Inhibition of Oxidized Low-density Lipoprotein-induced Apoptosis in Endothelial Cells by Nitric Oxide

PEROXYL RADICAL SCAVENGING AS AN ANTIAPOTOTIC MECHANISM*

Srigiridhar Katamraju‡, Neil Hogg‡‡, Joy Joseph‡, Larry K. Keefer§, and B. Kalyanaraman‡¶

From the ‡Biophysics Research Institute and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the ¶National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

Proatherogenic oxidized low-density lipoprotein (ox-LDL) induces endothelial apoptosis. We investigated the anti-apoptotic effects of intracellular and extracellular nitric oxide (NO) donors, iron chelators, cell-permeable superoxide dismutase (SOD), glutathione peroxidase mimetics, and nitrone spin traps. Peroxynitrite (ONOO⁻)----modified ox-LDL induced endothelial apoptosis was measured by DNA fragmentation, TUNEL assay, and caspase-3 activation. Results indicated the following: (i) the lipid fraction of ox-LDL was primarily responsible for endothelial apoptosis. (ii) Endothelial apoptosis was potently inhibited by NO donors and lipophilic phenolic antioxidants. Ox-LDL severely depleted Bcl-2 levels in endothelial cells and NO donors restored Bcl-2 protein in ox-LDL-treated cells. (iii) The pretreatment of a lipid fraction derived from ox-LDL with sodium borohydride or potassium iodide completely abrogated apoptosis in endothelial cells, suggesting that lipid hydroperoxides induce apoptosis. (iv) Metalloporphyrins dramatically inhibited ox-LDL-induced apoptosis in endothelial cells. Neither S-nitrosation of caspase-3 nor induction of Hsp70 appeared to play a significant role in the antiapoptotic mechanism of NO in ox-LDL-induced endothelial apoptosis. We propose that cellular lipid peroxy radicals or lipid hydroperoxides induce an apoptotic signaling cascade in endothelial cells exposed to ox-LDL, and that NO inhibits apoptosis by scavenging cellular lipid peroxy radicals.

Ample evidence supports the notion that oxidatively modified low-density lipoprotein (ox-LDL) plays a key role in the onset of atherogenic processes (1–8). Endothelial injury is considered to be one of the earliest atherogenic events (9–11). The cytotoxic effects of oxLDL are well established (12–15). Endothelial injury plays a prominent role in the increased adherence of monocytes and their migration into the subendothelial space of blood vessels (16–18). The adhesion of monocytes to the endothelium is a key atherogenic process (9, 10). Recently it has been shown that the activation of the cellular suicide pathway of the endothelial cell may be crucial to the development of atherosclerosis (19–26). Although the exact mechanism of oxLDL-induced apoptosis in endothelial cells remains unknown, published reports suggest a role for free radical intermediates (27–31). It has also been reported that the up-regulation of endotheial nitric-oxide synthase (eNOS) and copper-zinc superoxide dismutase and/or manganese superoxide dismutase protects endothelial cells against oxLDL-induced apoptosis (32). Collectively, these reports reveal an intriguing link between oxLDL, apoptosis, and nitric oxide (NO)/superoxide (O₂⁻) interaction in endothelial cells.

Nitric oxide has been reported to have a dual effect on cell-dependent LDL oxidation (33). NO acts as a pro-oxidant in the presence of O₂⁻ and an antioxidant in the presence of lipid peroxy radical (34–36). The reaction between NO and O₂⁻ to form peroxynitrite (ONOO⁻) is one of the most facile radical-radical recombination reactions in free radical biology (37, 38). NO also reacts with lipid peroxy radical (LOO⁻) at a nearly diffusion-controlled rate (k = 1–3 × 10¹⁰ M⁻¹ s⁻¹) (39). This rate constant is ~10⁸ times greater than that reported for the recombination reactions in free radical biology (37, 38). NO can act as a potent chain-breaking antioxidant. Consequently, the reaction between NO and O₂⁻ has the combined effect of removing an antioxidant such as NO, and generating the prooxidant, ONOO⁻. The role of these reactions in regulating downstream apoptosis cell signaling has not been fully investigated.

In vivo immunohistochemical studies in atherosclerotic tissues indicate the presence of oxidation and nitration marker products (i.e. lipid hydroperoxides and nitrotyrosine) that are diagnostic for reactive oxygen and nitrogen species (23, 37–40). In this study we tested the hypothesis that the propagation of lipid peroxidation is primarily responsible for oxLDL-induced apoptosis. Therefore, we investigated the effects of extracellular and intracellular NO donors (NONOates), cell-permeable SOD mimetics and ONOO⁻ scavengers (MnTBAP and FeTBAP), a cell-permeable mimetic of glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase (eb-selen), lipophilic phenolic antioxidant (probucol), and a nitrone...
spin trap α-phenyl-tert-butyl nitrore (PBN) (Fig. 1) on endothelial apoptosis induced by oxLDL. Results show that NO inhibits oxLDL-induced apoptosis as do MnTBAP/FeTBAP, eb-selen, Probucol, and PBN. These structurally diverse antioxid-ants share a common mechanism that is their ability to in-hbit the propagation of lipid peroxidation. Therefore, we propose that the major antiapoptotic mechanism of NO in-volves peroxyl radical scavenging.

EXPERIMENTAL PROCEDURES

Materials—Ebseelen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) and PBN were obtained from Sigma. PBN was also obtained from the Oklahoma Medical Research Foundation Spin Trap Source as a gift from Dr. Ronald Mason (NIHES, National Institutes of Health, Research Triangle Park, NC). Mn(III)-tetakis(4-benzoic acid) porphyrin (MnTBAP), FeTBAP, and S-nitrosoglutathione (GSNO) were synthe-sized according to the published methods (42, 43). Probucol was pur-chased from Sigma. Diethylenetriamine NONOate (DETA/NO) was obtained from Cayman Chemical Co. Anti-Hsp70 antibody and hamster anti-human Bcl-2 antibody and diethylenetriaminepentaacetic acid (DTPA) were purchased from Pharmingen.

Cell Culture—Bovine aortic endothelial cells (BAEC) harvested from thoracic aortas were maintained (37 °C, 5% CO2) in Dulbecco’s modified Eagles medium (1 g/liter of glucose, Life Technologies, Inc.) containing 2% fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, and 3 mg/ml bovine brain extract. Cells cultured between passages 5 and 10.

Human umbilical vein endothelial cells were obtained from Clonetics and cultured in endothelial basal medium (Clonetics) containing 2% fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, and 3 mg/ml bovine brain extract. Cells cultured between passages 2 and 4 were used in this study.

Preparation of LDL and LDL Lipid Extraction—LDL was isolated by sequential ultracentrifugation (d = 1.019–1.063) from freshly drawn, normolipidemic human plasma to which EDTA was added (44). LDL was oxidized by adding ONOO− (1 mM). In control experiments, LDL was added to a phosphate buffer (pH 7.4, 100 mM) containing pre-decomposed ONOO−. Medium containing 150 μg of the modified LDL was extracted by adding 2 volumes of ice-cold methanol followed by 2 volumes of chloroform (45). The mixture was centrifuged at 1800 × g for 10 min to separate the phases. The lipid phase was carefully removed and evaporated under a stream of nitrogen and re-dissolved in a minimum volume of methanol.

Treatment of BAEC with OxLDL—For all treatments, cells were washed twice with Dulbecco’s phosphate-buffered saline and incubated with serum-free Dulbecco’s modified Eagle’s medium in the presence or absence of reagents. Either native or oxidized LDL was added to a final concentration of 150 μg of LDL protein/ml.

Quantification of DNA Fragmentation by Gel Electrophoresis—DNA was isolated from BAEC. Culture medium was removed and centrifuged at 3000 × g for 5 min to collect any detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) and then pooled with the pellet made up of detached cells. After incubation at 4 °C for 15 min, lysates were incubated with 10 μl of 10 mg/ml RNase A for 1 h at 37 °C followed by 10 μl of 20 mg/ml protease K for 2 h at 50 °C. DNA was extracted using chloroform: phenol:isoamyl alcohol (25:24:1). It was then precipitated overnight with 1 volume of isopropyl alcohol at −20 °C, electrophoresed on 2% agarose gel, and then visualized under UV light after staining with ethidium bromide.

Terminal Deoxynucleotidyltransferase-mediated Nick-End Labeling Assay—Apoptosis was detected in BAEC’s using terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) assay (46). Labeling of 3’ free hydroxyl ends of the fragmented DNA with fluorescein-dUTP, was catalyzed by terminal deoxynucleotidyltransferase (TdT) using a commercially available kit (ApoAlert, CLONTECH) following the manufacturer’s directions. The areas of apoptotic cells were then detected by fluorescence microscopy equipped with rhodamine (for propidium iodide staining) and fluorescein isothiocyanate filters. The quantification of apoptosis was performed using Sigma Scan 5.0 Image Analysis package. Propidium iodide-stained cells (which represents the total number) were counted under rhodamine filter and the apoptotic (TUNEL positive) cells were counted under fluorescein isothiocyanate filter. Percentage of apoptosis was calculated from the ratio of these two measurements.

Caspase-3 Enzyme Activity—Cells were washed after treating with appropriate drugs with phosphate-buffered saline and resuspended in 50 μl of chilled lysis buffer (Caspase-3 assay kit, CLONTECH) and incubated on ice for 10 min. The cell lysates were centrifuged in a
Effect of antioxidants and antioxidant enzymes on oxLDL-induced caspase-3 activation

| Treatment | Caspase activity (nmol pNA/mg protein) |
|-----------|--------------------------------------|
| None      | 9.7 ± 1.2                            |
| Native LDL| 11.6 ± 2.3                            |
| OxLDL     | 50.2 ± 6.3                            |
| + Ebselen (50 μM) | 12.6 ± 2.1                        |
| + SOD (500 units) | 40.3 ± 5.9                          |
| + Catalase (500 units) | 42.6 ± 5.0                        |
| + MnTBAP (100 μM) | 14.9 ± 1.3                         |
| + FeTBAP (10 μM) | 16.4 ± 1.9                          |
| + TBP (100 μM) | 43.9 ± 5.2                           |
| + DTPA (10 μM) | 16.3 ± 2.4                           |
| + Desferal (10 μM) | 17.2 ± 2.6                          |
| + BHT (10 μM) | 11.9 ± 1.9                           |
| + PBN (1 mM) | 16.4 ± 3.4                           |

OxLDL was prepared by adding ONOO⁻ (100 μM) to native LDL (150 μg/ml) in 50 mM phosphate buffer after 2 h. The LDL particle was extensively dialyzed to remove excess ONOO⁻ and other oxidants. BAEC were treated with oxLDL for 16 h. Values are mean ± S.D. of three independent experiments.

The Effect of Small Molecular Weight Peroxyl Radical Scavengers, Cell-permeable Antioxidant Enzyme Mimetics, and Antioxidant Enzymes on OxLDL-induced Apoptosis—After incubating BAEC for 24 h with native LDL, no DNA fragmentation was observed (Fig. 2A, lane 2). However, if the LDL was pre-oxidized, either with copper (II) sulfate (100 μM) or with ONOO⁻ (1 mM), significant DNA laddering occurred (Fig. 2A, lanes 3 and 4). DNA laddering did not occur in the presence of decomposed ONOO⁻ (not shown). In addition, the dialysis of LDL after treatment with ONOO⁻ did not alter the ability of LDL to fragment cellular DNA (not shown). This indicates that ONOO⁻ caused the oxidation of a component of LDL to an intermediate or product that stimulated DNA fragmentation, and that this intermediate remained associated with the LDL particle during dialysis. This suggests that apoptosis is not caused by a decomposition product of ONOO⁻, nor by a low-molecular weight, soluble, LDL oxidation product, such as m-andidine, or 4-hydroxynonenal in cells exposed to ONOO⁻-modified LDL. OxLDL-induced DNA laddering was significantly inhibited in the presence of Probucol (Fig. 2B, lane 4), nitrene trap, PBN, or with metallocorphyrin antioxidant, FeTBAP (Fig. 2B, lane 7).

In addition to DNA fragmentation, the effect of oxLDL on the activation of caspase-3 was also investigated. Caspase-3, one of the downstream members of this enzyme family, is activated by proteolysis and is considered to be a committed step in several apoptotic pathways (46). As shown in Table I, native LDL did not enhance caspase-3 activity, whereas oxLDL stimulated caspase-3 activity by ~5-fold. This suggests that the oxLDL-mediated apoptosis acts through the activation of caspase-3.

In order to examine the mechanism of oxLDL-induced apoptosis, the effects of a range of antioxidants were examined (Table I). These compounds were chosen based on their wide range of targets and on their differential compartmentalization. BHT, a peroxyl radical scavenger, and DTPA, a metal chelator, both inhibited caspase-3 activation. This suggests that metal ion-dependent lipid peroxidation propagation reactions, involving the breakdown of LOOH and the formation of LOO⁻ are important steps in mediating the apoptotic cascade. Ebselen, a selenium-containing glutathione peroxidase mimetic abolished the activation of oxLDL-mediated caspase-3. Ebselen is able to access both the intracellular and extracellular compartments and cause degradation of both intracellular and extracellular hydroperoxides (48). However, the availability of thiols (which are required to mediate ebselen-dependent peroxide decomposition) may be limited in the extracellular environment. The antioxidant enzymes, SOD and catalase, had little effect on caspase-3 activation. As the actions of these enzymes are limited to the extracellular environment, this observation implies that neither superoxide nor hydrogen peroxide present in the extracellular compartment is involved in the initiation of apoptosis. In contrast, the cell-permeable metallocorphyrin SOD mimetics, MnTBAP and FeTBAP, inhibited caspase-3 activation to almost control levels (49–51). It is noteworthy that FeTBAP was effective at a 10-fold lower concentration than MnTBAP. Although the exact reasons for this
differential effect are not understood, it is consistent with the increased activity of FeTBAP as a catalyst of superoxide dismutation or ONOO$^-$$^2$ decomposition. The metal-free porphyrin, TBAP, had little effect on caspase-3 activation. Nitrone trap caused a 70% inhibition in caspase-3 activation as did desferal, a chelator of redox-active iron (Table I). The present data suggest that oxLDL-induced intracellular superoxide or chain-propagating lipid peroxyl radicals mediate the intracellular signal transduction mechanism leading to caspase-3 activation.

The Effect of Extracellular and Intracellular Nitric Oxide Donors on OxLDL-induced Apoptosis—We previously reported that NO donor compounds and S-nitrosothiols will inhibit the toxicity of oxLDL to endothelial cells in culture (52). We ascribed this effect to the ability of NO to scavenge LOO$^*$ radicals and so to prevent lipid hydroperoxide-mediated oxidation of the membranes (35, 36, 53). In the present study we examined whether nitric oxide donors and S-nitrosothiols could inhibit oxLDL-mediated apoptosis. Fig. 3 confirms the pro-apoptotic effect of oxLDL on BAEC, as treatment of cells with ONOO$^-$-modified oxLDL for 18 h results in significant TUNEL-positive staining (Fig. 3A). Both the nitric oxide donor, DETA/NO, and the S-nitrosothiol, GSNO, significantly prevented the TUNEL positive staining (Fig. 3, C and D), suggesting that these compounds inhibited DNA fragmentation. These results are shown quantitatively in Fig. 3E.

Caspase-3 is a cysteine protease that is synthesized in an inactive “pro” form. The apoptotic cascade results in the proteolytic activation of pro-caspase to generate active caspase-3. It has been demonstrated that caspase-3 can be inactivated by S-nitrosylation of the active site thiol (54, 55). In addition to DNA fragmentation, ONOO$^-$-treated LDL caused an almost 5-fold induction in caspase-3 activity, as measured by following the formation of $p$-nitroanilide (Table I). Both DETA/NO and GSNO substantially inhibited caspase-3 activation (Fig. 4A). This inhibition of caspase activity was not due to S-nitrosation as the addition of dithiothreitol, which will remove any S-nitrosothiols, did not alter the caspase activity (data not shown).

The time course of caspase activation by oxLDL is shown in Fig. 4B. Caspase-3 activity was stimulated between 4 and 8 h after adding oxLDL, and remained high for up to 24 h. In the presence of an NO donor, DETA/NO, only a slight increase in caspase-3 activity was observed over 24 h, but the large increase in activity between 4 and 8 h was abolished. DETA/NO, an extracellular NO donor, spontaneously decays within the cell culture medium. Consequently, NO released from these compounds is generated within the intracellular environment. As shown in Fig. 5, these compounds were able to suppress oxLDL-mediated caspase-3 activation at concentrations that were 50–100-fold lower than the extracellular NO donor, DETA/NO. With 1 $\mu$M of these esterase-sensitive intra-
caspase-3 activation. BAEC were treated with ONOO− or oxLDL (150 μg/ml) for 16 h in the presence and absence of cell-permeable 'NO donors. For structures of 'NO donors, see Fig. 1.

cellular 'NO donors, robust inhibition was observed, whereas 5 μM AcOM-DEA/NO or AcOM-PYRRO/NO completely suppressed caspase-3 activation (Fig. 5).

To determine the component of the LDL particle that was responsible for apoptosis, we isolated the lipid component of oxidized and native LDL and examined the effects of the extracts on caspase-3 activation. As shown in Fig. 6, exposure of cells to the lipid extract of native LDL resulted in only a minor increase in caspase activity, which was inhibited by DETA/NO and GSNO. In contrast, the lipid fraction of oxLDL caused a significant activation of caspase-3 activity to a level almost equivalent to that observed with whole oxLDL particle. This indicates that the lipid component of the LDL particle is largely responsible for the activation of caspase-3. Both DETA/NO and GSNO inhibited caspase-3 activation by oxLDL extract, indicating that the pro-apoptotic intermediate, that is inhibited by 'NO, is present in the lipid component of oxLDL. The influence of lipid hydroperoxides on caspase-3 activation was assessed by reducing the peroxides to alcohols using potassium iodide or sodium borohydride. Treatment of oxLDL lipid extract with these agents, before addition to the cells, ablated the ability of this lipid mixture to activate caspase-3 (Fig. 6). These results suggest that the lipid hydroperoxide component of oxLDL is responsible for caspase activation.

It has been previously reported that 7-ketocholesterol (7-KC), a known oxidation product of cholesterol, is pro-apoptotic (57–59). In Fig. 7, the effect of oxLDL was compared with 7-KC. Both agents enhanced caspase-3 activation, whereas only oxLDL-dependent caspase-3 activation was inhibited by DETA/NO. This suggests that 7-KC enhances caspase-3 activity, and hence apoptosis, by a mechanism that is distinct from oxLDL. Furthermore, the level of 7-KC (e.g. 40–100 μg/ml) required to promote apoptosis far exceeds the levels of 7-KC expected to be present in oxLDL used in these experiments.

The Effect of Hsp70 and Bcl-2 Induction on OxLDL-induced Apoptosis—It has previously been reported that the anti-apoptotic effects of 'NO can be correlated to the stimulation of heat shock protein 70 (Hsp-70) synthesis (60, 61). Fig. 8A demonstrates that control cells showed little or no Hsp-70 content as determined by the Western blot analysis. However, when cells with DETA/NO, GSNO, or native LDL were incubated, Hsp-70 synthesis was induced. We further observed that oxLDL induced a much lower level of protein expression, which was increased in the presence of DETA/NO or GSNO. This data suggested an inverse correlation between Hsp-70 content and caspase-3 activation. To examine if preinduction of Hsp-70 could protect against oxLDL, we pretreated cells for 6 h with DETA/NO, GSNO, or native LDL and then exposed these cells to oxLDL. After 16 h these cells showed a robust expression of Hsp-70 with a high level of caspase activation (Fig. 8B). Under these conditions we found no correlation between caspase-3 activation and Hsp-70 induction. Therefore the induction of Hsp-70 is not inhibitory to oxLDL-mediated apoptosis, but is a parallel process that is stimulated by exposure of cells to DETA/NO, GSNO, or native LDL.

Results described in Fig. 8B demonstrate that DETA/NO and GSNO must be present at the same time as the oxLDL in order to prevent caspase activation. Hence, these agents affect the cell directly and instantly rather than through the sustained and mediated induction of antioxidant or antiapoptotic enzymes in the system.

In search of the molecular mechanism(s) responsible for the protective effects, we also measured the relative amounts of Bcl-2 protein as described in Fig. 9. Bcl-2 was significantly inhibited in the oxLDL-treated group (62). OxLDL-induced depletion of Bcl-2 was restored in the presence of 'NO donors and other antioxidants (Fig. 9). Similar results were obtained using human umbilical vein endothelial cells (data not shown). This indicates that the antiapoptotic effect of 'NO in human umbilical vein endothelial cells is likely to be mediated by the same mechanism as occurs in BAEC.

**DISCUSSION**

**Mechanism of OxLDL-induced Endothelial Apoptosis**—The pathophysiological effects of oxLDL in vascular cells have pre-
Previously been investigated using oxLDL as a whole (1–10). It is also well known that cells are more vulnerable to oxLDL-induced toxicity if serum or other proteins are excluded from the media. Reports also indicate that oxLDL-induced endothelial apoptosis is markedly diminished in the presence of added serum (63). As our objective was to elucidate the mechanism of oxLDL-induced cellular injury, experiments were performed in the absence of serum.

The individual components of oxLDL responsible for mediating apoptosis are not known. OxLDL is a mixture of several cytotoxic components consisting of lipid hydroperoxides (e.g. 9- and 13-hydroperoxyoctadecadienoic acid, cholesteryl hydroperoxyctadecadienoate, aldehydes such as 4-hydroxynonenal and malondialdehyde, and oxysterols (7-ketocholesterol, 7β-hydroxycholesterol)). Individually these components are potent inducers of apoptosis in several cell types including bovine and human endothelial cells. In the present work, we have shown that the lipid extract of oxLDL containing lipid hydroperoxides induces endothelial apoptosis in BAEC or human umbilical vein endothelial cells exposed to ONOO\(^-\)-modified LDL. Possible proapoptotic candidates present in ONOO\(^-\)-modified LDL include hydroperoxy derivatives of cholesteryl linoleate, linoleate, and cholesteryl hydroperoxides. Pretreatment of endothelial cells with ebselen, a synthetic glutathione peroxidase/phospholipid hydroperoxide glutathione peroxidase mimic, has been shown to afford protection against copper oxLDL (64). Scheme 1 summarizes the reactions of pro-apoptotic reactive oxygen and nitrogen species and the anti-apoptotic mechanism(s) of several antioxidants including NO.

Based on the effects of PBN, ebselen, desferral, SOD mimetics, phenolic antioxidants, and NO donors as shown in the present study, we postulate that lipid peroxyl radicals trigger the apoptotic cascade (Scheme 1). This is linked to membrane lipid peroxidation which is presumably one of the earliest upstream apoptotic signaling events (65). Agents that inhibit this process are likely to negatively influence the apoptotic signaling process. Literature data also indicate that the metalloporphyrin class of SOD mimetics are efficient inhibitors of lipid peroxidation (66). The antioxidant effect of NO is linked to its ability to scavenge lipid peroxyl radicals (35, 36). Thus we propose that NO can potentially inhibit formation of intracellular reactive oxygen species. This antiapoptotic mechanism of

![Diagram](image-url)
‘NO will compete with the proapoptotic peroxynitrite-inducing reaction.

**Extracellular and Intracellular ‘NO Donors: Mechanism of Action**—In this study we used both intracellular and extracellular ‘NO donors to investigate the antiapoptotic mechanism of ‘NO. We used slow-releasing NONOates from which ‘NO was released at defined rates with known stoichiometry from a thermolytic decomposition (53). The disadvantage of using extracellular NONOates is that a substantial proportion of ‘NO generated extracellularly is oxidized before it enters the cell. As a result, we used relatively higher concentrations (~100 μM) of these compounds. In contrast, the advantage of using cell-permeable pro-‘NO donors is that they require activation by cytoplasmic esterases in order to release ‘NO. Consequently ‘NO will be released intracellularly (see Scheme 2). Thus, relatively low concentrations (1–5 μM) of esterase-specific ‘NO donors are needed for maximal results in apoptosis experiments.

**Scavenging of Lipid Peroxyl Radical by ‘NO: A Potential Antiapoptotic Mechanism?**—The collective works of our group (35, 52, 53) and other’s (36, 67) have demonstrated the following: (i) ‘NO is a highly sensitive inhibitor of copper- and azo-initiator-dependent LDL oxidation, (ii) ‘NO outcompetes vitamin E as an inhibitor of LDL lipid oxidation, (iii) the release rate of ‘NO from ‘NO donors is a critical determinant of the extent of inhibition of lipid oxidation, (iv) ‘NO inhibits cell-dependent LDL oxidation, and (v) ‘NO inhibits the toxicity of oxLDL to endothelial cells by a peroxyl radical scavenging mechanism. The oxLDL itself is unreactive to ‘NO. Published reports indicate that ‘NO does not directly react with lipid hydroperoxides, aldehydes, or with other associated products of oxLDL, nor with β-carotene and α- and γ-tocopherols (68). ‘NO, however, reacts with the lipid peroxyl radical associated with oxidizing LDL at a diffusion controlled rate (39). ‘NO is a potent inhibitor of lipid peroxidation and LDL oxidation. Lipid peroxidation, the central process in most mechanisms of LDL modification, is controlled by the steady-state concentrations of lipid peroxyl radicals. Any compound that scavenges peroxyl radicals (e.g. α-tocopherol, Probucol) to give a stable end product will inhibit lipid peroxidation. The peroxyl radical can be thought of as an organic analog of superoxide in the same way that lipid hydroperoxide is an analog of hydrogen peroxide. Accordingly, the chemistry between ‘NO and peroxyl radical is similar to that between ‘NO and superoxide in that they react at a diffusion-limited rate through radical-radical recombination (38, 39). Differences occur due to the fact that the product of this reaction, unlike ONOO−, decomposes to stable products that do not re-initiate the peroxidation reaction. Because the endothelium and subendothelial space (the physiological locus of LDL modification) are exposed to a constant supply of ‘NO due to basal NOS activity, these observations are of critical importance in determining the oxidative propensity of the artery wall. In addition, ‘NO is a highly sensitive controller of cellular signaling processes that require lipid oxidation. Published data suggest that oxLDL exposure to endothelial cells impairs eNOS activation through displacement of eNOS from caveolae and attenuates the capacity of the endothelium to produce NO (69). Exposure of endothelial cells to oxLDL was shown to cause a decrease in the expression of eNOS (70). We propose that lipid peroxyl radicals or lipid hydroperoxides are responsible for initiating the apoptotic cell signaling and that this cascade of events is regulated by ‘NO.

The increased formation of intracellular reactive oxygen species was suggested to be one of the mechanisms of endothelial apoptosis induced by oxLDL components (71). The intracellular source of ROS, however, remains to be established. OxLDL-induced cell membrane signaling leading to ROS generation is an active area of research. ROS can originate from endothelial nitric-oxide synthase, mitochondriald electron transport chain, xanthine oxidase, cyclooxygenase, and NADPH oxidases (72). Pritchard et al. (73), reported that endothelial cells incubated with atherogenic concentrations of native LDL released superoxide from eNOS. Wever et al. (41), also proposed an uncoupling of NOS activity as a mechanism of increased formation of superoxide and ONOO− under similar conditions. Clearly, the identification of the intracellular source of ROS is required for the potential antiapoptotic apolipoprotein.

In conclusion, we have shown that ‘NO can effectively inhibit endothelial apoptosis mediated by oxLDL. It is likely that the antiapoptotic mechanism of ‘NO is probably linked to its ability to scavenge the lipid peroxyl radicals that are presumably responsible for the apoptotic signaling cascade.

**Acknowledgment**—We thank Dr. Nalini Santanam, Emory University, for providing us with fresh LDL during the initial stages of this project.

**REFERENCES**

1. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) N. Engl. J. Med. 320, 915–924
2. Chisolm, G. M. I., Hazen, S. L., Fox, P. L., and Cathcart, M. K. (1999) J. Biol. Chem. 274, 25859–25862
3. Heinecke, J. W. (1998) Atherosclerosis 141, 1–15
4. Berliner, J. A., Navah, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., Watson, A. D., and Lusis, A. J. (1995) Circulation 91, 2388–2406
5. Berliner, J. A., and Heinecke, J. W. (1996) Free Radic. Biol. Med. 20, 707–727
6. Devaraj, S., and Jialal, I. (1996) J. Clin. Lab. Res. 26, 178–184
7. Steinbrecher, U. P., Zhang, H., and Loughheed, M. (1996) Free Radic. Biol. Med. 20, 155–168
8. Hajjar, D. P., and Haberland, M. E. (1997) J. Biol. Chem. 272, 22975–22978
9. Heinecke, J. W., Rosen, H., and Chait, A. (1984) J. Clin. Invest. 74, 1890–1894
10. Cathcart, M. K., Li, Q., and Chisolm, G. M. (1995) J. Lipid Res. 36, 1857–1865
11. Stein, C., Mitchinson, M. J., Aruoma, O., and Halliwell, B. (1999) Biochem. J. 266, 901–905
12. Darley-Usmar, V. M., Hogg, N., O’Leary, V., Wilson, M. T., and Moncada, S. (1992) Free Radic. Res. Commun. 17, 9–20
13. Graham, H., Hogg, N., Kalyanaraman, B., O’Leary, V., Darley-Usmar, V., and Moncada, S. (1993) FEBS Lett. 330, 181–185
14. Xing, X., Buffie, J., and Sparrow, C. P. (1996) J. Lipid Res. 37, 2201–2208
15. Ehrenwald, E., Chisolm, G. M., and Fox, P. L. (1994) J. Clin. Invest. 93, 1493–1501
16. Folch, V. A., Nivar-Aristoy, A. R., Krajewski, L. P., and Cathcart, M. K. (1995) J. Clin. Invest. 96, 904–910
17. Parthasarathy, S., Wield, E., and Steinberg, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1046–1050
18. Sparrow, C. P., Parthasarathy, S., and Steinberg, D. (1988) J. Lipid Res. 29, 745–753
19. Cathcart, M. K., McNally, A. K., and Chisolm, G. M. (1991) J. Lipid Res. 32, 625–630
20. Kühn, H., Belkner, J., Zains, S., Fahrenklemper, T., and Wohlfel, S. (1994) J. Exp. Med. 179, 1903–1911
21. Steinberg, D. (1999) J. Clin. Invest. 103, 1497–1498
22. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2075–2081
23. Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Ohiishi, S., Steinbrecher, U. P., and Heinecke, J. W. (1997) J. Biol. Chem. 272, 1433–1436
24. Podrez, E. A., Schmitt, D., Hoff, H. F., and Hazen, S. L. (1999) J. Clin. Invest. 103, 1547–1560
25. Zatarain, R., Pei, H., Gu, J. L., Sarma, J. M., and Nadler, J. (1999) Cardiovasc. Res. 41, 489–499
26. Haberland, M. E., Dong, F., and Cheng, L. (1988) Science 241, 215–218
27. Seelos, C., Gmeiner, B., Kapiotis, S., and Cerni, C. (1997) Biochem. Mol. Biol. Int. 42, 1015–1022
Inhibition of Oxidized Low-density Lipoprotein-induced Apoptosis in Endothelial Cells by Nitric Oxide: PEROXYL RADICAL SCAVENGING AS AN ANTIAPOPTOTIC MECHANISM
Srigiridhar Kotamraju, Neil Hogg, Joy Joseph, Larry K. Keefer and B. Kalyanaraman

J. Biol. Chem. 2001, 276:17316-17323.
doi: 10.1074/jbc.M011731200 originally published online February 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011731200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 31 of which can be accessed free at http://www.jbc.org/content/276/20/17316.full.html#ref-list-1