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Nitric oxide donor GEA 3162 inhibits endothelial cell-mediated oxidation of low density lipoprotein

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Abstract

The effect of a nitric oxide (NO) donor GEA 3162 on the endothelial cell (EC)-mediated oxidation of low density lipoprotein (LDL) was studied. In comparison to LDL incubated with EC without GEA 3162, the presence of GEA 3162 inhibited LDL oxidation by EC, as indicated by the following findings. (a) The degradation rate of LDL in macrophages was reduced to control levels. (b) The electrophoretic mobility of LDL decreased in a dose-dependent manner. (c) The concentrations of thiobarbituric acid-reactive substances and hydroperoxide-derived hydroxy fatty acids were lower. (d) The breakdown of apolipoprotein B was reduced. The results indicate that GEA 3162 prevents EC-mediated oxidation of LDL.

Key words: Atherosclerosis; Endothelial cell; Nitric oxide; Oxidized low density lipoprotein; SIN-1

1. Introduction

Oxidative modification of low density lipoprotein (LDL) may play an important role in the pathogenesis of atherosclerosis [1]. Oxidized LDL has been isolated from atherosclerotic lesions [2], and antioxidant therapy reduces atherogenesis in animal models [3,4]. Previous studies of cell-mediated LDL oxidation have demonstrated that all cell types present in atherosclerotic lesions can oxidize LDL [5–8]. The mechanisms whereby different cell types oxidize LDL particles are still unknown, although several mechanisms, such as lipoxygenases [9,10], superoxide anions (O_2^-) [7], and peroxynitrite ($ONOO^-$) [11] have been suggested to play roles in this process.

Oxidation causes significant changes in several properties of LDL [1,12]. Among these changes are increased electrophoretic mobility, increased contents of fatty acid peroxides and thiobarbituric acid-reactive substances (TBARS), breakdown of apolipoprotein B and increased degradation rate by macrophages. These properties of oxidized LDL were used in the present study as indicators of LDL oxidation.

NO is a short-lived, rapidly diffusable biological messenger molecule, which can cause many metabolic changes in target cells [13]. In EC, NO has been reported to lead to autocrine stimulation of cGMP [14] and to function as a negative feedback modulator of its own synthesis [15]. NO is also a free radical and when reacting with O_2^-, it can produce a potent oxidant peroxynitrite [16]. Protonated peroxynitrite has been proposed to decay to hydroxyl radical, Indeed, simultaneous generation of O_2^- and NO by SIN-1 has been demonstrated to initiate lipid peroxidation in human LDL [11,17,18] and to convert LDL to a form recognized by macrophage scavenger receptor [17].

The purpose of the present study was to investigate the effects of a potent NO donor GEA 3162 on the EC-mediated oxidation of LDL. It was found that the NO donor inhibited rabbit aortic EC-mediated oxidation of LDL.

2. Materials and methods

2.1. Materials

Carrier-free sodium ^{125}I was purchased from Amersham (Buckinghamshire, UK). Optimem-1, RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), Newborn calf serum, Penicillin-Streptomycin and Gentamycin were from Gibco laboratories (Paisley, Scotland). Endothelial cell-derived growth supplement, 4-chloro-1-naphthol, butylated hydroxytoluene, glyceryltriheptadecanoate and reference methyl esters of hydroxy fatty acids were from Sigma Chemical Co. (St. Louis, MO,

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Abbreviations: EC, endothelial cells; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; MDA, malondialdehyde; NO, nitric oxide; O_2^-, superoxide anion; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid-reactive substances; SIN-1,3-morpholino-sydnonimine-hydrochloride.

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USA). Agarose gels were purchased from Corning Diagnostics (Palo Alto, CA, USA). Cell culture dishes were from Nunclon (Roskilde, Denmark). NO donor GEA 3162 was kindly provided by GEA Ltd. (Copenhagen, Denmark). GEA 3162 is a mesoionic 3-aryl-substituted oxatriazole-5-imine derivative (Fig. 1) which produces NO upon decomposition [19,20]. NO donor 3-morpholino-sydnonimine-hydrochloride (SIN-1) was obtained from GEA Ltd. X-ray films were purchased from Eastman Kodak Co. (Rochester, NY, USA). DB-I columns were from J & W Scientific, Inc. (Rancho Cordova, CA, USA). Nitrocellulose membranes and molecular weight markers were from Bio-Rad Laboratories (Richmond, CA, USA). Peroxidase-conjugated goat anti-guinea pig antibodies were from Dakopatts (Copenhagen, Denmark).

2.2. Cell cultures

Rabbit aortic EC [21] were grown in RPMI-1640 medium supplemented with heat-inactivated 10% newborn calf serum, 1.6 mM L-glutamine, 10 mM HEPES, 300 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml endothelial cell-derived growth supplement. Cells were grown in a humidified atmosphere containing 5% CO_2 and fed three times a week. Peritoneal macrophages were harvested from male NMRI mice and washed two times with phosphate-buffered saline (PBS). The cells were added to tissue culture plates in serum-free DMEM and cultured for 2 h at 37°C. The non-adherent cells were washed off and monolayers were then placed in DMEM containing 10% newborn calf serum and cultured for 24 h. In all macrophage experiments the medium was replaced with Optimem-1 supplemented with 5% human lipoprotein-deficient serum (LPDS), glutamine and antibiotics.

2.3. LDL modification and degradation analyses

Human plasma LDL was isolated from healthy donors by sequential ultracentrifugation at a density of 1.019–1.063 g/ml [22]. Lipoproteins were labeled with carrier-free sodium ^{125}I-iodide as described [23]. LDL protein concentrations were determined according to Lowry et al. [24]. TBARS were analyzed as described [9] and expressed as nmol malondialdehyde (MDA)/mg LDL protein.

EC-modified LDL was made as follows: confluent monolayers of rabbit aortic EC were washed with Hank's solution, fed with fresh Optimem-1 without serum and exposed to 5–60 μg/ml of ^{125}I-LDL (100–200 cpm/ng protein) with or without the NO donor GEA 3162. Optimem-1 was used in all experiments, because it contains no copper and maintains EC functions better than most other media during serum-free incubations.

Cells were incubated at 37°C for indicated times. The NO donor GEA 3162 was introduced into the culture medium every 8 h. At the end of the incubation, culture medium was collected and sterilized by filtration (0.45 μm). To estimate the degree of LDL modification, an aliquot of the EC medium was used for 1% agarose electrophoresis (pH 8.6). LDL was visualized on agarose gels using either Fat Red 7B staining or autoradiography. LPDS was added to the EC conditioned medium at a final concentration of 5% (which prevents further oxidation) and the medium was incubated with macrophages for another 24 h. At the end of the incubation, culture medium was collected and LDL degradation was determined as trichloroacetic acid-soluble non-iodide radioactivity [25]. Macrophages were dissolved in 200 mM NaOH and assayed for protein [24]. Each cell culture experiment was performed at least three times using duplicate samples. No microscopic signs of toxicity were detected in EC or macrophage cultures during incubations with the NO donor GEA 3162.

2.4. SDS-PAGE and immunoblotting

Rabbit aortic EC were cultured with unlabelled LDL (60 μg/ml) for 48 h and the incubation medium was collected and adjusted to the density of 1.2 g/ml with solid KBr. Samples were centrifuged in the presence of 0.01% one mM EDTA at 40,000 × *g* for 48 h at +10°C (Beckman L-60, Palo Alto, Ca, USA). Lipoproteins were collected from the top of the tubes and dialyzed against lipoprotein-dialyzing buffer (0.9% NaCl/0.01% EDTA) for 48 h. SDS-PAGE was performed using 10% gels in 0.025 M Tris/0.19 M glycine buffer (pH 8.3) containing 0.1% SDS (100 V, 30 mA for 75 min) [2]. Before electrophoresis, the samples were heated for 3 min in a boiling water bath. The sample buffer contained 0.063 M Tris-HCl, 2% SDS, 10% glycerol, 10 μM butylated hydroxytoluene, and 0.001% bromophenol blue (pH 6.8). Twenty μg of proteins were loaded per lane.

Proteins were transferred to a nitrocellulose membrane in the same electrophoresis unit at +4°C for 80 min (25 V, 100 mA). Nonspecific binding sites on the membranes were blocked by incubation with 5% nonfat dried milk proteins [2]. The membranes were incubated for 12 h with a polyclonal guinea pig anti-LDL antiserum YE [26], followed by incubation with peroxidase-conjugated anti-guinea pig antibodies. 4-chloro-1-naphthol was used as a color substrate to visualize apolipoprotein B bands.

2.5. Analysis of hydroxy fatty acids

To a 500-μl aliquot of the cell culture supernatant, 25 μg butylated hydroxytoluene was added as an antioxidant and 25 μg triheptadecanoin as an internal standard. Lipids were extracted with chloroform/methanol (1:1) and purified according to Folch et al. [27], followed by reduction of the double bonds by hydrogen gas in the presence of platinum oxide for 15 min. After saponification in KOH-ethanol, the fatty acids were esterified with dry methanol containing 2% H_2SO_4, acetylated overnight with acetic anhydride-pyridine (5:1), and fractionated in a Hewlett Packard 5890 gas chromatograph fitted with a 30-m 0.5-mm i.d. fused silica column of DB-1, on-column inlet, flame ionization detector, and HP3365 ChemStation software. The temperature was programmed from 50 to 270°C. The peaks of hydroxyoctadecanoate and hydroxyeicosanoate were identified with the aid of reference methyl esters of hydroxy fatty acids. Nitrite generation was measured as described [28].

3. Results

We first compared nitrite generation from GEA 3162 and SIN-1. The initial rates of nitrite generation from 50 μM GEA 3162 and SIN-1 in DPBS at 37°C were 1.24 ± 0.04 and 0.49 ± 0.03 nmol/ml/min (mean ± S.E., $n = 6$), respectively. The rate of nitrite generation from GEA 3162 at 37°C was linear for about 5 min, while the linear rate of nitrite generation from SIN-1 continued for at least 20 min (data not shown).

The NO donor GEA 3162 (3.3 μM) effectively blocked the oxidation-dependent increase in LDL electrophoretic mobility after incubation with rabbit aortic EC (Fig. 2). The effect of GEA 3162 was different from that of another NO-donor, SIN-1, which under the same conditions increased LDL electrophoretic mobility (Fig. 2). GEA 3162 also decreased the breakdown of apolipoprotein B caused by EC-mediated oxidation of LDL (Fig. 3).

The amount of the linoleate-derived hydroxy fatty acids was clearly increased in the EC-oxidized LDL, but no increase was seen in the samples incubated with the NO donor GEA 3162 (Table 1). GEA 3162 also prevented an increase in LDL TBARS values after incubation with EC (Table 1).

The degradation rate of EC-modified LDL in mouse peritoneal macrophages was increased 3–4-fold as com-

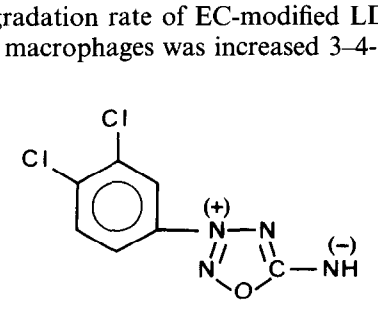

Fig. 1. The chemical structure of the NO donor GEA 3162.

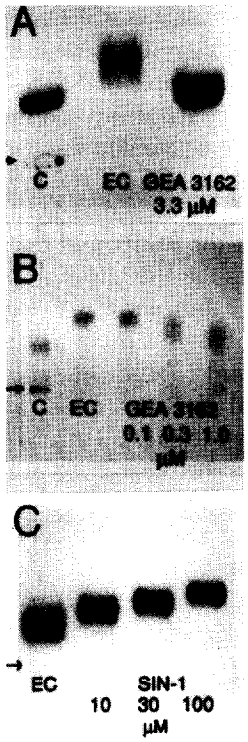

Fig. 2. Effect of the NO donor GEA 3162 on the electrophoretic mobility of LDL after endothelial cell-mediated oxidation. (A) Confluent rabbit aortic endothelial cells were incubated for 24 h with 5 μg/ml ^{125}I-LDL in serum-free Optimem-1 in the presence or absence of the NO donor GEA 3162 (3.3 μM) (B) Dose–response of the protective effect of the NO donor GEA 3162. (C) Effect of another NO donor SIN-1 on the endothelial cell-mediated oxidation of LDL. Samples were analyzed in 1% agarose electrophoresis, followed by autoradiography. C, control LDL; EC, endothelial cell-treated LDL; GEA 3162 and SIN-1, effects of various concentrations of GEA 3162 and SIN-1 on the endothelial cell-mediated oxidation of LDL. An arrow indicates the point of application.

pared to controls (Fig. 4A). When the NO donor GEA 3162 was present during the incubation with EC, the subsequent degradation rate of LDL in macrophages was decreased to control values. The same effect was seen in cell-associated radioactivity (Fig. 4B).

Since some of the NO donor could remain intact in the EC-conditioned medium after the incubations, we tested the direct effect of GEA 3162 on the degradation of acetylated LDL by macrophages. It appeared that at a 3.3 μM concentration GEA 3162 decreased the degradation rate of acetylated LDL only by 15%. Also, in a cell-free system the NO donor GEA 3162 (3.3 μM) alone was unable to oxidize LDL to a form recognized by macrophages nor did it inhibit copper-induced oxidation of LDL (data not shown).

4. Discussion

Oxidized LDL possesses several atherogenic properties: it can lead to the formation of foam cells, it is chemotactic for blood monocytes and cytotoxic to EC [1]. Various forms of oxidized LDL can also induce changes in arterial wall gene expression [29] and inhibit EC-derived relaxing factor-mediated vasodilation [30]. All cell types found in atherosclerotic lesions can modify LDL in vitro through oxidative processes [5–8]. Since exact mechanisms by which cells oxidize LDL remain uncertain, it would be important to identify conditions which either increase or decrease LDL oxidation. The use of compounds that generate NO provide one promising approach in this kind of studies.

Recently, Jessup et al. [18] reported indirect evidence that NO inhibits LDL oxidation by macrophages. They

Table 1
Effect of the NO donor GEA 3162 on the content of hydroxy fatty acids in LDL after endothelial cell-mediated oxidation

	Control LDL	Endothelial treated LDL	
		GEA 3162	
		–	+
Hydroxy fatty acids (μg/mg LDL)			
OH-octadecanoate (OH-18)	14.5	71.8	5.7
OH-eicosanoate (OH-20)	8.4	8.0	4.8
TBARS (nmol MDA/mg LDL)	16	64	10

LDL (80 μg/ml) was incubated with endothelial cells in serum-free Optimem-1 medium for 48 h in the presence (3.3 μM) or absence of the NO donor GEA 3162. The lipids were extracted from the incubation medium with chloroform/methanol, hydrogenated and saponified. The fatty acids were then esterified and analyzed with gas chromatography. Control LDL was kept under similar conditions at 4°C for 48 h. The table gives results of a representative experiment. Similar results were obtained after a 24 h incubation time (data not shown).

Fig. 3. Effect of the NO donor GEA 3162 on the breakdown of apolipoprotein B during endothelial cell-mediated oxidation of LDL. Unlabeled LDL (60 μg/ml) was incubated with rabbit aortic endothelial cells in serum-free Optimen-1 for 24 h in the presence or absence of the NO donor (3.3 μM). LDL was then reisolated using ultracentrifugation and subjected to 4–15% SDS-PAGE and immunoblotting. Immunoblot was done using a polyclonal anti-LDL antiserum YE (dilution 1:200). Lane 1: Molecular weight markers; lane 2: Control LDL; lane 3: LDL oxidized by endothelial cells; lane 4: LDL incubated with endothelial cells in the presence of the NO donor. An arrow indicates the position of intact apolipoprotein B-100. Each lane contains 20 μg protein.

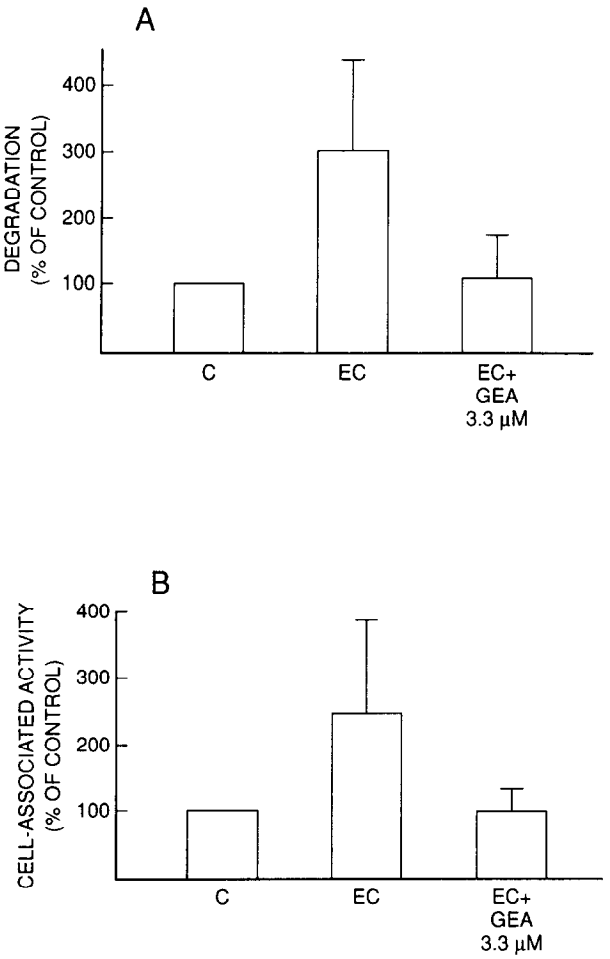

Fig. 4. Degradation of ^{125}I-LDL (5 μg/ml) in mouse peritoneal macrophages after incubation with endothelial cells in the presence or absence of the NO donor GEA 3162 as indicated in the legend for Fig. 2A. Endothelial cell incubation medium was filtered, supplemented with 5% lipoprotein deficient serum and incubated with macrophages for 24 h. Macrophages were then analyzed for (A) LDL degradation products and (B) cell-associated radioactivity. Results are expressed as percentages of control values ± S.E. (three separate experiments). C, control LDL; EC, endothelial cell-treated LDL; EC + GEA, the effect of GEA 3162 on the endothelial cell-mediated oxidation of LDL.

found that γ-interferon-stimulated macrophages, in which NO production is increased, did not oxidize LDL as well as unstimulated macrophages. Although γ-interferon induces several other changes in macrophages besides an increased NO production, their results are compatable with our findings and suggest a protective role of NO against cell-mediated LDL oxidation. Similar findings were also reported by Yates et al. [31].

Jessup et al. [18] also tested the effects of another NO donor, SIN-1, on the oxidation of LDL. SIN-1 undergoes an oxygen-dependent release of NO with the concomitant production of O_2^- [32]. They found that SIN-1 increased the content of LDL hydroperoxides but did not convert LDL into a form recognized by macrophages. However, the direct effects of SIN-1 on the macrophage-mediated oxidation of LDL were not tested. In a cell-free system, Darley-Usmar et al. [11] and Graham et al. [17] have demonstrated that SIN-1 is able to oxidize LDL, while NO generated from sodium nitroprusside or *S*-nitroso-*n*-acetylpenicillamine was virtually ineffective.

In the present study, GEA 3162 reduced lipid hydroperoxide formation (measured as hydroxy acids), which could indicate that the compound does not produce O_2^- simultaneously with the NO release. This agrees with our findings that SIN-1, but not GEA 3162, increases LDL electrophoretic mobility. Also, in a cell-free system SIN-1 (100 μM) increased LDL degradation by mouse macrophages 2-fold (Malo-Ranta et al., unpublished observations). In contrast to GEA 3162, SIN-1 also caused a strong spontaneous chemiluminescence in an oxygenated aqueous environment at biologically active concentrations [19]. The rate and kinetics of nitrite production from GEA 3162 and SIN-1 were quite different: GEA 3162 generated NO quickly and the nitrite production started to decline already in a few minutes, while the production of nitrite from SIN-1 was slower and more persistent. The rates are in a good agreement with the reported production of NO from GEA 3162 and SIN-1, as measured by the oxyhaemoglobin method [20]. Although the exact mechanism of NO release by GEA 3162 is unknown, these data indicate differences between SIN-1 and GEA 3162 with respect to the generation of NO.

The inhibitory effect of GEA 3162 on the EC-mediated oxidation of LDL could be related to the stimulation of soluble guanylate cyclase by NO [13] or to the inhibitory effect of NO on 15-lipoxygenase activity [33]. It is also possible that NO or a product derived from GEA 3162 can scavenge radicals [34] or chelate iron or other components needed for LDL oxidation [11,18,33]. Further studies are needed to define actual mechanisms responsible for the inhibitory effect of the NO donor GEA 3162 on the EC-mediated oxidation of LDL.

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