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# LOW DENSITY LIPOPROTEIN INHIBITS ACCUMULATION OF NITRITES IN MURINE BRAIN ENDOTHELIAL CELL CULTURES

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Endothelial cells produce nitric oxide which is considered to serve as a major source of endothelial derived relaxing factor activity. It has been demonstrated that activation of mouse brain endothelium by TNF-α and IFN-γ led to accumulation of nitrite which is presumably formed by oxidation of nitric oxide. A number of studies suggest that reactive oxygen species produced by cytokine-activated cells are involved in the conversion of nitric oxide to nitrites and nitrates. We investigated whether low density lipoprotein (LDL), acting as a radical scavenger, is able to inhibit nitrite accumulation in mouse brain endothelial cell cultures and in a cell-free system in which sodium nitroprusside was used as a source of nitric oxide. A comparison of these two models indicates the active involvement of LDL in suppressing nitrite accumulation in murine endothelial cultures. 

Several types of mammalian cells including macrophages (1, 2), endothelium (3, 4), and neutrophils (5) have been shown to be able to produce nitrogen oxides. The formation of nitric oxide radicals (NO·) from L-arginine is catalyzed by NO-synthase which can exist in at least two distinct forms: a constitutive, $Ca^{2+}$/calmodulin-dependent enzyme and a cytokine-inducible, $Ca^{2+}$/calmodulin-independent enzyme (6). Studies by Pollock et al. (6) and by Kilbourne and Belloni (4) suggest the existence of both NO-synthase forms in endothelial cells. Relatively little is known about metabolism of short living NO· either within cells or in solution. It has been proposed that oxidation of NO· by reactive oxygen species results in formation of nitrites ($NO_2^-$) and nitrates ($NO_3^-$) (7, 8). Therefore the measurement of $NO_2^-$ accumulation in cell cultures allows for indirect estimation of NO· production by cells. However the formation of $NO_2^-$ depends not only on the activity of NO-synthase but also on the availability of oxygen reactive species. Thus scavengers may significantly impair the formation of $NO_2^-$. The susceptibility of LDL to oxidative modifications by endothelial cells (9) suggests that LDL may act as a biological scavenger of oxygen radicals. We examined whether LDL may inhibit the production of $NO_2^-$ by microvascular endothelial cells by

Abbreviations: LDL, low density lipoproteins; Ac-LDL, acetylated-LDL; Ox-LDL, oxidized-LDL; Dil-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide labeled LDL; HDL, high density lipoprotein; SNP, sodium nitroprusside; FCS, fetal calf serum; FCS w/o LDL, LDL-deficient FCS; F10, F10 nutrient mixture (Ham).

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neutralization of radicals. Our results indicate that native LDL but neither modified LDL nor HDL actively inhibits $NO_2^-$ accumulation in endothelial cell cultures.

## MATERIALS AND METHODS

Reagents. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN) and recombinant murine IFN-γ from Amgen (Thousand Oaks, CA). LDL, Ac-LDL and Dil-LDL were obtained from Biomedical Technologies Inc. (Stoughton, MA). N-monomethyl-L-arginine (L-NMMA), HDL, SNP, LDL-deficient FCS were purchased from Sigma (St. Louis, MO). Dulbecco's PBS (without $Ca^{2+}$ and $Mg^{2+}$) was purchased from JRH Biosciences (Lenexa, KS).

Maintenance of cells. Murine brain microvascular endothelial cells (MME) were a gift from Dr. Robert Auerbach (Madison, WI). They were grown in medium consisting of DMEM (JRH Biosciences), 20% FCS (Biocell Laboratories, Rancho Dominguez, CA), endothelial cell growth supplement (30 μg/ml) (Sigma), 2 mM L-glutamine and antibiotics.

Copper ion-induced oxidation of LDL. Oxidized LDL was produced according to Aviram (10). The effectiveness of oxidation was evaluated by an increase in the electrophoretic mobility of LDL preparations (11).

Nitrite assay. MME cells were cultured in 96-well plates (Becton Dickinson, Lincoln Park, NJ) in 100 μL of culture medium. 2-3 days after the cells reached confluence, the medium was replaced with fresh medium containing factors described in each experiment. Nitrite concentration in the medium was determined after 48 h by a microplate assay according to Ding et al. (7). Briefly, 80 μL aliquots of the culture medium were incubated with equal volumes of Griess reagent (1% sulfanilamide/0.1% naphthylenediamine dihydrochloride (Sigma) in 2.5% $H_3PO_4$) at room temperature for 10 minutes. The absorbance at 570 nm was measured with a Dynatech ELISA reader. [$NO_2^-$] was determined by using dilutions of sodium nitrite in water as a standard. Background nitrite levels in control cultures (exposed to all experimental conditions except cytokines) were subtracted from the experimental values.

Cell-free model for nitrite production. Sodium nitroprusside (SNP) was used as a source of NO• instead of cytokine-stimulated MME cells. SNP solutions were incubated in 96-well plates (80 μL/well) at 37°C. [$NO_2^-$] was determined at the end of 24 h incubation by addition of an equal volume of Griess reagent directly to incubation solutions.

Flow cytometric analysis of Dil-LDL incorporation. MME monolayers, cultured in 6-well plates, were incubated for 24 h in RPMI 1640 (Gibco, Grand Island, NY) enriched with 5% LDL-deficient FCS and with different concentrations of LDL labeled with Dil, a fluorescent probe (12). The cells were washed 3X with medium alone, released from plates with trypsin and analyzed using the Consort 30 program on FACScan (Becton Dickinson).

## RESULTS

The stimulation of MME by TNF-α/IFN-γ for 24 or 48 h led to a dose dependent accumulation of $NO_2^-$ in culture medium (Fig. 1). In agreement with the results of Kilbourn and Belloni (4), we found that this process was completely inhibited when MME cells were exposed to the cytokines in the absence of L-arginine or in the presence of 1 mM L-NMMA, the competitive inhibitor of NO-synthase (data not shown). To examine whether extracellular pro-oxidative conditions and/or the presence of LDL affect the process of $NO_2^-$ accumulation, the stimulation of MME by cytokines was carried in F10 or RPMI medium supplemented with 5% FCS or 5% LDL-deficient FCS. Unlike RPMI, F10 contains transition metal ions ($Fe^{2+}$, $Cu^{2+}$) which promote oxidative processes (8). As shown in Fig. 1, the greatest accumulation of $NO_2^-$ occurred when MME monolayers were stimulated with cytokines in F10 medium in the absence of LDL. Similar differences in $NO_2^-$ accumulation (9.0 ± 0.6 nmoles in F10 and 3.6 ± 0.4 nmoles/1.6 x $10^4$ cell/48 h in RPMI) were observed when MME monolayers were

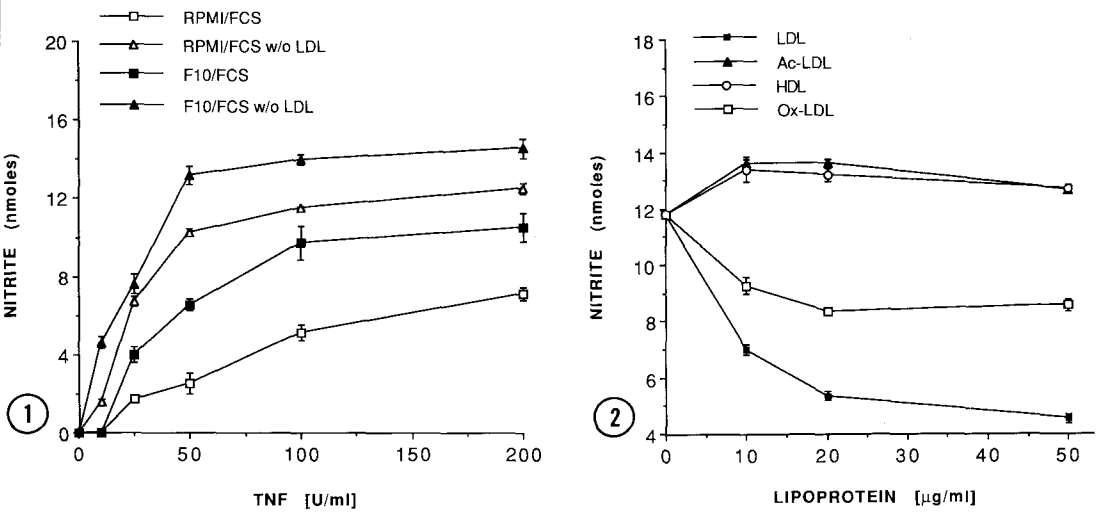


Figure 1. Accumulation of $NO_2^-$ in MME culture medium. MME (1.6 x $10^4$ cell/well) were stimulated with different doses of TNF-α in the presence of IFN-γ [400 U/ml] for 48 h. The amount of $NO_2^-$ was determined as described in Materials and Methods. Data points represent means ± SD of 3 experiments and each determination was done in triplicate.

Figure 2. Accumulation of $NO_2^-$ in MME culture medium. MME (1.6 x $10^4$ cell/well) were stimulated with TNF-α [100 U/ml] and IFN-γ [400 U/ml] for 48 h in RPMI medium supplemented with 5% LDL-deficient FCS and with different concentrations of indicated lipoproteins. The amount of $NO_2^-$ was determined as described in Materials and Methods. Data points represent means ± SD of triplicate determinations from 1 experiment (representative of 4 experiments).

stimulated with optimal concentrations of cytokines (TNF-α [100 U/ml], IFN-γ [400 U/ml]) in the absence of any serum.

Addition of exogenous LDL to MME culture medium (RPMI supplemented with 5% FCS w/o LDL, TNF-α [100 U/ml], IFN-γ [400 U/ml]) inhibited the accumulation of $NO_2^-$ in a dose dependent manner ($ED_{50}$~17 µg/ml) (Fig. 2). Similar results were obtained when LDL was added to F10 medium ($ED_{50}$~25 µg/ml) (data not shown). The ability to inhibit $NO_2^-$ accumulation could be attributed to native LDL since the oxidation of LDL significantly reduced its inhibitory effect. Ac-LDL as well as HDL caused an increase rather then an inhibition of $NO_2^-$ accumulation under the same conditions. To exclude the possibility of interference by low molecular substances (metal ions or chelators, free amino acids) in these experiments, all sera and lipoproteins were dialyzed against PBS. We did not observe different effects between dialyzed and nondialyzed preparations.

MME cells were incubated with various concentrations of Dil-LDL for 24 hours. As shown in Fig. 3 the cells were able to incorporate LDL in a dose-dependent manner. This led us to question whether the entire inhibitory effect of LDL on $NO_2^-$ accumulation is due to extracellular neutralization of radicals by LDL or whether increased concentration of intracellular cholesterol could also account for the diminished formation of $NO_2^-$ by MME cells.

To characterize the conditions for the conversion of $NO^\bullet$ to $NO_2^-$, we created a cell-free model in which we used sodium nitroprusside (SNP) as a donor of $NO^\bullet$ (13). As shown

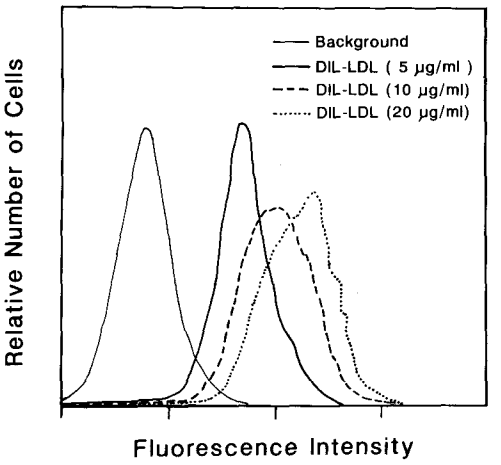


Figure 3. Flow cytometric analysis of Dil-LDL incorporation by MME cells. Cell labeling and analysis was performed as described in Materials and Methods.

in Fig. 4 A, the incubation of SNP for 24 h (0.5 - 5.0 mM in DPBS, $Ca^{2+}$-, $Mg^{2+}$-free) led to no detectable $NO_2^-$ formation in the absence of light. However when the reaction was carried out in full spectrum light, which allowed for photolysis of SNP, significant amounts of $NO_2^-$ were detected. Similar quantities of $NO_2^-$ were formed when the reaction was carried out in the dark but in the presence of 0.1 mM sodium ascorbate. This is in agreement with results showing that ascorbate promotes the formation of NO• from SNP (13, 14). The addition of $Fe^{3+}$ ion together with ascorbate to SNP solutions allowed for the generation of hydroxyl radicals (OH•) in a Fenton-type reaction (15) resulting in a significant increase in $NO_2^-$ formation. These results indicate that in our cell-free model the production of $NO_2^-$ from SNP can be considered a two-step reaction: 1/ the generation of NO• stimulated by light or reducing agents, and 2/ conversion of NO• to $NO_2^-$ by oxidizing agents such as OH•.

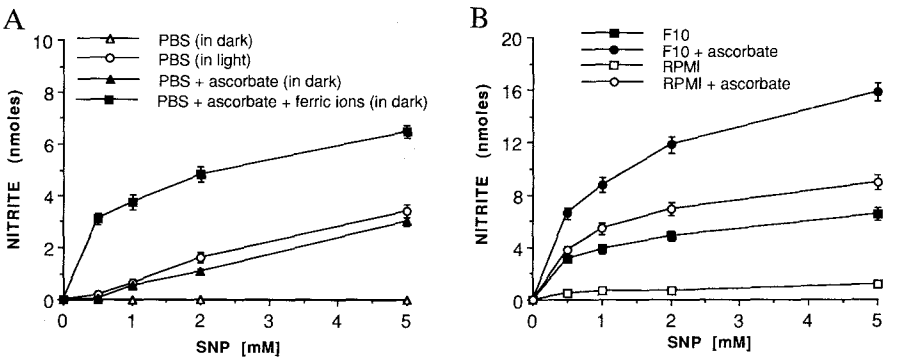


Figure 4. Formation of $NO_2^-$ in SNP solutions under different conditions. Solutions of SNP at the concentrations shown were prepared in PBS (A) or in F10 or RPMI medium (B). To some solutions sodium ascorbate (0.1 mM) or sodium ascorbate with $FeCl_3$ (1.0 mM) and EDTA (0.1 mM) were added. Incubation of SNP in PBS was performed in light or without light; incubations of SNP in F10 or RPMI were performed without light. After incubation of solutions for 24 h at 37°C the amount of nitrite was determined as described in Materials and Methods. Data points represent means ± SD of 3 experiments and each determination was done in sextuplicate.

The production of $NO_2^-$ from SNP was greater when the reaction was carried out in culture media in place of PBS. In both RPMI and F10 media, addition of ascorbate led to further increase in $NO_2^-$ formation (Fig. 4B). The pattern of increases in $NO_2^-$ accumulation resulting from the effect of F10 compared to RPMI were very similar in both the cell-free and MME models. However, LDL which had a strong inhibitory effect on $NO_2^-$ accumulation in MME cultures had a very limited effect on $NO_2^-$ production from SNP dissolved in F10 or RPMI medium. LDL, in concentrations from 10 to 100 μg/ml, caused less than 20% inhibition of $NO_2^-$ production from SNP.

## DISCUSSION

The production of $NO_2^-$ by MME cells is considered to be a process that requires three sequential steps including: 1/ stimulation of NO-synthase, 2/ synthesis of NO•, and 3/ intra- and/or extracellular conversion of NO• into nitrites/nitrates. The generation of NO• by NO-synthase depends on accessibility of the substrate (L-arginine) as well as of a number of cofactors in their reduced form (NADPH, FAD/FMN, tetrahydrobiopterin) (16, 17). Although NO• may react with various substrates (guanylate cyclase and other Fe-containing protein/enzymes, thiols, etc) (18, 19), we and others have demonstrated that stimulation of NO-synthase led to accumulation of $NO_2^-$. This indicates that NO• may also react with reactive oxygen species (8). In different cell models TNF-α was shown to stimulate the generation of reactive oxygen species, of which the most active is OH•. These radicals account, in part, for the cytotoxic effects of TNF-α (20, 21). The hypothesis that the major physiological function of NO is to act as a scavenger of cytotoxic oxygen radicals (22) has been partially confirmed by Rubanyi et al. (23). Our data suggest that the formation of $NO_2^-$ may result from the reaction of NO• with OH• according to the following hypothetical summary equation:

$$NO^\bullet + OH^\bullet \rightarrow NO_2^- + H^+$$

This process may lead to the neutralization of these radicals and the generation of a proton that can play a protective role against alkalization of cytoplasm in cells undergoing oxidative stress. It has been suggested that LDL may be peroxidized by reactive oxygen species produced by endothelial cells (9). We considered the possibility that LDL by scavenging reactive oxygen species would inhibit conversion of NO• to $NO_2^-$. However, our results demonstrated that although LDL suppressed the accumulation of $NO_2^-$, its effect in a cell-free system was much weaker than in MME cultures. This observation, together with the fact that MME cells are able to incorporate LDL in a dose-dependent manner suggests that LDL apart from a possible function as a scavenger may also play an active, regulatory role in the process of $NO_2^-$ formation. Our preliminary results suggest that cholesterol is at least partially responsible for the effect of LDL on MME cells (manuscript in preparation). With respect to the broad spectrum of LDL/cholesterol activities (24), their involvement at every step of the process of $NO_2^-$ formation by MME cells is possible.

It has been demonstrated that Ox-LDL but not LDL diminished the biological activity of NO/EDRF (25, 26). If the activity of LDL/cholesterol that we observed does not

affect the generation of NO• but only inhibits its conversion to $NO_2^-/NO_3^-$, then LDL/cholesterol in their native forms may play protective roles for biologically active NO•.

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