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A protective role for nitric oxide in the oxidative modification of low density lipoproteins by mouse macrophages

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Low density lipoproteins (LDL) oxidatively modified by macrophages have been shown to be atherogenic in ex vivo studies. We studied the potential role of nitric oxide (NO), a free radical produced by macrophages, in LDL modification. Human LDL (1 mg/ml) were incubated with mouse peritoneal macrophages in Ham's F-10 medium. The cells were then stimulated by interferon-γ and tumor necrosis factor-α to increase their production of NO from 1.3 to 12.2 μM in 24 h, as measured by nitrite. Lipid peroxidation of LDL, as measured by thiobarbituric acid-reactive materials (TBARS), was reduced in stimulated cells in a time-dependent manner. At 24 h, the decrease was about 27%. In the presence of an NO synthase inhibitor (N^G-aminophomoarginine), the generation of NO was diminished and the protection against LDL lipid peroxidation was reversed. The extent of LDL protein modification was also assessed by examining its electrophoretic mobility. It was found that macrophage NO reduced the change in LDL electromobility. These data indicate that the production of NO may inhibit the oxidative modification of LDL with cytokine-stimulated macrophages. We suggest that NO plays a protective role in limiting macrophage-induced LDL modification.

Low density lipoprotein; Oxidation; Nitric oxide; Macrophage; Atherosclerosis

1. INTRODUCTION

The accumulation of lipid-laden foam cells derived from macrophages in the aortic intima is an early event in atherogenesis [1–7]. Studies have suggested that lipid peroxidation of low density lipoproteins (LDL) induced by free radicals can increase the atherogenicity of LDL [8–14]. These oxidized LDL are recognized and taken up by macrophage scavenger receptors leading to foam cell formation [15,16]. In the presence of an antioxidant, such as probucol, the formation of foam cells is inhibited both in vitro and in vivo [17–21]. However, the sources and species of those free radicals responsible for the oxidation of LDL are not well established. The superoxide anion (O_2^-) produced by macrophages is known to be involved, since the pretreatment of macrophages with superoxide dismutase (SOD) attenuates LDL lipid peroxidation [22]. Macrophages have been shown to produce the nitric oxide (NO) radical via nitric oxide synthase. This enzyme converts arginine into citrulline and is induced by cytokine treatment [23,24]. Although NO can neutralize O_2^- to form a stable peroxynitrite anion ($ONOO^-$) [25], under some conditions the peroxynitrite can also rapidly decompose to form a strong oxidant with reactivity similar to the hydroxyl radical [26]:

$$O_2^- + NO^\cdot \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightleftharpoons ONOOH \rightarrow HO^\cdot + NO_2^\cdot$$

In addition, NO generation in vivo may lead to the mobilization of iron with a subsequent increase in the level of reactive oxygen species [27]. These observations could explain the cytotoxic action of NO that has been demonstrated in a number of studies [28,29]. The purpose of the present study was to determine the effect of NO production by macrophages on LDL lipid peroxidation. To this end we incubated LDL with macrophages in which the synthesis of NO was induced with interferon-γ and tumor necrosis factor-α, and followed the extent of oxidative modification of LDL.

2. MATERIALS AND METHODS

2.1. *Nitric oxide synthase inhibitor (MDL 100,248)*

S-Methylisothiosemicarbazide hydroiodide (17.8 g, 0.076 mol) was added to L-lysine monohydrate (9.3 g, 0.076 mol) in water (100 ml) and adjusted to pH 9.5 with 1 N NaOH. The reaction mixture was heated and stirred at 40°C for 30 h. The solution was then cooled to room temperature and acidified to pH 4.0 with acetic acid. Flavianic acid (20 g) was then added to the mixture and cooled to 0°C. The resulting precipitate was then filtered and recrystallized from water to yield 13.2 g L-N^G-aminohomoarginine flavinate. The solid was then placed on a bed of 200–400 mesh AG2-X8 chloride from ion-exchange resin in a soxhlet extraction thimble and eluted with hot water. The solution was lyophilized and the resulting hydroscopic solid taken up in methanol (75 ml) and precipitated as the free base by the addition of propylene oxide (10 ml).

The arginine analogue, MDL 100,248, was freshly prepared as a

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stock solution (100 mM) in dimethylsulfoxide (ME_2SO). The solution was then diluted to the appropriate concentration in medium and filter sterilized prior to use in the assay.

2.2. *Preparation of LDL*

Low density lipoproteins (LDL) were isolated from human plasma using sequential ultracentrifugation at a density of 1.019–1.063 g/ml [30]. The freshly prepared LDL were extensively dialyzed against 0.012 M phosphate-buffered saline (PBS) containing penicillin G (100 U/ml) and streptomycin (100 U/ml) and stored at 4°C. The protein concentration of LDL was determined by the method of Lowry et al. [31] with minor modification [30].

2.3. *Macrophage monolayer preparation*

Resident peritoneal macrophages were harvested from 24 male CD-1 mice (16–20 g) (Charles River) and washed 1× with Dulbecco's modified Eagle's medium (DMEM) and 1× with DMEM containing 10% fetal bovine serum (Gibco). The cells were added to 24-well tissue culture plates at 2×10^6 cells/well in DMEM containing 10% fetal bovine serum, and cultured for 8 h at 37°C in an incubator containing 5% CO_2 with 90% humidity. The non-adherent cells were removed and the monolayers were then placed in serum-free DMEM or Ham's F-10 medium (Gibco). In the stimulated macrophage cultures, recombinant interferon-γ (IFN-γ, Amgen) and tumor necrosis factor-α (TNF-α, Genzyme) were each added at 1,000 U/ml. In those cultures in which NO production was inhibited, cytokine treatment was preceded by the addition of the arginine analogue, L-N^G-aminohomoarginine (200 μM). Control monolayers were cultured without stimulating agents. Human LDL (100 μg) were immediately added to each culture in the appropriate medium with a final volume of 1 ml/well.

2.4. *Assessment of LDL modification*

At each period of 0, 5, 14 and 24 h the cultures were harvested in triplicate to assess the oxidative modification of LDL. Total lipid peroxidation in the cell supernatant (assaying 50 μg of LDL) was determined and expressed as thiobarbituric acid-reactive substances (TBARS) using malondialdehyde (MDA) as a standard [19]. Agarose gel electrophoresis was performed to evaluate the electrophoretic mobility of the LDL. Aliquots of the culture supernatants (250 μl) were concentrated five-fold using a centricon-30 microconcentrator apparatus (Amicon). This was performed by centrifugation at 4,000 rpm (2,500 × g) for 15 min at 4°C. 3 μl of the concentrated samples were applied to a 1% agarose gel and electrophoresis was performed with a 90B power supply for 35 min (Ciba-Corning). The gel was dried for 1 h at 60°C and stained with Fat red 7B (Sigma).

2.5. *Macrophage NO production*

Macrophage NO synthesis was measured indirectly as the nitrite concentration in the culture medium. 100 μl of the supernatant fluid were used to determine the level of nitrite in the manner described by Stuehr and Nathan [29].

3. RESULTS AND DISCUSSION

The purpose of this study was to explore the possible role of NO radicals produced from macrophages in LDL lipid peroxidation. Since LDL can be directly oxidized and modified by peroxyl radicals ($HOO^{\cdot}$) generated via the thermolysis of azo compounds [32], we tested whether or not the production of NO radicals by macrophages may also oxidatively modify LDL. First, we used cytokines to stimulate NO synthase in macrophages resulting in the overproduction of NO. Fig. 1 shows that there was a 25-fold increase in NO production in stimulated cells cultured in DMEM medium for 24 h. The elevation of NO levels did not enhance the

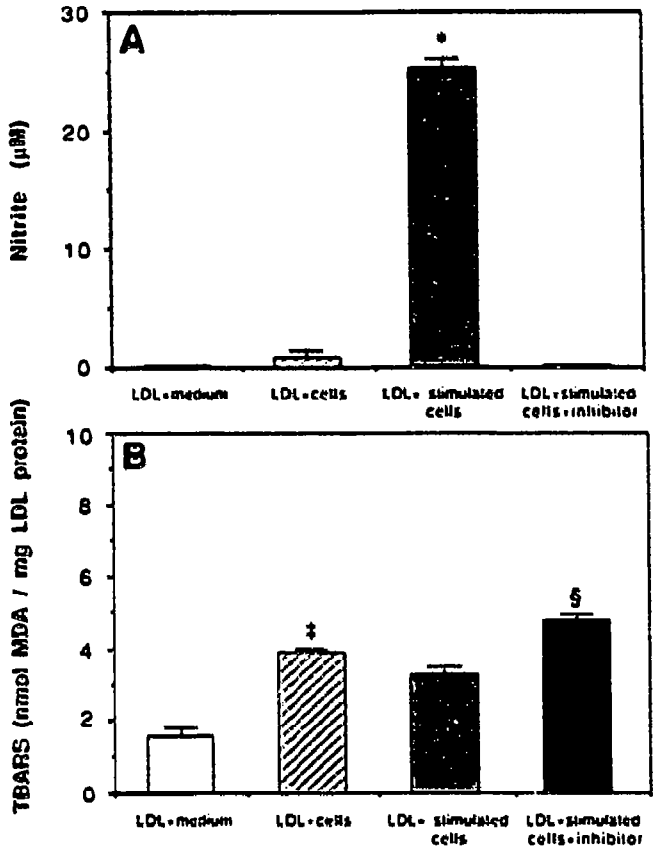

Fig. 1. Effect of NO production (A) on the lipid peroxidation (B) of LDL incubated with macrophages for 24 h in DMEM culture medium. NO production was stimulated by IFN-γ and TNF-α with and without an NO synthase inhibitor and measured indirectly as nitrite. NO was not detectable in medium containing LDL alone. TBARS were expressed using malondialdehyde (MDA) as a reference and were insignificant in cultured medium without LDL. Each bar represents the mean ± S.E.M. of three experiments. *$P < 0.005$ as compared to LDL + cells. ‡$P < 0.001$ as compared to LDL + medium. §$P = 0.03$ as compared to LDL + stimulated cells.

lipid peroxidation of LDL, as measured by TBARS. A slight, but not statistically significant, decrease was observed (Fig. 1B).

In theory, if NO protects against macrophage-mediated LDL oxidation, stimulated cells treated with a specific NO synthase inhibitor would demonstrate enhanced oxidation. We show that with the synthase inhibitor (L-N^G-aminohomoarginine) the production of NO was reduced below detectable levels (Fig. 1A), and that LDL oxidation was increased (P =0.03) compared to stimulated cells without the inhibitor (Fig. 1B). We also tested the possibility that the increase of lipid peroxidation of LDL was due to the interference of the inhibitor with TBA in the assay. It was found that the inhibitor (200 μM) neither changed the formation of the MDA–TBA complex nor acted as a pro-oxidant to LDL lipid peroxidation mediated by Cu^{2+} (data not

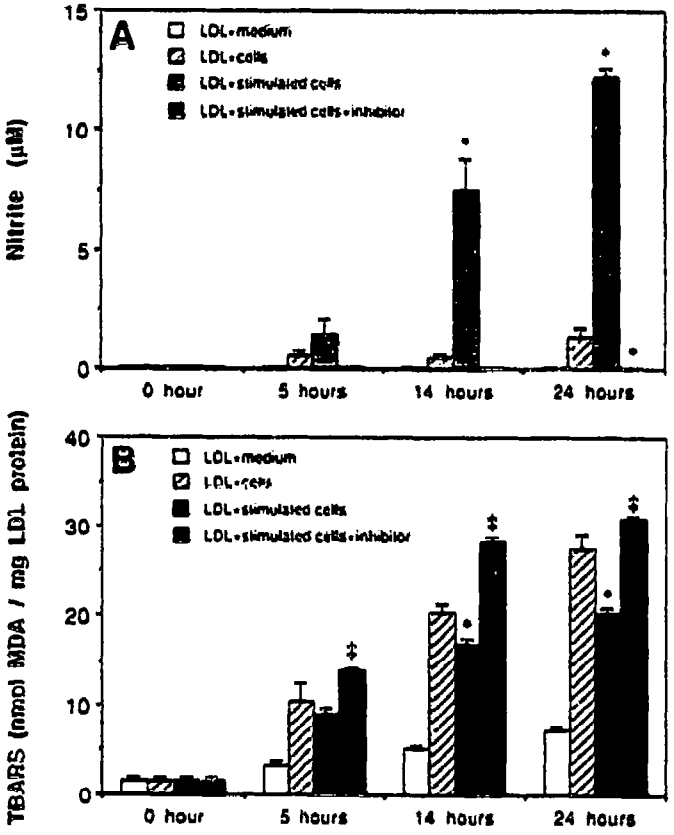

Fig. 2. Effect of NO production (A) on the lipid peroxidation (B) of LDL incubated with macrophages in F-10 culture medium. Experimental conditions were identical to those described in Fig. 1. TBARS were insignificant in cultured medium without LDL. Cells were stimulated by IFN-γ and TNF-α at 0 h. Each bar represents the mean ± S.E.M. of three experiments. *$P < 0.005$ as compared to LDL + cells. ‡$P < 0.005$ as compared to LDL + stimulated cells.

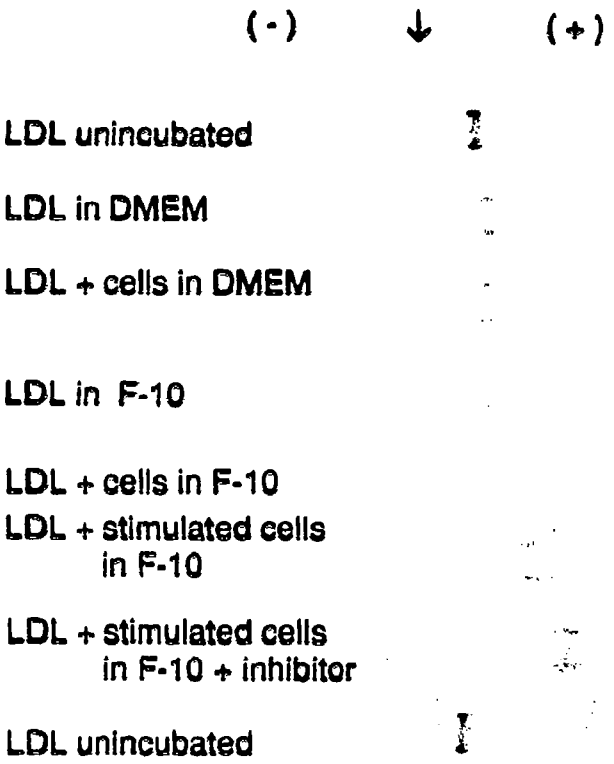

Fig. 3. Electrophoretic mobility of LDL before and after incubation with macrophages. All LDL samples, except for the top lane, were incubated at 37°C for 24 h under the conditions indicated. LDL were incubated with and without stimulated macrophages at 37°C. Culture medium containing LDL (original concentration 0.1 mg/ml) was concentrated 5× prior to application on a 1% agarose gel (3 μl) and followed by Fat red 7B lipid staining. Arrow indicates sample origin.

shown). The data suggest that NO radicals do not contribute to accelerated lipid peroxidation in our system.

In the next experiment, cells were cultured in Ham's F-10 medium, which has previously been shown to enhance macrophage-mediated LDL oxidation [12]. We show that TBARS were dramatically increased by the macrophages cultured in this medium in a time-dependent manner (Fig. 2), however, TBARS were significantly less ($P < 0.005$) when the macrophages were stimulated with cytokines for 14 and 24 h. The data indicate that the increase of NO in macrophages may actually inhibit the oxidation of LDL under our experimental conditions. In the presence of the NO synthase inhibitor, the production of NO in stimulated cells was again reduced below the endogenous unstimulated level (Fig. 2), and TBARS formation was significantly elevated as compared to stimulated cells without the inhibitor ($P < 0.005$). The present study demonstrated that the increase of NO protects against macrophage-mediated LDL lipid peroxidation and that the inhibition of NO production reversed the phenomenon. The results do not support the hypothesis that NO reacts with superoxide to form cytotoxic peroxynitrite ions [26] which propagate LDL damage in the presence of macrophages.

Although TBARS have been frequently used to measure the extent of LDL lipid peroxidation, the assay is not highly specific for peroxide measurement [12,18,21,27,33,34]. Therefore, we also examined the change in electrophoretic mobility of LDL in an agarose gel, a technique widely used for determining the extent of LDL lipid peroxidation [12,35]. Fig. 3 shows that the mobility change of LDL modified by cytokine-stimulated macrophages was less than that of LDL treated with unstimulated macrophages. The addition of the NO inhibitor prevented the effects of NO and restored the electromobility of LDL observed in the unstimulated cultures. The decreased mobility of LDL in stimulated macrophages is due to the reduced oxidative modification of apoB, the major protein moiety of LDL known to be modified by oxidized lipids [14].

NO, a free radical, has a number of biological activities and accordingly has attracted much attention recently. It is a potent vasodilator released from endothelial cells and, under certain conditions, from macrophages [23,24]. The role of NO in relation to athero-

sclerosis is not well understood. It is known that NO can react with and neutralize superoxide ions, and may hypothetically inhibit lipid peroxidation [36]. In a cell-free in vitro experiment, Dee et al. [37] have shown that peroxidation of LDL may be enhanced or suppressed by NO depending on the relative concentrations of NO and hydrogen peroxide. It was thought that the release of NO from endothelial cells and macrophages in coronary vessels may enhance the lipid peroxidation of LDL which have already been minimally oxidized inside the vessel [37–39]. Therefore, whether or not the NO radicals produced by macrophages enhance LDL lipid peroxidation is a subject of controversy. The present study using cytokines to stimulate NO production and an NO synthase inhibitor to suppress NO levels suggests that NO may protect against macrophage-induced LDL oxidation. The mechanism by which NO reduces LDL lipid peroxidation is not readily apparent from the present study. One possibility is that NO reacts with the superoxide ion and inhibits its activity [36]. NO may also attenuate metal-catalyzed lipid peroxidation, as demonstrated in the system of myoglobin radicals [38]. Furthermore, NO stimulates cellular guanylate cyclase to raise the concentration of cyclic guanosine monophosphate (cGMP) [40] which may up-regulate the level of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase. More recently, during the preparation of this study, Rubanyi et al. [41] and Heim et al. [42] reported that NO may either inhibit or reduce the production of O_2^- in other cell systems. These data support our finding that NO exerts a protective role in macrophage-mediated LDL peroxidation. This protection role may in part account for the pathogenesis of atherosclerosis in animals deficient in NO [43].

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