~200–300%, and ox-LDL further increases Fas protein expression by another 200%. Effects of H-R or ox-LDL + H-R on bcl-2 protein expression are opposite of those on Fas expression. Gel is representative of 3 separate experiments.
vations suggest that ox-LDL has a more important role than native LDL in apoptosis in human endothelial cells. In this process, ox-LDL-mediated activations of PKC and PTK appear to be important signal conduction pathways. Hypoxia-reoxygenation also decreased bcl-2 and increased Fas protein expression of cultured HCAEC, and ox-LDL further downregulated bcl-2 and upregulated Fas protein expression. We also observed that ox-LDL-mediated apoptosis of HCAEC is closely associated with lipid peroxidation and a decrease in SOD activity.

Persistent myocardial ischemia results in cell death (i.e., necrosis and apoptosis). The most effective method of limiting cell death is restoration of blood flow. However, reperfusion per se may lead to additional tissue injury (6, 29) caused by release of oxygen free radicals (28), activation and infiltration of neutrophils (11, 25), release of cytokines (11, 17), and calcium overload (31). Xanthine oxidase is a major determinant of postischemic free radical generation in human vascular endothelial cells (47). Direct exposure of vascular tissues to xanthine-xanthine oxidase leads to endothelial disruption and dysfunction (23). Oxygen free radical-mediated myocardial tissue damage results from lipid peroxidation, loss of integrity of cell membranes, cell swelling, interstitial edema, and cell death. Recent studies indicate that oxygen free radicals can induce apoptosis both physiologically and pathologically (27, 45).

In this study, we found that hypoxia-reoxygenation significantly increased the level of MDA, a product of lipid peroxidation, in the medium bathing the cultured HCAEC, and ox-LDL (20 µg/ml) further increased the level of MDA in the cultured cells. Meanwhile, hypoxia-reoxygenation markedly decreased SOD activity. Ox-LDL further increased lipid peroxidation and decreased SOD activity during hypoxia-reoxygenation. Because hypoxia-reoxygenation concurrently induced apoptosis in HCAEC, we believe that reoxygenation increases the release of oxygen free radicals, which, along with the loss of endogenous antioxidant defense, may be a mechanism of apoptosis and cell necrosis.

Recent studies show that ox-LDL, but not native LDL, induces apoptosis in cultured rabbit vascular smooth muscle cells and human macrophages (13, 32, 33, 46). Nishio et al. (32, 33) attributed ox-LDL-mediated apoptosis to oxysterols. They also reported that treatment of smooth muscle cells with oxysterols was followed by a rapid decrease in bcl-2 protein in the cells. Hardwick et al. (13) also showed that ox-LDL, but not native LDL, induces apoptosis of human monocytes-macrophages. They observed that ox-LDL-mediated apoptosis was time and concentration dependent. In the present study, we showed that ox-LDL (20 µg/ml) alone causes apoptosis and enhances apoptosis of cultured HCAEC during hypoxia-reoxygenation. In contrast, native LDL and low concentrations of ox-LDL (<5 µg/ml) alone do not influence apoptosis in HCAEC or enhance hypoxia-reoxygenation-mediated apoptosis.

Although molecular pathways controlling apoptosis in cardiac cells have not been precisely defined, it is known that bcl-2 expression inhibits apoptosis and that Fas expression promotes this process. The bcl-2 protooncogene is a 24- to 26-kDa protein that is localized in mitochondria, endoplasmic reticulum, and nuclear membrane (20). bcl-2 protein has been shown to prevent apoptosis induced by diverse stimuli by acting as an antioxidant (15, 20) or by mechanisms (7, 18, 30) unrelated to its effect on reactive oxygen radicals. Several studies have shown that downregulation of bcl-2 expression promotes apoptosis in human umbilical venous endothelial cells (12, 42). Enforced expression of the bcl-2 gene with the use of gene transfer techniques, on the other hand, inhibits apoptosis in rat smooth muscle cells (41) and murine aortic endothelial cells (22). In studies by Meikrantz et al. (30), overexpression of bcl-2 protein suppressed apoptosis and reduced the amount of cyclin A-dependent kinases Cdc2 and Cdk2 in the nucleus, implying a possible mechanism by which bcl-2 inhibits the chromatin condensation characteristic of apoptosis. In the present study, we demonstrated that hypoxia-reoxygenation decreased bcl-2 protein expression in cultured HCAEC. Ox-LDL further reduced bcl-2 protein expression in conjunction with an increase in lipid peroxidation and a decrease in antioxidant activity. Our observations suggest that reduction of bcl-2 protein and a decrease in antioxidant activity are associated with ox-LDL-induced apoptosis of HCAEC.

An increase in Fas expression predicts impending apoptosis (4, 16, 40). Tanaka et al. (40) showed that hypoxia induces apoptosis and that this is associated with enhanced expression of Fas mRNA in cultured neonatal rat cardiomyocytes. Dong et al. (4) observed concurrent Fas expression and apoptosis by immunohistochemistry in arterial tissues with atherosclerosis. Fas positivity was identified mainly in the endothelial cells and to a much smaller extent in the macrophages. The present study shows for the first time that hypoxia-reoxygenation increases Fas protein expression in cultured HCAEC. Ox-LDL further enhanced Fas protein expression beyond that caused by hypoxia-reoxygenation.

These observations collectively indicate that an increase in Fas protein expression and a decrease in bcl-2 may play a role in ox-LDL-induced apoptosis of HCAEC. However, the cause-and-effect relationship among ox-LDL, bcl-2 protein downregulation, Fas protein upregulation, and changes in oxidant-antioxidant activity during apoptosis remains to be elucidated.

Apoptotic cell death can result from either developmentally controlled activation of endogenous execution programs or transduction of death signals triggered by a wide variety of exogenous stimuli (39). Signaling pathways of ox-LDL-mediated apoptosis in cardiac cells are not well defined. The interleukin-1β-converting enzyme is a cysteine protease that is involved in apoptosis induced by various stimuli, including Fas-mediated apoptosis (3). Nishio et al. (33) showed that oxysterols induce apoptosis in vascular smooth muscle.
cells through CPP32 protease activation. A study by Karsan et al. (21) showed that endothelial cell death induced by tumor necrosis factor-α (TNF-α) is mediated via a PKC pathway and that the bcl-2 family member A1 inhibited TNF-α-mediated endothelial cell death. Ox-LDL results in endothelial dysfunction by inactivation of nitric oxide synthase, production of free radicals, activation of PKC, and signal transduction of G protein (38). Various studies have shown that signal pathways of apoptosis in endothelial cells involve activation of PKC (21) and PTK (42). In this study, we indeed found that ox-LDL-mediated apoptosis of cultured HCAEC was associated with a significant increase in PTK and PKC activity. Furthermore, PKC as well as PTK inhibitors significantly reduced ox-LDL-mediated apoptosis of HCAEC. These findings strongly indicate that PTK and PKC are involved in signal conduction of ox-LDL-mediated apoptosis of cultured HCAEC.

A recent study showed that ox-LDL acts synergistically with ischemia-reperfusion to promote leukocyte recruitment in postcapillary venules in the mesentery of normotensive Wistar-Kyoto or spontaneously hypertensive rats (24). Our study complemented these observations.

In summary, we have demonstrated that hypoxia-reoxygenation per se causes apoptosis in cultured HCAEC. A modest concentration of ox-LDL (20 µg/ml), but not native LDL, significantly enhances hypoxia-reoxygenation-mediated apoptosis of HCAEC. In this process, intracellular activation of PKC and PTK pathways, downregulation of bcl-2 protein expression, and upregulation of Fas protein expression appear to play critically important roles in this process. Finally, ox-LDL-mediated apoptosis of cultured HCAEC appears to be closely associated with an increase in lipid peroxidation and a decrease in SOD activity.

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