Role of oxidant injury on macrophage lipoprotein lipase (LPL) production and sensitivity to LPL

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Abstract We investigated, in the present study, the role of reactive oxygen intermediates (ROI) in the control of macrophage lipoprotein lipase (LPL) secretion. Exposure of murine macrophages to increasing concentrations of hydrogen peroxide (H_2O_2) resulted in enhanced basal LPL production and mRNA levels. The increase of LPL production was reduced in the presence of antioxidants. Oxidant stress also modulated the regulation of macrophage LPL production by tumor necrosis factor alpha (TNFα). While antioxidants accentuated the inhibition of LPL by TNFα, addition of H_2O_2 significantly attenuated TNFα-induced LPL inhibition. As LPL has been shown to induce macrophage TNFα release, the effect of reactive oxygen species on LPL-induced TNFα production was also examined. Simultaneous treatment of macrophages with LPL and H_2O_2 or pretreatment of macrophages with H_2O_2 prior to LPL stimulation decreased the LPL-induced TNFα release by macrophages to the same extent. Under these experimental conditions, LPL binding to macrophages was markedly decreased. These data indicate that ROI are effective enhancers of macrophage LPL production and modulate macrophage response to LPL. These effects may represent additional mechanisms through which oxidant stress may participate to the development of atherosclerosis.—**Renier, G., A-C. Desfaits, A. Lambert, and R. Mikhail.** Role of oxidant injury on macrophage lipoprotein lipase (LPL) production and sensitivity to LPL. *J. Lipid Res.* 1996. **37:** 799–809.

Supplementary key words reactive oxygen intermediates • oxidative stress • hydrogen peroxide • atherosclerosis

Mounting evidence suggests that oxidative processes contribute to the pathogenesis of atherosclerosis. In the early atherogenic lesion, oxidative stress is manifested by an elevated production of ROI leading to the oxidative modification of low density lipoprotein (LDL) (1, 2). Oxidative modification of LDL has been shown to be associated with increased atherogenicity (3–7) which includes cytotoxicity to arterial wall cells (8), stimulation of hemostatic and thrombotic processes (9–11), and secretion of cytokines and growth factors from cells of the arterial wall (12–14). In addition, the uptake of this modified form of LDL by macrophages is greatly enhanced, resulting in rapid accumulation of intracellular cholesterol and foam cell formation (3–7).

Beside their effect on lipoprotein metabolism, ROI also act as major regulatory signals in macrophage activation. It has been recently proposed that oxygen radicals represent intracellular messengers mediating NF-kB activation (15) and enhancing the production of several potentially proatherogenic cytokines such as TNFα by stimulated macrophages (16). Induction of protooncogene expression and replicative DNA synthesis in aortic smooth muscle cells is also observed after oxidant stress (17). Taken together these results indicate that ROI may affect the atherosclerotic process by several ways, including arterial lipid metabolism and macrophage activation.

Lipoprotein lipase (LPL), a key enzyme in the metabolism of lipoproteins (18), is constitutively expressed by macrophages (19). Beside its effect on macrophage lipid metabolism, LPL acts as an autocrine activator of macrophage function, inducing TNFα secretion (20). LPL is expressed in the intima of atherosclerotic lesion (21). In vitro experiments demonstrate that LPL increases lipoprotein association with proteoglycans (22) and thereby enhances LDL accumulation in vascular tissue (23). Taken together, these observations support the hypothesis that LPL could act as a proatherogenic factor. Despite the key role of macrophage LPL in the

Abbreviations: LPL, lipoprotein lipase; ROI, reactive oxygen intermediates; DMSO, dimethyl sulfoxide; TNFα, tumor necrosis factor alpha; LDL, low density lipoprotein; FCS, fetal calf serum; DMEM, Dulbecco's minimal essential medium; BSA, bovine serum albumin; LPS, lipopolysaccharide; HRP, horseradish peroxidase; NHS, N-hydroxysuccinimide; NAC, N-acetyl-L-cysteine; BHT, butylated hydroxytoluene; DES, deferoxamine; PMA, phorbol myristate acetate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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control of lipid metabolism in the arterial wall and macrophage function, the effect of oxidative stress on macrophage LPL has not been studied. This study was undertaken to evaluate the possibility that ROI may modulate macrophage LPL secretion and LPL effect on macrophage activation.

MATERIAL AND METHODS

Reagents

Fetal calf serum (FCS) was purchased from Hyclone Lab. (Logan, UT). Dulbecco's minimal essential medium (DMEM) was obtained from ICN Biochemicals Inc. (Costa Mesa, CA) and supplemented with 10% FCS, 2 mM L-glutamine (ICN, Biochemicals Inc.) and penicillin–streptomycin (Flow, McLean, VA). Recombinant murine TNFα was obtained from Genzyme (Cambridge, MA). Hydrogen peroxide (H_2O_2), catalase (1400 U/ml), dimethyl sulfoxide (DMSO), mannitol, butylated hydroxytoluene (BHT), N-acetyl cysteine (NAC), deferoxamine (DES), allopurinol, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), bovine serum albumin (BSA), heparin, phorbol myristate acetate (PMA), and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO). Calphostin C was obtained from Calbiochem (LaJolla, CA). Percoll was purchased from Pharmacia (Pleasant Hill, CA). Human LPL and affinity-purified anti-bovine LPL antibody were a generous gift from Dr. DeSanctis (Institute of Immunology, Caracas, Venezuela). Biotin-N-hydroxysuccinimide ester (NHS), avidin-conjugated horseradish peroxidase (HRP), and HRP substrate were purchased from Bio-Rad (Richmond, CA).

Murine and human macrophages

We used the ANA-1 macrophage line, established by infection of the normal bone marrow of C57BL/6 mice with the J2 recombinant retrovirus, as previously described (24). The ANA-1 cells have been characterized as a homogeneous population of macrophages on the basis of their characteristic morphology, lack of T- or B-cell markers, and cell surface expression of specific markers or antigens expressed strongly by macrophages. They did not constitutively express major histocompatibility complex class II I-A region antigens or exhibit constitutive tumoricidal activity indicating that they are not activated macrophages. The macrophage line was cultured in DMEM containing 10% FCS and treated for different time periods with the appropriate agents.

Preparation of human monocyte-derived macrophages was performed as follows. Human peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll, allowed to aggregate in presence of FCS, then further purified by rosetting technique. After density centrifugation, recovery of highly purified monocytes (85–90%) as assessed by FACS analysis was obtained. Differentiation of monocytes into macrophages was achieved by culturing the monocytes in RPMI 1640 medium supplemented with 10% (vol/vol) autologous serum and phorbol myristate acetate (PMA) (200 ng/ml). The medium was replaced every 3 days. Monocyte-derived macrophages were used for the experiments after 10 days of culture.

Purification of LPL

LPL was purified from 100 ml of postheparin plasma by two successive affinity chromatography steps on heparin-Sepharose 4B columns as previously described (25). The plasma was mixed with 50 mM NH_4OH-HCl, pH 8.5, and applied to a preequilibrated heparin-Sepharose column. After washing, the lipoprotein lipase was eluted with the same buffer containing 2.0 M NaCl. The LPL collected in the effluent was further purified on a second heparin-Sepharose column and the enzyme was eluted using an NaCl gradient ranging from 0.3 to 2.0 M. The fractions containing enzyme activity were pooled, dialyzed against 25 liters of 0.005 M ammonium bicarbonate, and lyophilized. Purity of the protein was assessed by Western blot analysis (26).

Biotinylation of LPL

Human LPL was biotinylated as reported by Sivaram et al. (27). Briefly, 100 μl of a solution containing 1.33 mg/ml N-hydroxysuccinimide ester of biotin was incubated at 4°C for 10 min in presence of 900 μg purified LPL. The sample was then loaded onto a 4-ml heparin-agarose column equilibrated previously with 10 mM Tris-HCl, pH 7.4. After washing, the column was eluted with the same buffer containing 1.5 M NaCl. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Biotinylated LPL was visualized by avidin development.

LPL binding to macrophages

Biotinylated LPL binding to macrophages was done at 4°C for 2 h in DMEM containing 3% BSA as previously described for endothelial cells (28). Unbound LPL was removed by extensive washing and cell surface-associated LPL was released by incubation in PBS containing 100 units/ml heparin. Biotinylated LPL in the heparin-released fractions was analyzed by SDS-PAGE. Samples were transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 volts for 60 min. The membrane was incubated in PBS containing 3%

BSA (P-BSA) for 1 h at 37°C, followed by incubation in P-BSA containing avidin-horseradish peroxidase for 1 h at room temperature. The membrane was washed five times with PBS containing 0.3% BSA and 0.05% Tween 20. Biotinylated proteins were visualized by incubation in a solution containing 30 mg 4-chloro-1-naphthol, 10 ml ice-cold methanol, 40 ml PBS, and 30 μl H_2O_2 for 5–10 min.

Determinations of endotoxin concentrations

Endotoxin contents of the chemicals used in the present study were determined by a quantitative limulus amebocyte lysate assay (Whittaker, Walkersville, MA). Chemical preparations were all found to be endotoxin-free (endotoxin content lower than 6 pg/ml) except catalase solutions which contained high LPS amounts. Removal of endotoxins from catalase preparations was operated by passing those samples through Detoxi-gel Affinity Pak columns (Pierce Chemical Co., Rockford, IL).

RNA extraction

Ten million macrophages were plated in plastic petri dishes (100 × 20 mm), (Falcon, Lincoln Park, NJ). After treatment of macrophages with appropriate agents, macrophages were lysed with guanidine isothiocyanate. Total RNA was purified by centrifugation through a cesium chloride gradient as previously described in detail by Chirgwin et al. (29).

Northern blot analysis

Fifteen μg of total RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde as previously described (30). The blots were prehybridized for 18 h in prehybridization buffer. The mRNA expression was analyzed by hybridization with [^{32}P]dCTP- (sp act approx. 3000 Ci/mmol, Amersham Corp., Arlington Heights, IL) labeled LPL, TNFα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA inserts. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). RNA expression was quantified by high resolution optical densitometry (SciScan 5000, USB).

DNA probes

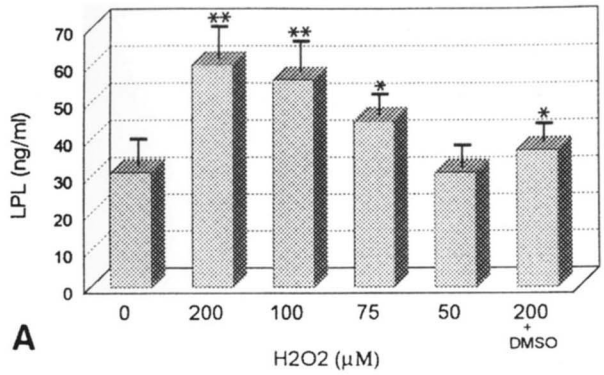
The cDNA probe for murine tumor necrosis factor (TNFα) was kindly provided by Dr. A. Cerami (Rockefeller University, NY). The probe for detection of human LPL was prepared by polymerase chain reaction. cDNA was obtained from total RNA using a reverse transcription reaction. Two synthetic primers spanning bases 255–287 and 1,117–1,149 of the LPL cDNA were used to enzymatically amplify a 894-bp region of the LPL probe. The murine GAPDH probe was obtained similarly by enzymatically amplifying a 456 bp of the GAPDH cDNA using two synthetic primers (5′-CCCTTCATTGACCTCAACTACATGG-3′; 5′-AGTCTTCTGGGTGGCAGTGATGG-3′). The identity of polymerase chain reaction-generated probes was verified by DNA sequencing. The LPL and GADPH probes were subsequently purified on a low-melting agarose gel.

Determination of TNFα protein level

A double-sandwich ELISA was used to determine the quantity of TNFα in the culture supernatants of macrophages as described in detail by Sheehan, Ruddle, and Schreiber (31). Hamster monoclonal antibody to murine TNFα was purchased from Genzyme, (Boston, MA) and rabbit polyclonal anti-murine TNFα was prepared and purified by standard procedures. Briefly, 2 μg per well of a monoclonal antibody against TNFα was absorbed in 96-well plates and incubated in the presence of different dilutions of the test sample or with the murine recombinant TNFα standards (Genzyme). After washing, the polyclonal antibody to TNFα was added. Anti-rabbit IgG peroxidase was added to the wells and incubated for 1 h. The peroxidase reaction was developed by adding peroxidase substrate and analyzed in an automated plate reader (Dynatech, Chantilly, VA).

Determination of the LPL immunoreactive mass

LPL immunoreactive mass was measured by ELISA as previously described (32). Murine standards were prepared and purified as follows. Murine macrophages were plated at a density of 5×10^6 cells per flask and maintained for 3 days in DMEM containing 10% FCS. The cells were washed with the same medium containing 0.5% BSA and 0.1 U/ml heparin. After 12 h, these media were mixed with an equal volume of cold loading buffer (10 mM Na phosphate, pH 7.0, 0.15 M NaCl, and 30% glycerol) and loaded onto a heparin-Sepharose affinity column equilibrated with loading buffer. The column was washed with loading buffer and LPL was eluted with the same buffer containing 2 M NaCl. Protein concentration of the purified enzyme was determined according to the Bradford method (33). Purity of the murine standards was assessed by SDS-PAGE which showed a major band at a molecular mass of 55 kDa. Affinity-purified antibody against bovine LPL was used: the affinity of this antibody to bovine and murine LPL was very similar (26). Different dilutions of the sample or murine LPL standards were absorbed in 96-well plates (Immulon II) and incubated overnight at 37°C. The wells were then washed and blocked before addition of affinity-purified anti-LPL (1.5 μg/ml). After further washing, anti-rabbit IgG-peroxidase was incubated for 3 h. The peroxidase reaction was developed by adding peroxidase substrate and results were read in an



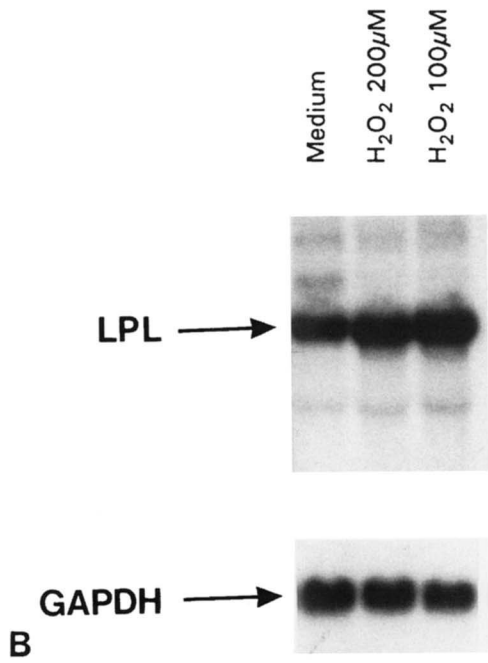


Fig. 1. Panel A: stimulatory effect of H_2O_2 on macrophage LPL production; effect of DMSO on H_2O_2-induced LPL production. ANA-1 cells were grown in medium containing increasing concentrations of H_2O_2. Cells treated with 200 μM H_2O_2 were incubated in the presence or absence of DMSO 1%. Supernatants were collected after 18 h and assayed for LPL mass. Data represent the mean ± SEM of five experiments. *$P < 0.05$ vs. samples incubated with medium or 200 μM H_2O_2 alone; **$P < 0.01$ vs. samples incubated in medium alone. Panel B: autoradiograph (exposure time: 18 h) showing that H_2O_2 increases macrophage LPL mRNA levels. ANA-1 cells were exposed to H_2O_2 (100–200 μM) for 30 min. Total cellular RNA was analyzed for LPL mRNA and GAPDH mRNA expression as described in

automated plate reader (Dynatech, Chantilly, VA).

Determination of protein concentrations

Total protein content was estimated according to the Bradford method (33) using a colorimetric assay (Bio-Rad, Mississauga, ONT).

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). Data were analyzed by Student's *t*-test for single comparisons and by Student-Newman-Keuls test for multiple comparisons.

RESULTS

Effect of oxidant stress on the constitutive macrophage LPL secretion and gene expression

To examine the role of oxidant stress in the control of basal macrophage LPL secretion, we tested the effect of H_2O_2 on LPL production by ANA-1 cells. Incubation of macrophages with increasing concentrations of H_2O_2 (50–200 μM) for 18 h led to a dose-dependent augmentation of LPL mass in the culture medium (**Fig. 1,** panel a). Maximal effect was observed at 200 μM H_2O_2. Stimulation of LPL production by H_2O_2 was reduced in presence of DMSO 1% (Fig. 1, panel a) and of several other antioxidants (**Table 1**). In contrast to the stimulatory effect of H_2O_2 on LPL production, agents that generate intracellular ROI such as menadione (100 μM) or pyrogallol (10 μM) did not significantly enhance macrophage LPL production (medium: 41.5 ± 2 ng/ml; menadione: 49.7 ± 6 ng/ml; pyrogallol: 44.4 ± 5 ng/ml; $P > 0.05$ vs. medium). The possibility that the increase in LPL production observed in H_2O_2-treated cells may result from an augmentation of LPL steady state mRNA concentrations was next assessed by measuring LPL mRNA levels in H_2O_2-treated macrophages. Exposure of ANA-1 cells to 100 μM and 200 μM H_2O_2 enhanced LPL mRNA levels by 3.1- and 3.5-fold, respectively, as judged by densitometric scanning compared with the untreated macrophages. The intensities of the bands estimated by densitometry were corrected by comparison with those of GAPDH mRNA bands (Fig. 1, panel b). Maximal effect was found after 0.5 to 3 h exposure of the cells to 100 μM H_2O_2. Evidence that the H_2O_2

TABLE 1. Effect of antioxidants on H_2O_2-stimulated LPL production

Treatment	Concentration	H_2O_2-Stimulated LPL Production (% increase over basal values)
H_2O_2	200 μM	254 ± 21
DMSO	1%	141 ± 7[a]
BHT	250 μM	138 ± 5[a]
DES	5 mM	143 ± 13[a]
Mannitol	10 mM	104 ± 17[a]
Allopurinol	10 mM	115 ± 4[a]
Catalase	20 μg/ml	113 ± 22[a]
NAC	10 mM	246 ± 25

Data shown represent the mean ± SEM of five experiments. H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene; DES, deferoxamine; NAC, N-acetyl-L-cysteine.

[a]Denotes a significant difference ($P < 0.05$) between the levels of LPL measured in the treated samples and samples stimulated with H_2O_2 alone.

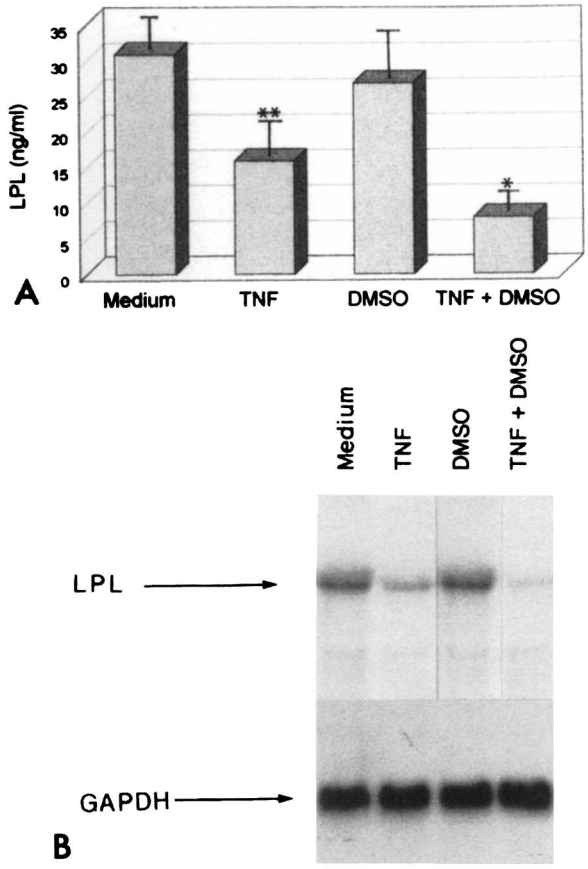


Fig. 2. Panel A: DMSO enhances the inhibitory effect of TNFα on LPL mass production. Macrophages were treated with TNFα (500 U/ml) in the presence or absence of 1% DMSO. After a 24-h incubation period, LPL mass was determined in the medium. Data represent the mean ± SEM of three experiments. *$P < 0.05$ vs. TNFα-treated samples; **$P < 0.01$ vs. samples incubated with medium alone. Panel B: autoradiograph (exposure time: 6 h) showing that DMSO accentuates the suppressive effect of TNFα on LPL steady state mRNA concentrations. ANA-1 cells were exposed to TNFα (500 U/ml) in the presence or absence of 1% DMSO for 24 h. mRNA levels of TNFα and GAPDH were determined as described in Methods.

Role of endogenous and exogenous oxidant stress in the regulation of LPL production

To evaluate the role of oxidant stress in the control of macrophage LPL secretion, we investigated the effect of antioxidants and H_2O_2 on the suppressive effect of TNFα on macrophage LPL production. The possibility that endogenous release of reactive oxygen species may contribute to the inhibitory effect of TNFα on LPL production was first determined. Culture of ANA-1 cells in presence of TNFα alone led to a significant decrease of macrophage LPL secretion (**Fig. 2,** panel a) (**Table 2**) and reduced LPL mRNA levels by 2.9-fold (Fig. 2, panel b). Addition of DMSO, in the absence of added radical generators, resulted in an accentuation of the TNFα inhibitory effect on macrophage LPL production (Fig. 2, panel a). Under these experimental conditions, a 4.6-fold decrease of LPL mRNA levels was observed (Fig. 2, panel b). Exposure of the cells to other antioxidants led to a similar increase of the TNFα suppressive effect on macrophage LPL production (Table 2). To demonstrate the effectiveness of DMSO as inhibitor of endogenous free radical production by macrophages, H_2O_2 production by TNFα-activated cells in the presence or absence of DMSO was measured. While undetectable in unstimulated cells, the amount of H_2O_2 released in the medium by TNFα-treated macrophages was significantly enhanced reaching 3.3 nmol/ml. Addition of DMSO totally abolished TNFα-induced H_2O_2 release, as reflected by the undetectable production of H_2O_2 observed under these conditions.

Evidence has been presented that H_2O_2 reduces cell sensitivity to TNFα and decreases TNFα binding to blood monocytes (34). Based on these data and on previous results demonstrating the ability of TNFα to inhibit macrophage LPL production (35), we tested whether or not exogenous oxidant stress may alter LPL effects observed on the ANA-1 macrophages were not restricted to this cell line was provided by results showing that oxidative stress also enhances LPL production by human monocyte-derived macrophages (medium: 73 ± 5 ng/ml; H_2O_2 (200 μM): 120 ± 13.5 ng/ml; $P < 0.02$ vs. medium). Activation of protein kinase C (PKC) was required for the stimulatory effect of H_2O_2 on LPL production as blockade of PKC activity by calphostin C (1 μg/ml) or H7 (20 μM) led to a complete suppression of H_2O_2 effect on LPL release by human monocyte-derived macrophages (calphostin: 51 ± 3 ng/ml; H7: 75 ± 7 ng/ml; H_2O_2 (200 μM): 120 ± 13.5 ng/ml; calphostin + H_2O_2: 55 ± 6 ng/ml, $P < 0.001$ vs. H_2O_2; H7 + H_2O_2: 79 ± 3 ng/ml, $P < 0.001$ vs. H_2O_2).

TABLE 2. Effect of antioxidants on the regulation of macrophage LPL production by TNFα

Treatment	Concentration	Percentage of LPL Inhibition
TNFα	500 U/ml	48 ± 8
DMSO	1%	74 ± 3[a]
BHT	250 μM	64 ± 2[a]
DES	5 mM	40 ± 7
Mannitol	10 mM	62 ± 2[a]
Allopurinol	10 mM	64 ± 5[a]
NAC	10 mM	37 ± 1

Data shown represent the mean ± SEM of three experiments. TNFα, tumor necrosis factor alpha; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene; DES, deferoxamine; NAC, N-acetyl-L-cysteine.

[a]Denotes a significant difference ($P < 0.05$) between the levels of LPL measured in the treated samples and samples stimulated with TNFα alone.

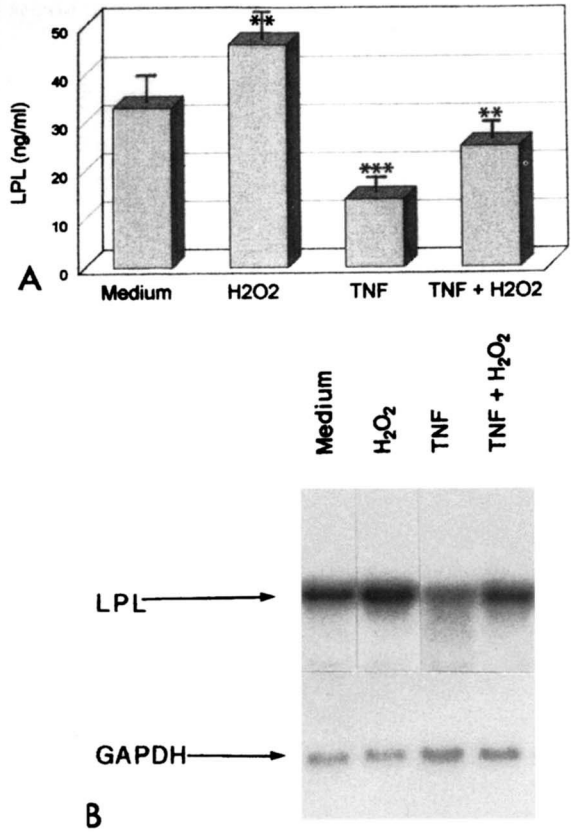


Fig. 3. Panel A: H_2O_2 decreases the inhibitory effect of TNFα on macrophage LPL secretion. Macrophages were treated with 500 U/ml TNFα in the presence or absence of 200 μM H_2O_2. After a 24-h incubation period, LPL mass was determined in the medium. Data represent the mean ± SEM of three experiments. $^{**}P < 0.01$ vs. respective controls (samples incubated with medium or TNFα alone); $^{***}P < 0.001$ vs. samples incubated with medium alone. Panel B: representative autoradiograph (exposure time: 6 h) showing LPL and GAPDH mRNA levels in cells exposed to TNFα (500 U/ml) in the presence or absence of 200 μM H_2O_2 for 24 h.

regulation by TNFα. Incubation of TNFα-treated cells in presence of H_2O_2 (200 μM) significantly reduced the inhibitory effect of TNFα on LPL production (**Fig. 3,** panel a). In contrast to the 3.9-fold decrease of LPL mRNA levels observed in TNFα-treated cells, a 1.8-fold decrease of LPL mRNA levels was found in cells exposed to TNFα and H_2O_2 (Fig. 3, panel b). None of the treatments had any significant effect on the expression of the macrophage GADPH gene (Fig. 3, panel b). To determine whether such effect of H_2O_2 is specific for the TNFα stimulus, LPS-stimulated macrophages were treated with or without H_2O_2. Exposure of macrophages to 10 ng/ml LPS induced a dramatic decrease of LPL secretion (**Fig. 4,** panel a) and led to a 4.5-fold decrease of LPL mRNA levels (Fig. 4, panel b). In presence of H_2O_2, a marked reduction of the LPS inhibitory effect on LPL production (Fig. 4, panel a) and steady state RNA concentrations (1.7-fold decrease) (Fig. 4, panel b) was observed.

Effect of exogenous and endogenous oxidant stress on macrophage response to LPL.

Endogenous generation of radical species has been shown to contribute to TNFα production in response to LPS. Moreover, imposing external stress has been demonstrated to enhance TNFα release from LPS-stimulated macrophages (16). As we have previously shown that LPL induced macrophage TNFα production, we evaluated the role and effect of endogenous and exogenous oxidant stress on LPL-stimulated TNFα mRNA levels and production. To test the possibility that endo-

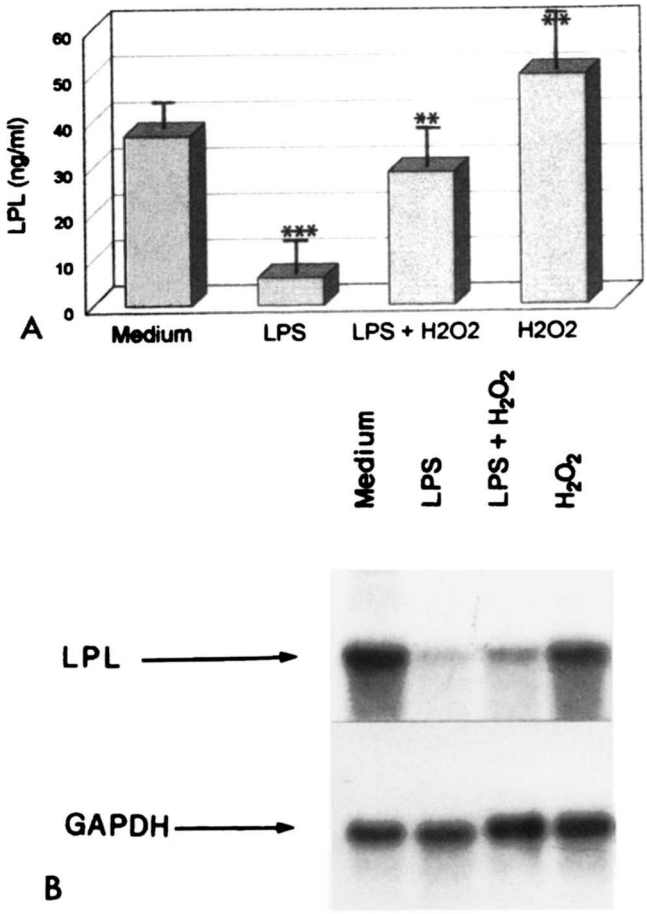


Fig. 4. Panel A: modulatory effect of H_2O_2 on macrophage LPL production in response to LPS. Macrophages were treated with LPS (10 ng/ml) alone or in combination with H_2O_2 (200 μM) for 24 h. At the end of the incubation period, media were collected and assayed for LPL mass. Data represent the mean ± SEM of four experiments. $^{**}P < 0.01$ vs. respective controls (samples incubated with medium or LPS alone); $^{***}P < 0.001$ vs. samples incubated with medium alone. Panel B: autoradiograph (exposure time: 18 h) illustrating the effect of H_2O_2 on LPL steady state mRNA concentrations by LPS-treated cells. ANA-1 cells were treated with LPS (10 ng/ml) alone or in combination with H_2O_2 (200 μM) for 24 h. Total cellular RNA were analyzed for LPL and GAPDH mRNA levels as described in Methods.

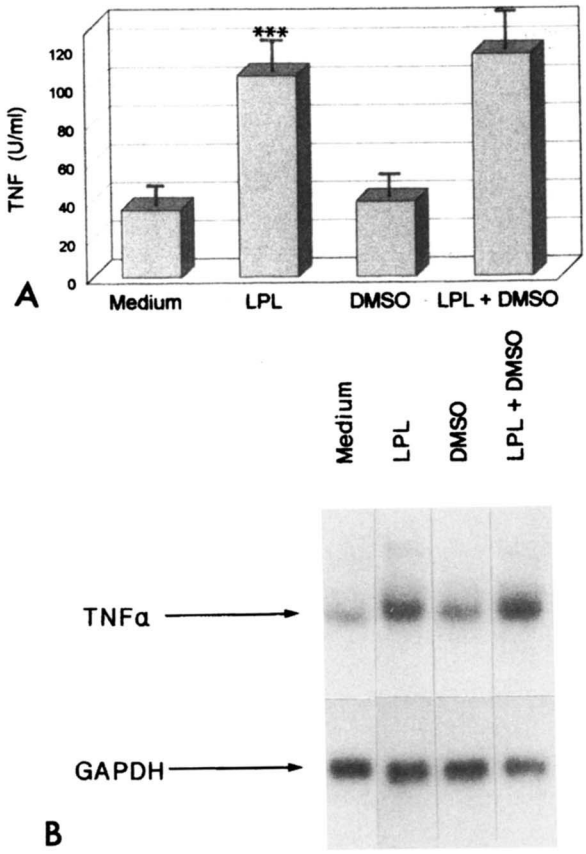


Fig. 5. Panel A: effect of DMSO on LPL-induced TNFα production by ANA-1 cells. Untreated or LPL-stimulated macrophages were cultured in the presence or absence of 1% DMSO. Supernatants were collected after 18 h and assayed for TNFα content. Data represent the mean ± SEM of three experiments. ***$P < 0.001$ vs. samples incubated with medium alone. Panel B: autoradiograph showing TNFα and GAPDH mRNA levels in ANA-1 cells treated or not with LPL (1 μg/ml) in the presence or absence of 1% DMSO for 3 h.

genously generated radical species may contribute to LPL-induced TNFα secretion, DMSO (1% final concentration) was added at the time of macrophage stimulation with LPL. Addition of DMSO affected neither the ability of LPL to enhance TNFα production (**Fig. 5,** panel a) nor TNFα RNA concentrations (fold increase over basal values: LPL: 2.3; LPL + DMSO: 2.4) (Fig. 5, panel b). Similar results were obtained when catalase was substituted for DMSO (data not shown). The effect of exogenous oxidant stress on LPL-induced TNFα production by macrophages was next evaluated. ANA-1 cells were incubated with 1 μg/ml LPL in the absence or presence of 200 μM H_2O_2. After 18 h, supernatants were collected and assayed for TNFα content. When expressed as percent increase over control basal values, LPL-induced TNFα release from macrophages exposed to H_2O_2 was significantly lower than that produced by the cells in the absence of H_2O_2 (**Fig. 6,** panel a). To determine whether the diminution of LPL-induced TNFα production by H_2O_2 was due to a reduction of cell sensitivity to LPL, ANA-1 cells were pretreated for 1 h with H_2O_2 (200 μM), washed, and then stimulated with 1 μg/ml LPL. The similar decrease of LPL-induced TNFα production that we found under these experimental conditions (Fig. 6, panel b) as compared to that observed after simultaneous treatment of the cells with LPL and H_2O_2 supported the hypothesis that decreased macrophage sensitivity to LPL is responsible for the diminished TNFα response to LPL.

To assess whether the inhibitory effect of H_2O_2 on macrophage sensitivity to LPL was indeed related to oxidant stress, the effects of various antioxidants on H_2O_2-induced decrease in LPL-induced TNFα production were tested. When added at the time of stimulation with H_2O_2, DMSO (1%) and several other antioxidants

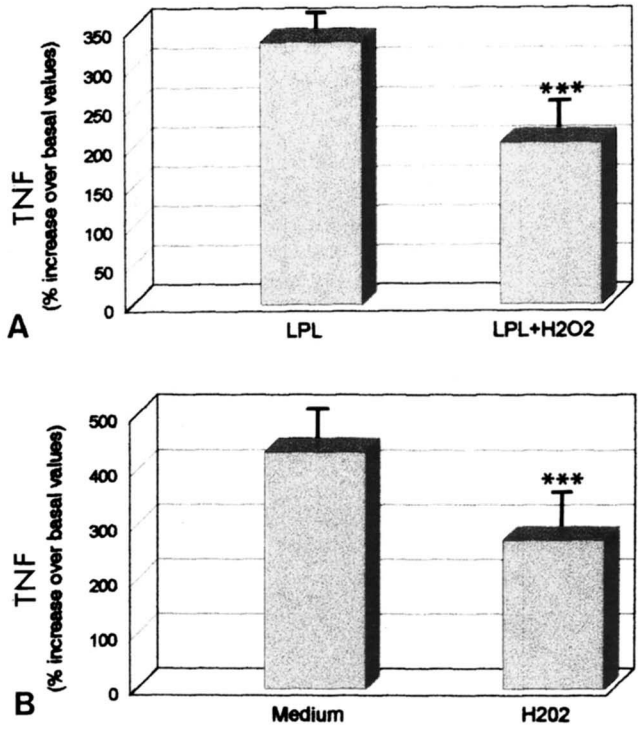


Fig. 6. Panel A: H_2O_2 reduces the stimulatory effect of LPL on macrophage TNFα production. Macrophages were incubated with medium, LPL alone (1 μg/ml), H_2O_2 alone (200 μM), or with LPL plus H_2O_2. After 18 h, LPL-stimulated TNFα production was measured in the supernatants. Results are expressed as percent increase over basal values (LPL vs. medium: 333 ± 16%; H_2O_2 + LPL vs. H_2O_2: 205 ± 9%; ***$P < 0.002$). Data represent the mean ± SEM of four experiments. Panel B: preincubation of ANA-1 cells with H_2O_2 decreases TNFα production response to LPL. Macrophages were exposed or not to 200 μM H_2O_2 for 1 h, washed, and stimulated for an additional period of time with LPL (1 μg/ml). LPL-induced TNFα production was measured after an 18 h incubation period in the medium. Results are expressed as percent increase over basal values (medium/wash/LPL vs. medium/wash/medium: 433 ± 8%; H_2O_2/wash/LPL vs. H_2O_2/wash/medium: 276 ± 17%; ***$P < 0.002$). Data represent the mean ± SEM of 10 experiments.

TABLE 3. Effect of antioxidants and protein kinase C inhibitor on the suppressive effect of H_2O_2 macrophage TNFα production

Pretreatment 1 h	Treatment 24 h	TNFα Production (% increase over basal values)	Control Basal Values
Medium	LPL	433 ± 8	medium/medium
H_2O_2	LPL	276 ± 17[a]	H_2O_2/medium
DMSO (1%)	LPL	450 ± 10	DMSO/medium
DMSO + H_2O_2	LPL	407 ± 11	DMSO + H_2O_2/medium
Catalase (20 μg/ml)	LPL	374 ± 16	catalase/medium
Catalase + H_2O_2	LPL	389 ± 9	catalase + H_2O_2/medium
Mannitol (10 mM)	LPL	403 ± 18	mannitol/medium
Mannitol + H_2O_2	LPL	423 ± 15	mannitol + H_2O_2/medium
Calphostin C (1 μg/ml)	LPL	392 ± 20	calphostin/medium
Calphostin C + H_2O_2	LPL	243 ± 14[a]	calphostin + H_2O_2/medium

Values are the means ± SEM of percent increase over basal values and are derived from determinations of ten experiments. The concentrations of H_2O_2 and LPL used are 200 μM and 1 μg/ml, respectively. LPL, lipoprotein lipase; TNFα, tumor necrosis factor alpha; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide.
[a] *P* values less than 0.001 (Student's *t* test) indicate a significant difference between samples pretreated with H_2O_2 and those untreated.

restored LPL-induced TNFα production to control values (**Table 3**). In contrast, no effect of the PKC inhibitor, calphostin C (1 μg/ml), was observed (Table 3).

In view of previous observations indicating an inhibitory effect of H_2O_2 on TNFα binding capacity (34), we tested the possibility that the decreased macrophage response to LPL in H_2O_2-treated cells could be associated with diminished LPL binding to the macrophage surface. ANA-1 cells were preexposed or not to 200 μM H_2O_2, washed, and treated for 2 h at 4°C with 10 μg biotinylated LPL. Cell surface-associated LPL released by heparin was analyzed on an SDS gel. As shown in **Fig. 7,** biotinylated LPL migrated at an apparent molecular mass of 55 kDa. A same mobility for native unlabeled LPL was assessed by Western blot analysis (data not shown). Incubation of the cells with biotinylated LPL led to a major increase of heparin-releasable cell surface LPL. Such heparin-binding properties of LPL at the macrophage surface were markedly reduced in H_2O_2-treated cells as compared to untreated cells (Fig. 7).

DISCUSSION

ROI are believed to play a significant role in various disorders, including ischemia/reperfusion injury and atherosclerosis. Elevated production of ROI has been reported in blood vessels and myocardium in response to a variety of injury-related conditions such as ischemia, thrombosis, and angioplasty (36, 37). Generation of ROI by vascular cells led to the formation of oxidatively modified LDL (38), which are thought to play a major role in the development and progression of atherosclerosis.

It has been recently shown that reactive oxygen metabolites, beside their effect on lipid metabolism, also possess immunomodulatory properties. Free radical

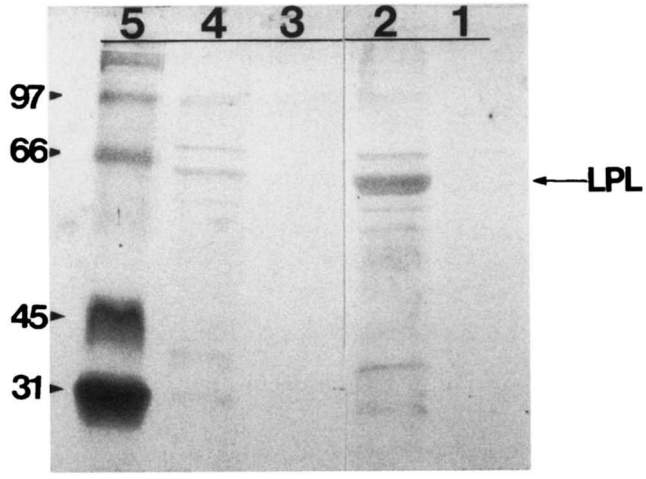


Fig. 7. Effect of H_2O_2 on the heparin binding properties of LPL to macrophage cell surface. ANA-1 cells were pretreated for 1 h with medium or 200 μM H_2O_2, washed, and further incubated in the presence or absence of 10 μg/ml biotinylated LPL for 2 h at 4°C. Unbound LPL was removed and cell surface LPL was released by incubating ANA-1 cells with medium containing 100 units/ml heparin. Heparin-releasable cell surface biotinylated LPL was visualized with avidin-horseradish peroxidase as described in Methods. Lanes 1 and 2: cells pretreated with medium and then incubated in the absence (lane 1) or presence (lane 2) of LPL. Lanes 3 and 4: cells pretreated with H_2O_2 and then incubated in the absence (lane 3) or presence (lane 4) of LPL. Lane 5: molecular weights of protein standards indicated in kDa.

scavengers have been reported to inhibit in vitro T cell proliferation (39, 40) while antioxidants have been demonstrated to dramatically decrease LPS-induced production of several cytokines including TNFα, IL-1, IL-6, and IL-8 (16, 41, 42). Conversely, H_2O_2 has been shown to directly stimulate the LPS-induced release of TNFα (16) and IL-8 (42). These findings support the possibility that ROI may interact with the immune cells present in the arterial wall and that their ability to affect the atherogenic process may be, in part, secondary to their capacity to increase the release of some proatherogenic molecules.

LPL has been identified as a constitutive secretory product of macrophages (19). The observations that increased macrophage LPL expression is associated with susceptibility to atherosclerosis (26) and that lesion macrophages express LPL mRNA and protein (21) are consistent with the hypothesis that LPL may play an important role in atherogenesis. The secretion of LPL in the arterial wall could potentiate the atherosclerotic process by facilitating the conversion of macrophages into foam cells and by modulating macrophage function.

The present report provides evidence that release of LPL by macrophages can be enhanced by imposing external oxidant stress. Such activity is likely to depend on free radical species generated by transition metal-catalyzed reduction of H_2O_2, as it is reduced by free radical scavengers and by the iron chelator, DES. The similar effectiveness of membrane-permeable and non-permeable scavengers to decrease the H_2O_2-induced LPL production and the lack of LPL stimulation by agents that generate intracellular ROI indicate that intracellular localization of ROI does not play a key role in the induction of LPL. Evidence that PKC-dependent mechanisms are involved in transduction of H_2O_2 signal is supported by our results that demonstrate, in human monocyte-derived macrophages, a complete suppression of the stimulatory effect of H_2O_2 on LPL production in presence of PKC inhibitors. These conclusions are in accordance with previous results demonstrating a role of PKC as mediator of cell response to H_2O_2 (43) and as inducer of LPL activity in preadipocytes (44).

In addition to demonstrating a direct stimulatory effect of H_2O_2 on macrophage LPL secretion, our data also provide convincing evidence for a modulatory effect of ROI in the regulation of macrophage LPL production by the proinflammatory cytokine, TNFα. Our finding that macrophage exposure to antioxidants, in the absence of any added radical generators, leads to an accentuation of the suppressive effect of TNFα on LPL secretion suggests that ROI generated upon macrophage stimulation by TNFα (45) may provide a feedback control to restrict LPL inhibition and that such an effect could account for the incomplete inhibition of LPL documented in TNFα-treated macrophages (35). Previous observations showing that adipocytes that do not produce ROI exhibit a complete inhibition of LPL production upon TNFα stimulation seem to support such hypothesis. Further evidence for a role of ROI in the regulation of macrophage LPL production is provided by our finding that the TNFα suppressive effect on LPL production is reduced in H_2O_2-treated cells. The reduction of TNFα effect in cells exposed to H_2O_2 may result from a decrease of TNFα activity and/or from a reduction of cell sensitivity to this cytokine. Preliminary results generated in our laboratory indicate that concomitant treatment of macrophages with TNFα and H_2O_2 or preexposure of the cells to H_2O_2 prior to TNFα treatment lead to a similar decrease of LPL response to this cytokine. These observations suggest that reduction of macrophage sensitivity to TNFα may represent at least one mechanism whereby oxidant stress could affect the regulation of LPL production. In contrast to the decrease of macrophage responsiveness to TNFα and LPL that we documented in presence of H_2O_2, increase of LPS-induced release of TNFα and IL-8 by ROI has been previously reported (16, 42). While reduced LPL and TNFα binding to the surface of H_2O_2-treated cells that we and others (34) documented seems to account for the altered macrophage responsiveness to these agents, the mechanisms responsible for the increase of LPS-induced cytokine production after oxidant stress are presently unclear (16).

Although our data indicate a stimulatory effect of H_2O_2 on human monocyte-derived macrophage LPL secretion, the physiological relevance of our findings remains uncertain, precise data about the oxidative stress and LPL levels encountered by macrophages in vessel walls being presently not available. Despite the lack of such information, several pieces of evidence seem to support the importance of our observations. H_2O_2 concentrations that we found effective in stimulating LPL production are observed in the vicinity of activated human granulocytes in vitro (46) and may therefore be reached in the inflammatory atherogenic lesion. In addition, as other LPL stimulatory factors are produced in the atherosclerotic lesion (47), the concerted effect of ROI and of these agents on macrophage function may result in significant accumulation of LPL in the arterial wall. In view of the key role of this enzyme in the control of lipid metabolism, it is tempting to postulate that such enhanced LPL production in the artery wall may have functional consequences on the progression of atherosclerosis.

In conclusion, the present report provides evidence for a role of ROI in the control of LPL production by macrophages. The recognition of such an effect of ROI

may provide new insights into the mechanisms underlying the deleterious effect of ROI on the atherogenic process and could partly explain the efficacy of antioxidants in the prevention of the atherosclerotic process.

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