Nitric oxide donor compounds inhibit the toxicity of oxidized low-density lipoprotein to endothelial cells

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Abstract Photo-oxidized low-density lipoprotein is cytotoxic to bovine aortic endothelial cells in a concentration-dependent manner. Total cell killing occurs at a concentration of 600 μmol/l lipid hydroperoxide (LOOH). Selenium deficiency enhances the toxicity of LOOH such that 300 μmol/l LOOH is cytotoxic. This toxicity is inhibited by desferrioxamine, a transition metal ion chelator, and by butylatedhydroxytoluene, a potent inhibitor of lipid peroxidation. Toxicity is also inhibited by the nitric oxide donors *S*-nitrosoglutathione and spermine NONOate but not by reduced or oxidized glutathione and spermine. We propose that nitric oxide, released from these compounds, is inhibiting the toxicity of LOOH to selenium-deficient endothelial cells. Furthermore we hypothesize that the mechanism for this inhibition of toxicity is the scavenging of the propagatory peroxyl and alkoxyl free radicals, by nitric oxide, that are generated during peroxidation of cell membranes.

Key words: Nitric oxide; Low-density lipoprotein; Endothelial cell; Toxicity; Lipid peroxidation; Atherosclerosis

1. Introduction

The interactions between low-density lipoprotein (LDL) and vascular endothelial cells are likely to play a key role in the underlying pathology of atherosclerosis. Endothelial cells act as a barrier between the blood and the thrombogenic connective and smooth muscle tissues that are present in the vessel wall. In atherosclerotic lesions the integrity of the endothelium can be compromised and such areas of damage may become the foci of thrombus formation and growth factor release [1]. It is of great interest to understand the molecular mechanisms that lead to endothelial cell damage and to explore ways in which this damage can be inhibited. The discovery that oxidized LDL, a form of LDL that is thought to be generated in an atherosclerotic lesion [2], is toxic to endothelial cells in culture indicates a possible mechanism for endothelial cell damage [3]. It is widely believed that such damage occurs by free radical-mediated oxidation of cellular phospholipids, leading to loss of integrity of the cell membrane [4–6].

The role of nitric oxide in the atherosclerotic process has become a subject of interest. It has been shown that macrophages, activated to generate nitric oxide, have a reduced ability to oxidize LDL, and that this effect is counteracted by nitric oxide synthase inhibitors [7,8]. We have previously shown that nitric oxide donors can inhibit both chemical [9] and cell-dependent LDL oxidation [10] by a process likely to involve the scavenging of propagatory lipid-derived free radicals.

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The recent observation that chronic treatment of hypercholesterolemic rabbits with inhibitors of nitric oxide synthase enhance lesion formation [11,12], together with earlier observations that L-arginine supplementation is potentially antiatherogenic [13,14], support the hypothesis that nitric oxide is an endogenous antiatherogenic molecule [15]. In this paper we demonstrate that nitric oxide can inhibit another potentially atherogenic process; namely the toxicity of oxidized LDL to endothelial cells. The mechanism of this inhibition may involve the scavenging of cellular lipid-derived radicals in a process similar to nitric oxide-mediated inhibition of LDL oxidation.

2. Materials and methods

2.1. Chemicals and reagents

S-Nitrosoglutathione (GSNO) was synthesized following the method of nitrosothiol production described by Field et al. [16]. Spermine NONOate (SNN) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Butylatedhydroxytoluene (BHT) was purchased from Fisher (Itasca, IL). Desferrioxamine (DFO) was obtained from Ciba-Geigy (Suffern, NY). Aluminium phthalocyanine tetrasulfonate ($AlPcS_4$) was kindly provided by Dr. A.W. Girotti (Medical College of Wisconsin, Milwaukee, WI). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), DME-Ham's F-12 mix medium (F12), insulin, transferrin, L-glutamine, 10×-trypsin-EDTA, penicillin-streptomycin solution, reduced glutathione (GSH), oxidized glutathione (GSSG), and spermine were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS), defined grade, was purchased from Hyclone Laboratories Inc. (Logan, UT). All other chemicals were of the highest purity available and aqueous solutions were prepared with deionized, glass-distilled water.

2.2. Cultured cells

Bovine thoracic aortic endothelial cells (BAEC) were obtained from American Type Culture Collection. Cells were obtained at the third passage, transferred to 75 cm^2 filter vent flasks (Costar, Cambridge, MA) and grown to confluence (5.3×10^6 cells/75 cm^2) in F-12 containing 10% FCS, insulin (10 μg/ml), transferrin (5 μg/ml), L-glutamine (4 mmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml), incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed in a similar medium containing 2% FCS. Cells were passaged as described by Balla et al. [17] and used between passages 6 and 13.

Selenium-deficient cells were prepared as described by Thomas et al. [16]. Serum concentration was decreased from 10% to 5%, and one population of cells, designated Se(−), was grown without added selenium while a separate population, designated Se(+), was grown in the presence of sodium selenite (10 ng/ml). Serum content of both cell populations was maintained at 5% in 75 cm^2 flasks for each consecutive passage. Cells were then passaged to 96-well (0.32 cm^2) tissue culture plates. Typically, BAEC grew to confluence (0.023×10^6 cells/0.32 cm^2) 2–3 days after passaging. Confluent Se(−) and Se(+) cells did not differ in their total protein content, as determined by the method of Lowry et al. [18], using serum albumin as the standard.

2.3. Cell viability

MTT staining was used as an indicator of cell viability. This assay

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is based upon the reduction of MTT to a colored formazan crystal by mitochondrial dehydrogenases [19]. MTT stock solution was made up at 5 mg/ml in PBS and stored at 0°C. Following experimentation, the medium was removed and cells were incubated with a MTT (10% of stock) solution in F-12 with 1% penicillin/streptomycin for 4 h. The medium was then carefully removed so as not to disturb the monolayer of stained cells. The formazan crystals were solubilized in isopropanol and the absorbance at 570 nm recorded. A close correlation between the MTT method and the clonogenic method of determining cell viability has been reported [20]. The term 'cell viability' in this paper refers to the total mitochondrial activity of a treated population of cells normalized to that of a population of untreated cells and expressed as a percentage.

2.4. Preparation of LDL and photooxidized LDL (LDLox)

Plasma was obtained from fasted human donors; EDTA (1 mmol/l) was present during all stages of isolation. LDL was isolated by ultracentrifugal flotation in KBr (density range: 1.019–1.063 g/ml) [21]. All steps were carried out with chelex-treated and argon-purged solutions. Before use, LDL were dialyzed against chelex-treated PBS to remove EDTA, and stored under argon at 4°C. Protein was determined by the method of Lowry et al. [18]. Preparations of LDL typically contained less than 5 nmol lipid hydroperoxide (LOOH)/mg protein (determined iodometrically) and less than 1 nmol malondialdehyde (MDA)/mg protein (determined by reaction with thiobarbituric acid). LDL was subjected to $AlPcS_4$-sensitized photoperoxidation [6]. A solution containing LDL (1.0 mg protein/ml) was aliquoted into two tubes. One tube received no treatment and was immediately placed on ice, under argon (LDLn), and the other tube received 20 μmol/l $AlPcS_4$. A small volume of this was set aside before irradiation as a 'dark control' (LDLdc). The remainder was irradiated at 10°C in a 5 ml vial placed within a larger thermostatted beaker which was covered with a clear piece of glass to block UV light [6]. The light source was a 90-W quartz-halogen lamp positioned ~15 cm above the reaction vial. Incident light intensity ~30 mW/cm^2, as measured with a YSI radiometer (Yellow Springs, OH). Solutions were continuously stirred and left open to the air during irradiation. Just before use and following filter-sterilization (0.22 μm) stock preparations of LDLox, LDLdc and LDLn were analyzed for LOOH content by the iodometric method [22].

2.5. Experimental conditions

Se(−) or Se(+) BAEC were grown to confluence and overlaid with fresh medium containing 2% FCS. LDL and other compounds were added before incubation at 37°C in 5% CO_2/95% air. In each experiment the total LDL content (LDLn plus LDLox) was kept constant. Thus, the concentration of LDL-lipid and LDL-protein were constant at all LOOH concentrations. After incubation the medium was removed and the viability was determined by the MTT assay. In some experiments the medium was assayed for LOOH. BHT was made up in ethanol and added to give a final concentration of 0.5% ethanol. All other compounds were dissolved in medium and filtered immediately before use.

3. Results

Photooxidation of LDL in the presence of $AlPcS_4$ (20 μmol/l) resulted in the accumulation of LOOH but no detectable increase in TBARS. An identical sample that was kept in the dark did not accumulate LOOH. Aliquots of LDLox were mixed with aliquots of LDLn in order to generate a range of concentrations of LOOH while maintaining the same concentration of total LDL. A parallel set of samples were prepared in which LDLdc was added instead of LDLox. Fig. 1. shows the effect of incubating BAEC with increasing concentrations of LOOH. LOOH is not toxic to BAEC grown in selenium-replete medium at concentrations less than 500 μmol/l. However, if the cells are grown in conditions which deplete the selenium content of the cell (BAEC(Se−)), toxicity is apparent at 200 μmol/l LOOH, and complete loss of cell viability occurs at 300 μmol/l LOOH.

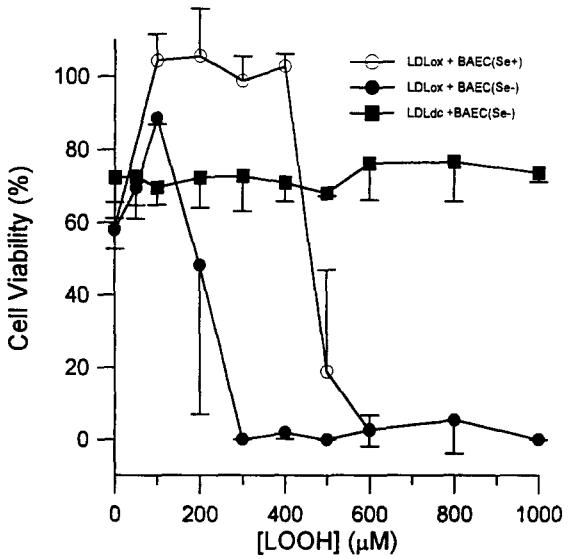

Fig. 1. The effect of selenium deficiency on the toxicity of LOOH to BAEC. Increasing amounts of LDLox were mixed with decreasing amounts of LDLn to achieve a range of LOOH concentrations while maintaining a constant protein concentration. These mixtures were added to either BAEC(Se+) (○) or BAEC(Se−) (●). Identical amounts of LDLdc were used in place of LDLox and added to BAEC(Se−) (■). Cells were incubated for 18 h after which viability was determined by the MTT assay. Data points represent mean ± S.D. (n = 5).

This agrees well with previous data using lactate dehydrogenase as an index of viability [6]. Loss of cell viability was not due to MDA formation as MDA levels remained unchanged throughout exposure and authentic MDA was not toxic to BAEC(Se−) at concentrations of ≤5 mM (data not shown).

Addition of LDLdc to BAEC(Se−) by an identical protocol produced marginal toxicity that was independent of the LDLdc concentration (Fig. 1) and was not affected by DFO (20 μmol/l) or BHT (100 μmol/l) (data not shown). This toxicity was also apparent when LDLn containing no $AlPcS_4$ was used and incubation with an identical volume of PBS resulted in no loss of viability (data not shown). This probably represents an inherent toxicity of LDLn which is independent of LOOH concentration. The zero point toxicity was reversed by sub-lethal concentrations of LOOH. The mechanism whereby low levels of hydroperoxide are able to reverse and/or protect against LDLn-mediated toxicity is unclear and is presently under investigation. This effect may be related to the known proliferative effect of sub-lethal concentrations of peroxide [23].

The effects of the nitric oxide donors GSNO and spermine NONOate on the toxicity of LDLox to BAEC(Se−) are shown in Fig. 2. Fig. 2A shows the effect of GSNO on LOOH-dependent endothelial cell death. As in Fig. 1, toxicity was observed in controls at 200 μmol/l LOOH with a complete cell kill at 300 μmol/l LOOH. The breakdown products of GSNO, namely GSH (500 μmol/l) and GSSG (500 μmol/l) had little effect on the toxicity of LOOH. GSNO (250 μmol/l), however, afforded protection against toxicity at up to 400 μmol/l LOOH. Pretreatment of the cells with GSNO (250 μmol/l) for 5 h followed by removal of GSNO before the addition of LOOH afforded no protection. This indicates that the inhibition of toxicity by GSNO was not due to a stable phenotypic change in cellular

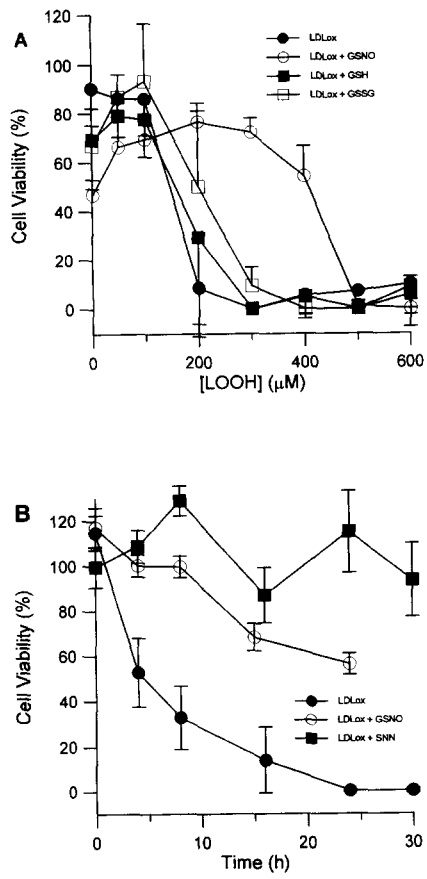

Fig. 2. The effect of GSNO and spermine NONOate on the toxicity of LOOH to BAEC(Se−). (A) Increasing amounts of LDLox were mixed with decreasing amounts of LDLn to achieve a range of LOOH concentrations while maintaining a constant protein concentration. These mixtures were added to BAEC(Se−) in the absence (●) or presence of GSNO (○, 500 μmol/l), GSH (■, 500 μmol/l) and GSSG (□, 500 μmol/l). Cells were incubated for 18 h after which viability was determined by the MTT assay. (B) LDLox containing LOOH (500 μmol/l) was incubated with BAEC(Se−) cells in the absence (●) and presence of GSNO (○, 500 μmol/l) and spermine NONOate (■, 500 μmol/l). Viability was determined as a function of time using the MTT assay. Data points represent mean ± S.D. (n = 5).

resistance to oxidative stess. The inhibition of LDLox toxicity by GSNO was not due to the destruction of endogenous LOOH by NO or GSNO as exposure of LDLox to GSNO did not affect the concentration of LOOH in the presence and absence of BAEC(Se−) (data not shown).

The protective effect of GSNO may be ascribed to either the GSNO itself or to NO released by GSNO over the course of the experiment. To assess this, a second nitric oxide donor, spermine NONOate was used as the source of nitric oxide. Fig. 2B shows the kinetics of LOOH (500 μmol/l)-induced cell killing. The majority of cell death occurs within the first 5 h of incubation. However, it is not until 24 h that complete toxicity is observed. In the presence of spermine NONOate (250 μmol/l), a dramatic inhibition of LOOH toxicity was observed over 24 h (Fig. 2B) whereas spermine (500 μmol/l) alone had no effect on LOOH toxicity over this time range (data not shown). GSNO (250 μmol/l) also inhibited toxicity at all time points throughout the experiment. Spermine NONOate, spermine, GSNO, GSSG and GSH had no intrinsic toxicity to BAEC(Se−) at the concentrations used in this study.

The effects of BHT and DFO on the toxicity of LOOH were examined. Experiments were performed in an identical manner to Fig. 1 with total LDL remaining constant. The addition of the transition metal ion chelator DFO (200 μmol/l) afforded significant protection such that complete toxicity was not apparent until concentrations of LOOH greater than 500 μmol/l were used (data not shown). BHT (50 μmol/l), a lipophilic free radical scavenger, afforded protection up to 600 μmol/l LOOH, and total cell death was only achieved at 800 μmol/l LOOH (data not shown). These are in agreement with previous reports detailing the effects of inhibiting lipid peroxidation on the toxicity of hydroperoxides to endothelial cells [4–6].

4. Discussion

In this study, we have deliberately sensitized endothelial cells to peroxide-dependent cell damage by selenium depletion. The effects of selenium depletion are clearly observed in Fig. 1. One major result of selenium depletion is the reduction of GPx and PHGPx activities [6]. This may be responsible for the dramatic enhancement in the toxicity of oxidized LDL in selenium-deficient cells. Our experiments were designed so as to minimize the number of components that vary with lipid hydroperoxide. Thus total phospholipid, protein and cholesterol are constant at all concentrations of LOOH. However, the unavoidable loss of LDL antioxidants during photo-oxidation means that these molecules (e.g. vitamin E, β-carotene) also varied with LOOH concentration.

GSNO and spermine NONOate are structurally unrelated compounds that release nitric oxide by different mechanisms. Spermine NONOate spontaneously decays to spermine and liberates two molecules of nitric oxide per molecule of spermine NONOate. The half life of decomposition has been measured as 39 min [24]. GSNO decomposition is more complex as the molecule is inherently stable and probably requires trace amounts of catalytic transition metal ions to release nitric oxide [25]. This process is still slow, but accelerated nitric oxide release can occur if transnitrosylation to form a more unstable nitrosothiol occurs [26], e.g. the formation of S-nitrosocysteine from cysteine. Upon decomposition GSNO gives GSSG, but after transnitrosylation GSH is the product. For this reason both GSSG and GSH were used as controls.

Both GSNO and spermine NONOate dramatically inhibit the toxicity of LDLox to BAEC(Se−). The exact mechanism of this inhibition is at present unknown, although we postulate that nitric oxide is acting as an inhibitor of lipid peroxidation in cells. Wink et al. [27] have shown that nitric oxide released from compounds with the NONOate moiety reduced the cytotoxic effects of superoxide/hydrogen peroxide in lung fibroblasts and neuronal cells. The investigators have suggested that the cytoprotective effect of nitric oxide could be due to radical scavenging. We have previously suggested [9] that nitric oxide is able to inhibit lipid oxidation by scavenging lipid peroxyl radicals, thus terminating the lipid peroxidation chain reaction. Recently, Rubbo et al. [28] have demonstrated the formation of nitric oxide–lipid adducts during the oxidation of linolenic acid by xanthine/xanthine oxidase/iron in the presence of nitric oxide, and that nitric oxide inhibited this oxidation.

It has been previously reported that the cytotoxic effects of

modified LDL to endothelial cells can be prevented by inclusion of antioxidants in the culture medium [4,29–31]. Inhibition of the toxicity of LOOH by BHT and DFO implies a role for the propagation of lipid peroxidation in the toxicity process. Lipid oxidation occurring at the level of the LDL particle may generate toxic aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal [32]. MDA, at least, is not responsible for the observed toxicity as little MDA is present or is generated during the course of the toxicity experiment and MDA is not toxic to BAEC(Se−) at concentrations (> 5 mmol/l) far in excess of what could conceivably be generated by LDL oxidation. During the time course of oxidation, the levels of LOOH remained unaltered indicating that no direct reaction between nitric oxide and LOOH was occurring and that the LDL in the medium was not being significantly oxidized.

Lipid peroxidation occurring at the level of the cell membrane could potentially disrupt cellular integrity by changing the permeability of the lipid bilayer to solutes and by damaging membrane transport systems. This mechanism would require the transfer of lipid hydroperoxides from LDL to the cell membrane. The fraction of LOOH transferring to the cell membrane may only be 1–2% of total LOOH and would thus not be detectable as a loss of LDL-LOOH. The effects of selenium depletion strongly suggest that transfer is occurring as GPx and PHGPx are intracellular enzymes and unlikely to be available to extracellular LDL. Transfer of peroxide to the cell could occur either by an active uptake mechanism or by passive transfer upon collision. It is also possible that the hydroperoxide itself exhibits direct toxicity by interfering with a vital cellular process and that BHT and DFO act by inhibiting propagation in the cell membrane, thereby lowering the net accumulation of peroxides.

The inhibition of the toxicity of oxidized LDL to endothelial cells by nitric oxide represents another mechanism whereby nitric oxide may play an anti-atherogenic role. Endothelial cell-derived nitric oxide may represent a continuously generated vascular antioxidant that suppresses the harmful oxidative reactions involved in the formation and progression of vascular pathologies such as atherosclerosis.

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