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FEBS Letters 342 (1994) 171–175

FEBS 13854

FEBS LETTERS

Oxidation of LDL by nitric oxide and its modification by superoxides in macrophage and cell-free systems

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Received 28 January 1994; revised version received 24 February 1994

Abstract

Oxidative modification of low density lipoproteins (LDL) can increase the atherogenicity of LDL. Here we demonstrate the potential role of nitric oxide (NO) in LDL modification. Dose-dependent alterations of LDL including an increase in electrophoretic mobility, generation of peroxides and degradation of apoprotein B occurred during incubation with NO. Sodium nitroprusside was also shown to increase the electromobility of LDL. Superoxide dismutase (SOD) and catalase did not affect this result. NO treatment also increases the negative charges on other lipoproteins. In addition, we have demonstrated that NO plays a protective role in macrophage or superoxide-induced LDL modification through its neutralizing action.

Key words: Nitric oxide; Superoxide; Macrophage; Lipoprotein; Lipid peroxidation

1. Introduction

Oxidative modification of human lipoproteins, especially the low density lipoprotein (LDL), is considered to be an important step in the development of atherosclerosis [1]. Lipoproteins can be modified by cell-free systems and by free-radical-generating cells [2–4]. This modification is accompanied by a number of striking changes, including a marked increase in electrophoretic mobility [5], hydrolysis of lecithin to lysolecithin, degradation of apoprotein B [6], and the generation of peroxides [7]. Even during the early stages of oxidation, cytotoxic products are released and may impair the function of the endothelium and other cell types. But, in the presence of α-tocopherol or butylated hydroxytoluene, which are effective free radical scavengers, the formation of peroxides and most of the other changes are inhibited both in vitro and in vivo [7].

The species of those free-radicals responsible for the oxidation of LDL are not well established. One of the reactive radicals which always produces conflicting results is nitric oxide (NO). Bruckdorfer et al. [8] reported that NO alone had no direct oxidative action on LDL or on erythrocyte membranes. In contrast, Jessup et al. [9] reported that incubation with NO alone caused oxidation of LDL and accumulation of small amounts of lipid hydroperoxide. Furthermore, NO was known to inhibit the oxidation of LDL in the presence of myoglobin and H_2O_2. However, in the presence of excess H_2O_2, the action of NO is totally different and appears to enhance the oxidation of the LDL [10]. Recently, NO was thought to enhance the lipid peroxidation of LDL in macrophages [9], but was then found to exert a protective role in macrophage-mediated LDL peroxidation [11].

In this study, we first ascertained that oxidative modification by NO occurs in vitro in VLDL, LDL, HDL_2 and HDL_3. We also demonstrated that NO could exert a protective role in superoxide-mediated oxidation of LDL.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma Chemical Co. (St Louis, MO): potassium superoxide, hemoglobin, sodium dithionite, SOD, catalase, nitroprusside, polyacrylamine and sodium dodecylsulfate (SDS). Sodium nitrate, thiobarbituric acid and trichloroacetic acid were obtained from E. Merck (Darmstadt). Protein markers were purchased from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin (BSA), Penicillin G and Streptomycin were obtained from Boehringer Mannheim Biochemical (Indianapolis, IN).

2.2. Preparation of lipoproteins

Very low density lipoprotein (VLDL) ($\rho < 1.019$), LDL ($\rho = 1.019$–1.063), high density lipoprotein (HDL_2) ($\rho = 1.085$–1.125), and HDL_3 ($\rho = 1.125$–1.21) were isolated through sequential ultracentrifugations of plasma from healthy human subjects. Lipoproteins used for oxidative modification were extensively dialyzed against 0.012 M phosphate-buffered saline containing penicillin G (100 units/ml) and streptomycin (100 units/ml) and stored at 4°C. Bio Rad protein assay solutions were used for protein determinations, employing BSA as a standard.

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SSDI 0014-5793(94)00239-R

2.3. Preparation of the NO saturated solution and treatment of LDL

Distilled water was aerated with pure nitrogen for 2 h before the preparation of the NO solution and was kept sealed until use. NO gas (Linde Air Products Co.) was then passed through a trap containing saturated solutions of NaOH to remove traces of NO_2^- and N_2O_3, and then the gas was passed through oxygen-free, nitrogen-treated water for 30 min to produce an NO-saturated solution. The conversion of hemoglobin to methemoglobin determined by a double-beam spectrophotometer (Hitachi Model U-3210) was used to analyze the NO content of the stock solution [12]. The NO solution was prepared in vials sealed with a rubber septum, and samples were removed anaerobically using a gastight syringe. LDL solutions were dispensed into sealed and deoxygenated vials. Aliquots of the saturated NO solution were then added directly to the LDL solution to give the indicated final concentrations.

2.4. Preparation of the superoxide anion radical solution

Pieces of potassium superoxide, approximately 75 mg, were dissolved under a swirling motion in 25 ml of ice-cold 50 mM NaOH plus 0.5 mM diethylenetriamine pentaacetic acid. The resulting solution initially contained approximately 15 mM H_2O_2 and 8 mM superoxide anion [13].

2.5. Assessment of lipoprotein modification

The extent of lipid peroxidation was measured in terms of thiobarbituric acid-reactive substances (TBARS) using malondialdehyde as a standard [14]. The electrophoretic mobility of the lipoproteins was evaluated by the Paragon lipo electrophoresis system (Beckman analytic, Milan, Italy). In addition, non-enzymatic oxidative cleavage of peptide bonds in apoprotein B-100 was performed by SDS-polyacrylamine gradient gel electrophoresis [6]. The LDL samples were dissolved in sample buffer containing 3% SDS, 10% glycerol and 5% 2-mercaptoethanol, and incubated in boiling water for 5 min. Vertical gel electrophoresis was then performed using a 3–12% gradient mini gel at a constant current of 30 mA for 3 h in a BDH electrophoresis unit. Proteins of known molecular weight were used as standards (phosphorylase B, 106 kDa; BSA, 80 kDa). Staining was performed using silver nitrate in water containing 0.18 M NH_3 and 20 mM NaOH.

3. Results

3.1. Oxidative modification of LDL by NO in cell-free systems

Concentration-dependent and marked alterations in the physicochemical properties of LDL occurred when LDL was exposed to NO concentrations comparable to those produced by macrophage cultures (NO_3^-/NO_2^-, 17.4 ± 0.4~271.2 ± 4.9 μM, [15]) and by C_3H/HeJ mice (urinary nitrate, 3~94 μmol per mouse, [15]) over a 24 h period. These alterations comprised of an increase in electrophoretic mobility and in the generation of thiobarbituric acid-reactive substances (TBARS) (Fig. 1A,B). Non-enzymatic breakdown of LDL apoprotein B-100 was also seen (Fig. 1C). The 514 kDa band of intact apo B gradually diminished with increasing radical doses. All silver staining of oxidized samples showed a diffuse, dark background plus several broad protein bands. This fragmentation correlated well with the degree of lipid peroxidation induced and the net negative charge. Fig. 2. shows that LDL can be oxidized by NO, but not by other factors such as the pH variation (pH 7.4 to 6.2), NO_2^-, or both combined. A deactivated NO solution was also used here to exclude the participation of divalent metals. Moreover, it was evident that the oxidative effects of NO added as a bolus were not decreased by the slow continuous infusion of NO over time which mimics the conditions that occur in vivo (Fig. 3).

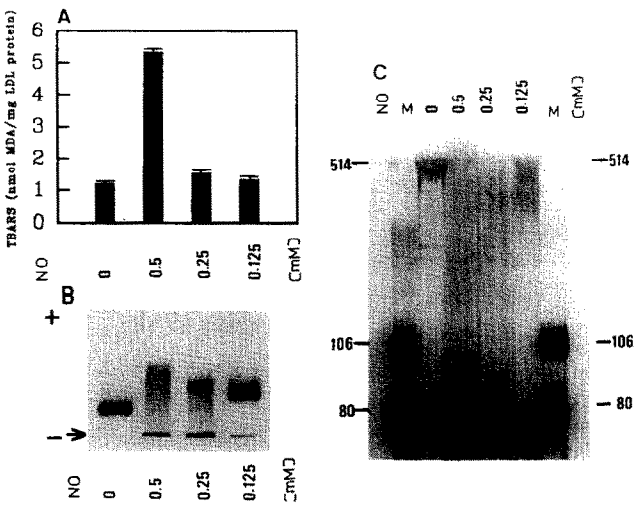

Fig. 1. The changes in LDL (0.4 mg/ml) after incubation with NO for 24 h were measured by the generation of peroxides (TBARS) (A), the increase of electromobility (B), and the degradation of apoprotein B-100 (C). The arrow indicates sample origin. Molecular masses are indicated in kDa. M, molecular mass markers.

Sodium nitroprusside, which usually acts as a source of NO in cell-free systems [16], was also used in this study. A dose-dependent increase in the electromobility of LDL was observed (Fig. 4). SOD and catalase did not affect the movement of LDL.

3.2. Modification of other lipoproteins by NO in a cell-free system

Other lipoproteins which were isolated through sequential ultracentrifugation of plasma were also used. We tested whether there was any difference between native and NO-oxidized lipoproteins. When measured using Beckman paragon lipo agarose gel electrophoresis, the negative charge on VLDL increased after NO treatment as compared with the native form. The electrophoretic mobility of two treated forms of high density lipoproteins, HDL_2 and HDL_3, were also increased (Fig. 5). The results clearly show that oxidative modification occurred in HDL and VLDL as well as in LDL.

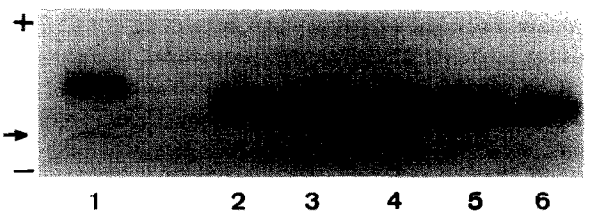

Fig. 2. The influence of pH variation (lane 2), the content of NO_2^- (0.25 mM) (lane 3), or both (lane 4) on the oxidative modification of LDL. Lane 5, LDL without any treatment. Deactivated NO solution (lane 6) (deactivated by air exposure for 24 h) was also tested. NO solution (0.25 mM) was used here for positive control (lane 1).

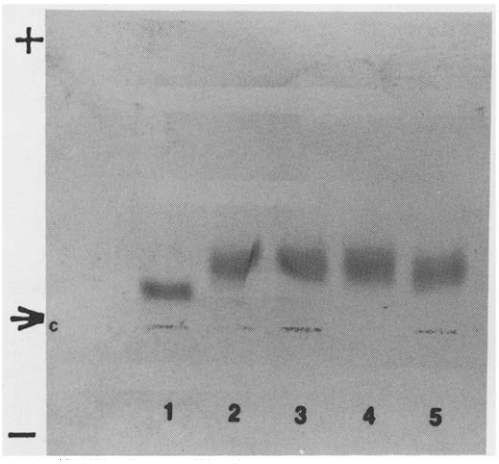

Fig. 3. The influence of the slow continuous infusion of the NO solution on the oxidative modification of LDL. Slow continuous infusions of NO were performed by adding a definite amount every 4 h (lane 2), 1 h (lane 3), or 10 min (lane 4) to make a final concentration (0.125 mM) equal to that of the bolus added (lane 5). Lane 1, LDL without any treatment.

3.3. *Oxidative modification of LDL by superoxide*

The alterations of LDL were examined when an NO stimulator or NO inhibitor was added to stimulate macrophages. Our data (not shown) support the finding by Yates et al. [11] that the increase of NO protects against macrophage-mediated lipid peroxidation and that the inhibition of NO production reverses the phenomenon.

A conflicting result is indicated here that NO seems to accelerate the oxidation of LDL in a cell-free system but inhibits it when stimulated macrophages are present. One possibility is that NO alone has direct oxidative action on LDL, but NO can neutralize the oxidative effects of superoxide ions which are also produced by macrophage cells. This hypothesis is demonstrated in Fig. 6. The superoxide anion-induced increase of anodic mobility was reversed or partially restored by co-incubation with NO. H_2O_2 did not change the electromobility.

4. Discussion

LDL has been shown to be oxidized by NO-generating cells such as macrophages, monocytes or endothelial cells [2,3,17]. Moreover, LDL modification by endothelial cells was not influenced by SOD, catalase, or single oxygen or hydroxyl radical scavengers [3]. In addition, although ferryl-myoglobin-mediated peroxidation of LDL may be enhanced or suppressed by NO depending on the relative concentration of NO and hydrogen peroxides [10], the action of NO on enhancing LDL oxidation suggests that under certain conditions NO may have disadvantageous effects [8]. Jessup et al. [9] also reported that incubation with NO alone caused oxidation of LDL's ubiquinol-10 and accumulation of small amounts of lipid hydroperoxides. In this study, we observed dose–response alterations in the physiological properties of LDL when LDL was exposed to an NO concentration comparable to those produced by macrophage cultures over a 24 h period [15].

A mechanism now widely accepted is that monocytes develop into macrophages in the subendothelial spaces where LDL is considered to be oxidatively modified. Oxidized LDL is then taken up by macrophages, leading to foam cell transformations [1,5]. In contrast, HDL is involved in the amelioration of the atherosclerotic lesion in vivo by way of stimulating a cholesterol efflux from the foam cells [18]. However, HDL loses its ability to stimulate an efflux of cholesterol from foam cells after oxidative modification [19]. The results in Fig. 5 clearly show that oxidative modification by NO occurs in HDL as well as in LDL. This finding implies that NO may be actively involved in the development of atherosclerosis.

The present study [20] reveals that SOD increases the half-life of NO, but NO does not bind directly to the copper of SOD [21], suggesting that the potency of NO as a vasodilator is decreased in the presence of superoxides. Wolin et al. [22] also reported that, by extracellular generation of superoxide anions, Methylene blue inhibited vasodilation due to nitric oxide. It is probable that NO can accept electrons and react with superoxides to form peroxynitrite anions and thereby inactivate the superoxides. This reaction has a high yield (>85%) [23]. Although Beckman et al. [24] proposed that a homolytic fission of the peroxynitrite anion produces a strong oxidant with reactivity similar to the hydroxyl radical, Hogg

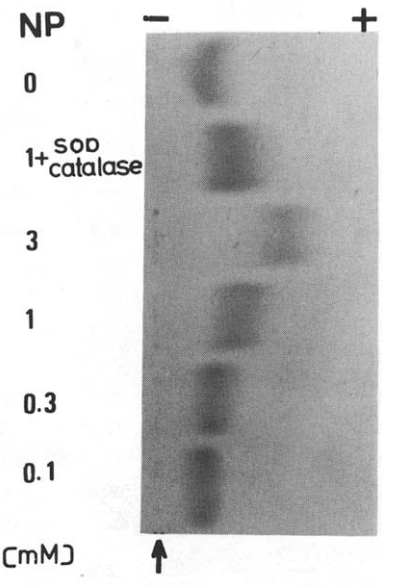

Fig. 4. Electrophoretic mobility of LDL (0.4 mg/ml) after incubation with nitroprusside for 24 h. The influence of SOD (600 units/ml) and catalase (600 units/ml) on the net negative charge of LDL was also tested. The arrow indicates sample origin. NP, nitroprusside.

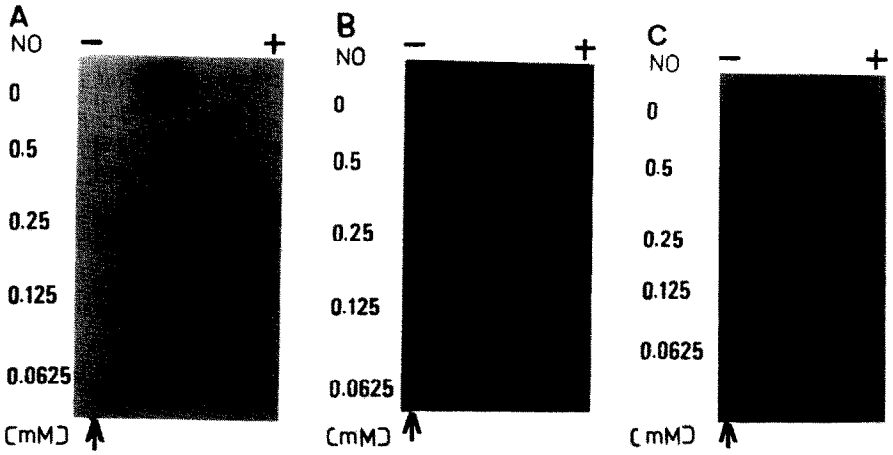

Fig. 5. Electrophoretic mobility of VLDL (0.4 mg/ml) (A), HDL_2 (0.4 mg/ml) (B), and HDL_3 (0.4 mg/ml) (C) were measured after incubation with NO for 24 h. Arrows indicate sample origins.

et al. [25] showed that there was low efficiency in production of hydroxyl radicals from the simultaneous presence of superoxides and NO. The maximum yield of hydroxyl radicals produced were about 3% of the concentration of 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1) decomposed. In addition, although it is not yet known what level of hydroxyl radical generation can be regarded as deleterious, the effects of several oxygen-centered free radicals on human LDL were examined by Bedwell et al. [26] who found that the hydroxyl radical alone had a low ability to produce modified LDL.

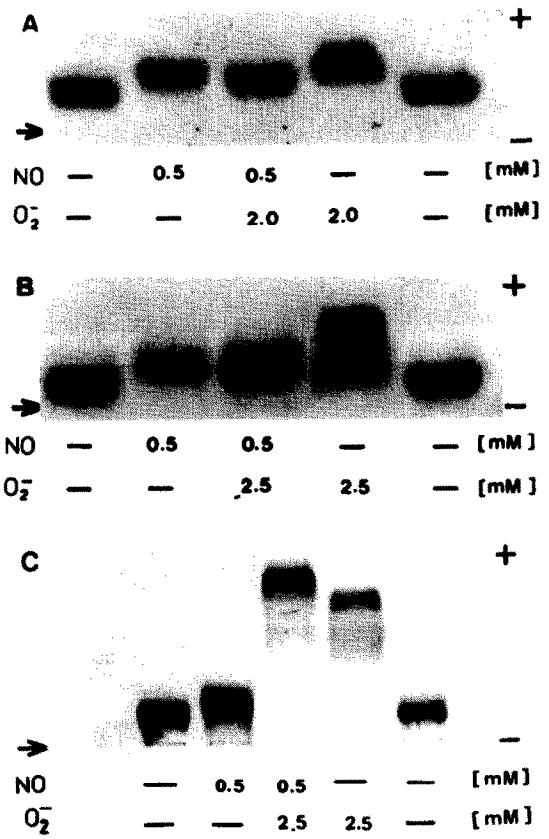

Fig. 6. The influence of NO on superoxide-mediated oxidation of LDL was measured by agarose gel electrophoresis. NO and superoxide were added at the same time (A,B). A 30-min time lag between the addition of NO and superoxide (C). Arrows indicate sample origins.

Hogg et al. [27] and Darley-Usmar et al. [28] have recently demonstrated that the simultaneous generation of superoxides and NO by the xanthine oxidase/*s*-nitroso-*n*-acetylpenicillamine system or the SIN-1 compound resulted in the formation of an oxidant with 'hydroxyl-radical like' reactivity capable of initiating the oxidation of LDL. We also observed a similar result when 1.2.4-benzentriol/nitroprusside system was used to generate these two radicals in the presence of LDL (data not shown). However, there is poor evidence that the hydroxyl radical itself mediated this modification. Furthermore, it was found that SIN-1 is attacked by molecular oxygen and is converted to a radical while oxygen is reduced to a superoxide, and the resulting highly unstable radical is stabilized by NO splitting off and deprotonating [29]. This finding implies that the time interval between superoxide formation and NO formation may be sufficient for the highly unstable superoxide radical to react with LDL first. Therefore, it is possible that the xanthine oxidease/*s*-nitroso-*n*-acetylpenicillamine, 1.2.4-benzentriol/nitroprusside or SIN-1 dependent peroxidation is mediated by the additive effect of these two radicals. In this study, gas-tight syringes were used to add the NO-saturated solution and superoxide stock solution simultaneously to the LDL solution. Fig. 6 clearly indicates that the superoxide-induced modification was reversed when co-incubated with NO, but this modification was additive when there is a 30-minute time lag in the addition of NO and superoxide. Thus, NO reacts with the superoxide and thereby inactivates it.

Acknowledgements: This study was supported by the National Taiwan University Research NTUH 81-040-A13 and the National Science Council NSC 82-0412-B002-202, Taipei, Taiwan, ROC.

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