Effect of Modified LDL on the Release of NO and PGI₂ from Rat Peritoneal Macrophages

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Nitric oxide (NO) and prostacyclin (PGI₂) have vasodilative and anti-proliferative effects on smooth muscle cells (SMC) and an anti-aggregating action on platelets. The present study was designed to elucidate the influence of modified low density lipoprotein (LDL) on the release of NO and PGI₂ from rat peritoneal macrophages. Cholesteryl ester (CE) content in macrophages markedly increased on incubation with acetylated LDL (ac-LDL), while NO release did not change. Although incubation with mildly oxidized LDL (m-ox-LDL) and highly oxidized LDL (h-ox-LDL) increased CE content in macrophages, only incubation with h-ox-LDL reduced NO release. PGI₂ release from macrophages was not affected by incubation with ac-LDL, m-ox-LDL or h-ox-LDL. These results indicate that the degree of suppression of NO release in macrophages by modified LDL is related to the extent of oxidative modification of LDL itself, but not to the extent of the accumulation of CE in macrophages. Although the role of NO released from macrophages in atherosclerosis is still unclear, the observation of reduced production of NO from macrophages in response to ox-LDL may provide new insight into the role of ox-LDL in the pathogenesis of atherosclerosis. *J Atheroscler Thromb*, 1995 ; 2 : 41-45.

Key words : Acetylated LDL, Oxidized LDL, TBARS, Cholesteryl ester

Circulating blood monocytes migrate into the sub-endothelial area and take up oxidized low density lipoprotein (ox-LDL), leading to foam cell formation in atheromatous plaques (1). It is known that macrophages can release nitric oxide (NO) and prostacyclin (PGI₂) (2, 3). NO inhibits smooth muscle cell (SMC) proliferation (4) and platelet aggregation (5). PGI₂ also has inhibitory effects on the functions of SMC and platelets (6, 7). Thus, through the production of these bioactive substances, macrophages may play a crucial role in the progression of atherosclerosis.

Cellular components of atheromatous lesions including SMC, macrophages and endothelial cells can oxidize LDL (8). Indeed, several lines of evidence indicate that ox-

LDL is formed in atherogenic lesions (9, 10). Ox-LDL is a chemotactic factor for monocytes (11) and a modulator of cytokine production of macrophages (12, 13) and, therefore, macrophage function could be modified during the exposure to ox-LDL.

It has been shown that NO release is reduced in atherosclerotic vessel walls (14). PGI₂ production is also impaired in atherosclerosis (15). Ox-LDL has been shown to alter NO and PGI₂ production in various cells such as SMC and endothelial cells (16, 17). It is, therefore, possible that ox-LDL may have an effect on the release of NO and/or PGI₂ from macrophages. It has been reported that the degree of lipid peroxidation of ox-LDL is an important determinant of the effects of LDL on prostanoid synthesis by SMC (17). The present study was designed to determine the influence of ox-LDL with different degrees of oxidation on the release of NO and PGI₂ from rat peritoneal macrophages.

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Received May 2, 1994.

Accepted for publication October 3, 1994.

Materials and Methods

Materials

RPML-1640 medium and fetal bovine serum (FBS) were from GIBCO (New York, NY, USA). Cholesterol was from Sigma Chemical Co. (St. Louis, MO, USA). Radioimmunoassay kit for 6-keto-prostaglandin $F_{1\alpha}$ (6-k-PGF $_{1\alpha}$) was purchased from Amersham plc. (Amersham, England). Sodium nitrite, sulfanilamide, N-1-naphthylethylene diamine dihydrochloride, sodium acetate, KBr, acetic anhydride, FeSO $_4$, gentamicin and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of macrophages

Peritoneal macrophages were prepared from six-week-old male Wistar rats as described previously (18). Briefly, 30 ml of phosphate-buffered saline [Ca $^{++}$, Mg $^{++}$ -free, PBS (—)] was injected into the rat peritoneal cavity after decapitation. Peritoneal fluid was then collected after gentle massaging and macrophages in the fluid were washed several times with PBS (—) by centrifugation at 120×g for 10 min. Finally, they were resuspended in RPML-1640 medium containing 5% (v/v) FBS and 100 μ g/ml gentamicin. One ml of cell suspension (2×10^6 cells) was put into each well of 24-well plastic culture dishes and incubated at 37°C in 5% CO $_2$ and 95% air for 2 h. Each dish was then rinsed with PBS (—) and the same culture medium was added to the dish.

Preparation of LDL

LDL was prepared from human blood as described previously (19). Human plasma was collected after overnight fasting from healthy volunteers, and LDL (s.g. 1.019–1.063) was obtained by sequential ultracentrifugation. LDL was pooled and dialyzed extensively against PBS (—) at 4°C. LDL prepared under these conditions was referred to as native LDL (n-LDL).

Modification of LDL

Acetylated LDL (ac-LDL), mildly oxidized LDL (m-ox-LDL) and highly oxidized LDL (h-ox-LDL) were prepared. Ac-LDL was prepared using acetic anhydride according to the method of Basu *et al.* (20). LDL oxidation was carried out by dialysis against isotonic saline containing 1 μ M FeSO $_4$ at 4°C. The degree of oxidation was controlled by changing the duration of dialysis, which for the preparation of m-ox-LDL and h-ox-LDL was 2–3 days and 5–7 days, respectively (21). Oxidation was stopped by dialyzing the samples against PBS (—) supplemented with 0.5 mM EDTA at 4°C for 48 h. Oxidative modification of n-LDL, ac-LDL and ox-LDL was evaluated measuring thiobarbituric acid reactive substances (TBARS) as described previously (22). TBARS values are expressed as malondialdehyde equivalents (nmol of malondialdehyde/mg of LDL cholesterol). N-LDL and

modified LDL were subjected to agarose gel electrophoresis (23). Cholesterol and protein contents of native and modified LDL were determined as described below.

Release and assay of NO $_2^-$, PGI $_2$ and LDH

After macrophages were pre-cultured for 24 h at 37°C in 5% CO $_2$ and 95% air, cells were further cultured for 24 h in RPML-1640 medium containing 2% FBS and various concentrations of native or modified LDL. Culture medium was collected and centrifuged to remove particulate fractions.

Amount of NO release was determined spectrophotometrically as nitrite (NO $_2^-$) in culture medium at 550 nm using Griess reagent (3). PGI $_2$ release was assessed by measuring 6-k-PGF $_{1\alpha}$, a stable metabolite of PGI $_2$, in culture medium using a specific RIA kit.

Lactic dehydrogenase (LDH) activity in culture medium was assayed with an autoanalyzer (24). Cell viability was assessed by the trypan blue dye exclusion test.

Determination of cholesterol and protein contents

After collecting medium for the assay of NO $_2^-$ and 6-k-PGF $_{1\alpha}$, macrophages on the culture dish were washed three times with PBS (—) and lysed with 0.1% (v/v) Triton-X 100. Cell lipids were extracted with chloroform : methanol solution (2 : 1, v/v), and the chloroform was evaporated under N $_2$ gas. The residue was redissolved in a small volume of ethanol, and the content of CE was assayed according to the method of Heider and Boyett (25). Cholesteryl ester (CE) content was determined by subtracting free cholesterol (FC) from total cholesterol content. Determination of protein contents was conducted according to the method, described by Lowry *et al.* (26).

Statistical analysis

Data are expressed as mean $\pm$ SE. Significance of differences were determined using the non-paired Student's *t*-test. The level of significance was defined as $p < 0.05$.

Results

Oxidative modification of LDL

TBARS level was 0.26 ± 0.02 (n=6) nmol MDA/mg chol for n-LDL, 0.11 ± 0.01 (n=3) nmol MDA/mg chol for ac-LDL, 3.96 ± 0.45 (n=7) nmol MDA/mg chol for m-ox-LDL and 12.99 ± 1.69 (n=6) nmol MDA/mg chol for h-ox-LDL.

Results of agarose gel electrophoresis of native and modified LDL are shown in Fig. 1. Ac-LDL demonstrated increased mobility, while both m-ox-LDL and h-ox-LDL showed only slight migration. The relative electrophoretic mobilities, defined as the ratio of migration distance of modified LDL to n-LDL, were 1.10, 1.27 and 5.0 for m-ox-LDL, h-ox-LDL and ac-LDL, respectively. This indicates that m- and h-ox-LDL both showed a significant but a subtle increment in negative charge compared to

Fig. 1. Agarose gel electrophoresis of native and modified LDL. n-LDL: native LDL, ac-LDL: acetylated LDL, m-ox-LDL: mildly oxidized LDL, h-ox-LDL: highly oxidized LDL.

ac-LDL.

CE and FC contents in LDL

CE content of n-LDL was 1.74 ± 0.09 (n=6) mg/mg protein, 1.80 ± 0.36 (n=3) mg/mg protein for ac-LDL, 1.51 ± 0.06 (n=7) mg/mg protein for m-ox-LDL and 1.56 ± 0.07 (n=5) mg/mg protein for h-ox-LDL. FC content of n-LDL was 0.22 ± 0.02 (n=6) mg/mg protein, 0.35 ± 0.07 (n=3) mg/mg protein for ac-LDL, 0.19 ± 0.03 (n=7) mg/mg protein for m-ox-LDL and 0.20 ± 0.02 (n=5) mg/mg protein for h-ox-LDL.

CE accumulation in macrophages

As shown in Fig. 2, CE content increased markedly during incubation with ac-LDL in a dose-dependent fashion. An increment of nearly 7-folds was observed at the concentration of $50 \mu\text{g}$ protein/ml. H-ox-LDL also induced significant increases (1.5-fold) at a concentration of $100 \mu\text{g}$ protein/ml. In contrast, "a lesser" but statistically significant increment was observed after incubation with n-LDL and m-ox-LDL.

NO release

Nitrite concentration in cell-free culture medium with or without native or modified LDL was less than 0.5 nmol/ml . NO_2^- level in 24 h cell culture medium without LDL was $40.0 \pm 2.2 \text{ nmol/mg cell protein}$. NO_2^- level after incubation with n-LDL, ac-LDL or m-ox-LDL was the same as the control. In contrast, as indicated in Fig. 3, h-ox-LDL significantly inhibited NO release in a dose-dependent manner. Nearly 30% inhibition was observed at a concentration of $100 \mu\text{g}$ protein/ml.

After 24 h in cell culture, LDH level in medium without LDL was 106.8 ± 4.1 (n=4) IU/l, 106.0 ± 3.7 (n=4) IU/l for medium with m-ox-LDL and 102.3 ± 3.3 (n=4) IU/l for medium with h-ox-LDL. Cell viability was more than 95%. The levels of endotoxin in culture medium with or

Fig. 2. CE accumulation in macrophages. After macrophages were pre-cultured for 24 h at 37°C in 5% CO_2 and 95% air, cells were cultured for a further 24 h in RPMI-1640 containing 2% FBS and various concentrations of native or modified LDL. Cell lipids were extracted with chloroform: methanol solution, and cholesterol was assayed as described by Heider and Boyett (25). n-LDL: native LDL, ac-LDL: acetylated LDL, m-ox-LDL: mildly oxidized LDL, h-ox-LDL: highly oxidized LDL. (*: $p < 0.05$, **: $p < 0.01$ vs. control, n=6)

Fig. 3. Effects of highly oxidized LDL on NO release in macrophages. The experimental conditions were the same as in Fig. 2. NO release was determined spectrophotometrically as NO_2^- at 550 nm using Griess reagent. (**: $p < 0.01$ vs. control, n=8).

without modified LDL were less than 0.1 ng/ml .

PGI_2 release

The concentration of 6-k-PGF $_{1\alpha}$ in cell-free culture medium with or without native or modified LDL was below the limit of detection. The concentration of 6-k-PGF $_{1\alpha}$ in 24-h cell culture medium without LDL was $24.6 \pm 2.6 \text{ ng/mg cell protein}$. The level of 6-k-PGF $_{1\alpha}$ was not changed after incubation with either n-LDL or modified LDL.

Discussion

Both m-ox-LDL and h-ox-LDL dose-dependently increased CE content in macrophages. However, only h-ox-LDL showed a significant and dose-dependent inhibition of NO release from macrophages. In addition, ac-LDL induced marked accumulation of CE in macrophages compare to m-ox-LDL and h-ox-LDL, while NO release from macrophages was not changed. These results indicate that the degree of suppression of NO release from macrophages by modified LDL may be related to the extent of oxidative modification of LDL itself, but not to the extent of the accumulation of CE in macrophages. The suppressive effect of ox-LDL on cytokine production has been reported to be reproduced with lipid extracts of ox-LDL (13, 27). The inhibitory effect of h-ox-LDL on NO release might be partly ascribed to oxidatively modified lipids, although other components of ox-LDL could also be contributing factors.

The mechanism of the decrease in NO release from macrophages by ox-LDL remains unclear. There are several possibilities for the mechanism; ox-LDL might interfere directly with the NO synthase activity, and/or may also decrease the availability of enzymatic cofactors or L-arginine (28). Expression of interleukin-1 α , interleukin-1 β , interleukin-6 (13) and TNF- α mRNA (27) have been reported to be suppressed by ox-LDL in murine peritoneal macrophages. This suggests that ox-LDL may interfere with the expression of NO synthase mRNA. Further studies are required to test this possibility.

Ox-LDL has been reported to be toxic to a variety of cultured cells under certain conditions (21). In our study, after incubation with h-ox-LDL, LDH level in culture medium was not elevated and cell viability was more than 95%. Thus, it is unlikely that NO release from macrophages was reduced by the cytotoxic effect of ox-LDL.

NO has vasodilative (29) and anti-proliferative effects on SMC (4), and an anti-aggregating effect on platelets (5). Although the role of NO released from macrophages in atherosclerosis is still unclear, the reduced production of NO from macrophages by ox-LDL may provide new insight into the role of ox-LDL in the pathogenesis of atherosclerosis.

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