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Involvement of endotoxins or tumor necrosis factor-α in macrophage-mediated oxidation of low density lipoprotein

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Abstract Oxidation of low-density lipoprotein (LDL) can be mediated in vitro by cultured cells, including macrophages. This cellular oxidation is dependent on the production of free thiols by the cells in the presence of transition metal ions. We now report that the production of thiols by macrophages is greatly enhanced when cells are cultured with lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). Oxidation of LDL by macrophages is markedly augmented by pre-treatment of the cells with LPS or TNF-α. The results suggest that activation by endotoxins or TNF-α is a necessary step for macrophages to mediate oxidation of LDL.

Key words: Low-density lipoprotein; Macrophage; Lipopolysaccharide; Tumor necrosis factor-α; Cystine

1. Introduction

Recent evidence strongly suggests that low-density lipoprotein (LDL) is heavily implicated in the pathogenesis of atherosclerosis [1–4]. Oxidized LDL probably participates in the formation of the cholesterol-laden foam cells in the atherosclerotic lesion, predominantly derived from macrophages [5]. Oxidized LDL is taken up avidly by macrophages via scavenger receptors, leading to cholesterol accumulation in the arterial wall, while native LDL is not recognized by the scavenger receptor [6].

The mechanism involved in LDL oxidation is still unclear. Some types of cells can mediate the LDL oxidation in the presence of transition metals [7]. Although several mechanisms have been proposed, evidence to date is most consistent with the involvement of thiols generated by the cells [8–10]. Thiols of L-cysteine are unstable in the medium and exist as a disulfide form L-cystine. In the absence of cells culture medium contains very little thiols [11]. Some kinds of cells, e.g. fibroblasts are able to take up L-cystine from the medium via a transporter and reduce intracellularly to L-cysteine, which is incorporated into glutathione or proteins [12,13]. However, a large part of L-cysteine thus produced is released into the medium and therefore thiols are accumulated there during the culture [11]. The transport system for the uptake of L-cystine was identified and designated as System x_c^-, which mediates the exchange between the extracellular L-cystine and the intracellular L-glutamate [13,14]. We reported previously that in mouse peritoneal macrophages the activity of System x_c^-, which is negligibly low immediately after preparation, is strongly induced by LPS or by TNF-α [15]. In this paper we report that pretreatment of macrophages with LPS or TNF-α potently enhances the production of thiols and oxidation of LDL.

2. Materials and methods

2.1. Materials

Thioglycollate broth (Brewer's formula) and lipopolysaccharide (from *Salmonella typhosa* 0901) was obtained from Difco Laboratories, Detroit, MI, USA. Recombinant mouse interleukin 1β (IL-1β) was from Boehringer Mannheim. Recombinant human TNF-α was a kind gift from Suntory Biomedical Research Laboratory, Osaka, Japan.

2.2. Isolation of LDL

Plasma was obtained from fasted healthy normolipidemic male volunteers, 20–30 years old, and LDL was isolated by standard procedures with a slight modification [16]. Plasma was centrifuged at 100×10^3 rpm for 2 h at 4°C in a Beckman TL-100.3 rotor. The infranatants were collected, adjusted to $d=1.063$ g/ml with KBr solution, and centrifuged at 100×10^3 rpm for 4 h at 4°C. The supernatants (about 1.5 mg protein/ml) were collected and dialyzed 3 times against 200 volumes of phosphate-buffered saline (PBS) containing 10 μM EDTA. The solution was sterile-filtered, stored at 4°C and used within 2 weeks.

2.3. Cell culture

Macrophages were collected by peritoneal lavage from female C57BL/6N mice weighing 20∼25 g, that had been given an intraperitoneal injection of 2 ml of 4% thioglycollate broth 4 days previously. The cells were plated in 24-well tissue culture plates at a density of 0.4×10^6 cells/well in 0.4 ml RPMI 1640 medium containing 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin, and were incubated at 37°C in 5% CO_2 incubator.

Fetal bovine serum lots with endotoxin content of less than 0.5 ng/ml were used to minimize the contaminated endotoxin in the culture medium. After 1 h the medium was replaced by fresh medium with and without LPS, TNF-α or IL-1β and the cells were incubated for the indicated time.

2.4. Determination of thiol concentration in the medium

Thiol concentration in the medium was measured with 5,5′-dithiobis(2-nitrobenzoic acid) as described previously [11]. Cells in the well were washed twice with PBS and then incubated with 200 μl/well of phenol red-free minimum essential medium buffered with 10 mM HEPES (MEM-HEPES(−)PR). After incubation for 2 h, 180 μl of the medium was carefully removed and mixed rapidly with 180 μl of 0.2 M potassium phosphate-10 mM EDTA, pH 7.2. Then 15 μl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) was added. After about 5 min the increase in absorbance at 412 nm was measured. The thiol content was calculated from the increase, by using GSH as a standard.

2.5. Assay of oxidative modification of LDL

To prepare chemically oxidized LDL, LDL (100 μg protein/ml) was

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Abbreviations: LDL, low density lipoprotein; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MEM-HEPES(−)PR, phenol red-free minimum essential medium buffered with 10 mM HEPES; TBARS, thiobarbituric acid-reactive substances

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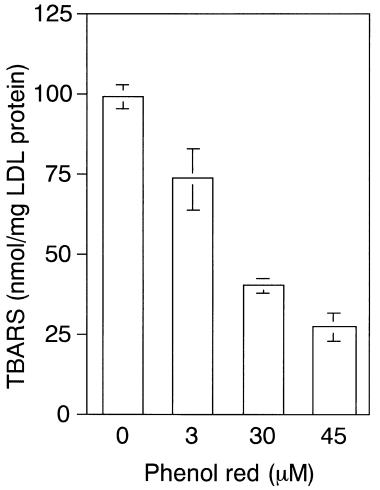

Fig. 1. Effect of phenol red on Cu^{2+}-mediated LDL oxidation. LDL (100 μg protein/ml) was incubated for 3 h in PBS containing 2 μM $CuSO_4$ in the presence of phenol red as indicated. Aliquots of the solution were assayed for TBARS assay. Values are means ± S.D. of 6 experiments.

incubated at 37°C for 3 h in the presence of 2 μM $CuSO_4$. After the incubation butylated hydroxyanisol was added at the final concentration of 20 μM and estimation of oxidative modification of LDL was performed immediately.

Macrophage-mediated oxidative modification of LDL was performed as follows. Cells in the well were washed twice with PBS and incubated with 200 μl/well of MEM-HEPES(−)PR containing LDL (100 μg protein/ml) with or without $FeSO_4$. Small amounts of EDTA that were present in the LDL solution carried over into the incubations (less than 0.7 μM). This level of EDTA had no effect on the results, as judged by experiments in which EDTA was removed by dialysis. After incubation the culture medium was collected and butylated hydroxyanisol was added at the final concentration of 20 μM. The mixture was stored at 4°C and estimation of oxidative modification of LDL was performed within 24 h after preparation of the samples.

Oxidation of LDL was examined by thiobarbituric acid-reactive substances (TBARS) assay [17] and by agarose gel electrophoresis [18]. Aliquots (80 μl) of the culture medium containing LDL (about 8 μg of protein) were mixed with 150 μl of 0.8% thiobarbituric acid, 150 μl of 20% acetic acid (adjusted to pH 3.5 by NaOH) and 20 μl of 8% SDS. The mixture was boiled for 30 min and the absorbance at 540 nm was measured immediately after cooling. Electrophoresis of LDL was carried out on 0.8% agarose gel in 68.8 mM barbital buffer, pH 8.6. About 10 μl of the culture medium containing LDL (1 μg of protein) was applied onto the gel.

3. Results

3.1. Antioxidant action of phenol red

Culture media routinely used contain phenol red as a pH indicator. This compound has a structural similarity to phenolic antioxidant and may have inhibitory effect on the oxidative modification of LDL. We investigated the effect of phenol red on the cell-free oxidation of LDL mediated by Cu^{2+}. As shown in Fig. 1, LDL oxidation, which was assessed by TBARS production, was inhibited by phenol red; the inhibition was slight at 3 μM which is close to the concentration in Ham's F-10 medium, and very strong at 30 and 45 μM which are equivalent to the phenol red concentrations in MEM and Dulbecco's MEM (DMEM), respectively. Therefore, we used phenol red-free medium in the following experiments.

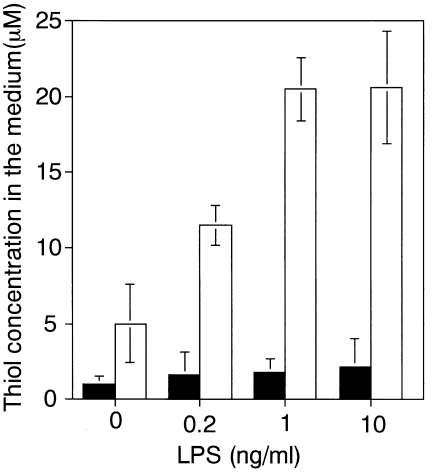

Fig. 2. The thiol production by macrophages pretreated with various concentrations of LPS. Macrophages were incubated for 1 h and the medium was replaced by fresh medium containing LPS as indicated. The cells were incubated for 12 h and then the medium was replaced by MEM-HEPES(−)PR (open bar) or by L-cystine-free MEM-HEPES(−)PR (filled bar). After incubation for 2 h, thiol content in the medium was measured. Values are means ± S.D. of 6 experiments.

3.2. Thiol production by macrophages pretreated with LPS

Macrophages were incubated for 12 h with various concentrations of LPS, then the medium was changed and the ability of macrophages to generate thiols in the medium was investigated (Fig. 2). The ability to generate thiols was estimated by measuring thiol concentrations in the medium after 2 h incubation. The generation of thiols depended on the concentration of LPS with which macrophages had been treated. Thiols were significantly generated at null concentration of LPS, probably due to the contaminated LPS-like endotoxins in the medium. Although we used serum with low endotoxin content, it was possible that the medium for the macrophage

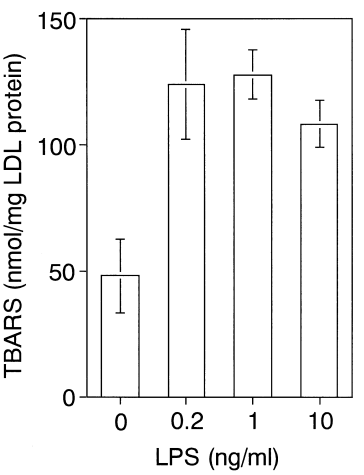

Fig. 3. LDL oxidation by macrophages pretreated with LPS: TBARS assay. Macrophages were incubated for 1 h and the medium was replaced by fresh medium. The cells were incubated for 12 h in the presence of various concentrations of LPS, washed twice with PBS and then incubated in MEM-HEPES(−)PR containing 1 μM $FeSO_4$ and LDL (100 μg protein/ml). After incubation for 7 h, aliquots of the medium were assayed for TBARS. Values are means ± S.D. of 6 experiments.

Table 1
Effect of L-cystine and $FeSO_4$ on the LDL oxidation by macrophages

Pretreatment with LPS	Cystine (μM)	$FeSO_4$ (μM)	TBARS (nmol/mg LDL protein)
−	0	0	0.6 ± 1.1
−	0	1	19.0 ± 7.8
−	100	0	2.1 ± 2.4
−	100	1	48.2 ± 9.8
+	0	0	6.5 ± 6.4
+	0	1	31.1 ± 8.0
+	100	0	15.1 ± 4.4
+	100	1	128.0 ± 9.78

Macrophages were incubated for 1 h and the medium was replaced by fresh medium. The cells were incubated for 12 h in the presence or absence of 1 ng/ml LPS and then the medium was replaced by MEM-HEPES(−)PR containing 1 μM $FeSO_4$ and LDL (100 μg protein/ml). In some experiments $FeSO_4$ was omitted or cystine-free MEM-HEPES(−)PR was used. The incubation was continued for 7 h and then aliquots of the LDL were assayed for TBARS. As cell-free control, TBARS was measured in the normal or cystine-free MEM-HEPES(−)PR containing LDL and 1 μM $FeSO_4$ after standing for 7 h at 37°C. Values are means ± S.D. of 6 experiments.

culture contained endotoxins at the concentration less than 0.05 ng (LPS equivalent)/ml (see Section 2). The maximal effect was observed at 1 ng/ml LPS. The activity for L-cystine uptake is induced by LPS at concentrations higher than 0.01 ng/ml and reached maximal level at 1 ng/ml LPS [15]. Therefore the thiol generation seemed to be correlated with the L-cystine uptake activity. Fig. 2 also shows that the generation of thiols was very low in the L-cystine-free medium indicating that it depends almost absolutely on L-cystine in the medium. We have determined the chemical nature of thiols generated in the medium. Thiols were derivatized by monobromobimane and analyzed by HPLC with a fluorescence detector [19]. We found that almost all thiols generated were those of cysteine, with a trace of GSH, in both LPS-pretreated and non-treated macrophages.

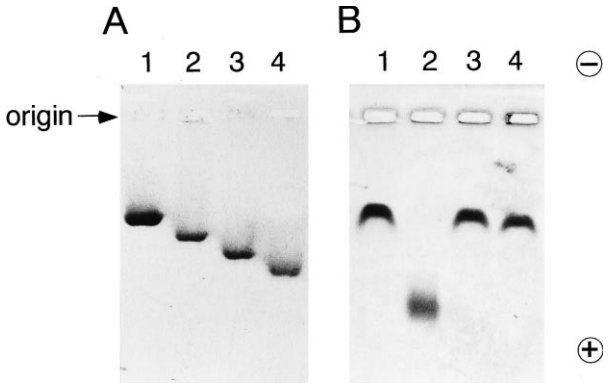

Fig. 4. LDL oxidation by macrophages pretreated with LPS: electrophoretic analysis. Macrophages were incubated for 1 h and the medium was replaced by fresh medium containing LPS as indicated. The cells were incubated for 12 h, washed twice with PBS and then incubated in MEM-HEPES(−)PR containing 1 μM $FeSO_4$ and LDL (100 μg protein/ml). The incubation was continued for 12 h and then aliquots of the medium were loaded on agarose gel electrophoresis (A). Lane 1: native LDL; lanes 2, 3 and 4: LDL incubated with macrophages pretreated with 0, 0.2 and 1 ng/ml of LPS, respectively. As cell free control, LDL was incubated in the absence of macrophages with or without $FeSO_4$ for 12 h. Then aliquots of the medium were loaded on agarose gel electrophresis (B). Lane 1: native LDL; lane 2: chemically oxidized LDL (LDL incubated with $CuSO_4$ as described in Section 2); lane 3: LDL incubated in MEM-HEPES(−)PR without $FeSO_4$; lane 4: LDL incubated in MEM-HEPES(−)PR with $FeSO_4$.

3.3. LDL oxidation by macrophages pretreated with LPS

Since there is convincing evidence that thiols can cause the oxidation of LDL in the presence of iron [20], we tested whether oxidation of LDL by macrophages is enhanced when they are pretreated with LPS. After 12 h preincubation of the cells with various concentrations of LPS, the medium was replaced by MEM-HEPES(−)PR containing 1 μM $FeSO_4$ and LDL. After 7 h incubation the TBARS formation in the medium was examined (Fig. 3). TBARS formation was significantly enhanced in macrophages pretreated with LPS. Pretreatment with 0.2 ng/ml LPS seems to be enough to induce maximal TBARS formation under these experimental conditions. We also investigated the requirement of L-cystine and iron ion for the TBARS formation from LDL by LPS-pretreated macrophages. The results in Table 1 clearly show that both L-cystine and $FeSO_4$ are required for the maximal oxidation of LDL evaluated by TBARS formation.

TBARS are composed of reactive aldehydes and although TBARS assay is widely used as a reliable index of lipid oxidaton, the amount of TBARS does not necessarily represent the exact degree of lipid peroxidation because TBARS are lost by the reaction with protein amino groups. Thus in order to evaluate the cumulative oxidation of LDL, we measured its anodic electrophoretic mobility. As shown in Fig. 4, the electrophoretic mobility of LDL after 12 h incubation with macrophages was significantly increased, depending on the LPS concentration employed for the pretreatment. These results indicate that the ability of macrophages to oxidize LDL is enhanced by pretreatment with LPS. Fig. 4 also shows that in the absence of macrophages oxidation of LDL was almost undetectable even though LDL was incubated for 12 h in the presence of 1 μM $FeSO_4$.

3.4. Thiol production and LDL oxidation by macrophages pretreated with TNF-α

L-Cystine transport activity of macrophages is induced not only by LPS but also by TNF-α [15]. Thus the generation of thiols and the oxidation of LDL by macrophages pretreated with TNF-α were investigated (Table 2). The results demonstrated that the ability of macrophages to generate thiols in the medium and to produce TBARS from LDL was strongly enhanced by pretreatment with TNF-α at 100 units/ml or higher. On the other hand, IL-1β, which could not induce L-cystine transport activity [15], had little effect on thiol generation and LDL oxidation by macrophages.

Table 2
Thiol production and LDL oxidation by macrophages pretreated with TNF-α and IL-1β

Additions (units/ml)	Thiol concentration (μM)	TBARS (nmol/mg LDL protein)
None	3.4 ± 3.1	48.2 ± 14.6
TNF-α		
100	14.2 ± 2.4	128.9 ± 4.0
1000	17.9 ± 1.2	130.0 ± 0.8
IL-1β		
100	4.4 ± 2.0	43.7 ± 4.5
1000	8.6 ± 1.6	48.2 ± 14.6

Macrophages were incubated for 1 h and the medium was replaced by fresh medium containing TNF-α or IL-1β as indicated and incubated for 12 h. To measure thiol production, the medium was then replaced by MEM-HEPES(−)PR. After incubation for 2 h, thiol content in the medium was measured. To estimate oxidation of LDL, the medium was then replaced by MEM-HEPES(−)PR containing 1 μM $FeSO_4$ and LDL (100 μg protein/ml). After incubation for 7 h, the aliquots of LDL were assayed for TBARS. Values are means ± S.D. of 6 experiments.

4. Discussion

The oxidative modification of LDL by cultured cells requires a medium containing transition metal ions [18,21,22]. The medium most commonly used to support cellular oxidation of LDL is Ham's F-10 [9], which is formulated with 3 μM $FeSO_4$ and 0.01 μM $CuSO_4$. Macrophages were able to oxidize LDL in F-10 medium and the inclusion of $FeSO_4$ was thought to be essential to the oxidation. However, there is conflicting evidence showing that macrophages could not modify LDL in DMEM even when 3 μM $FeSO_4$ was added to it [23]. The present study demonstrated the antioxidant activity of phenol red. The reason why LDL oxidation is blocked in DMEM is probably that DMEM contains phenol red at a concentration (43 μM) much higher than F-10, i.e. 3.4 μM. F-10 is unique with its metal ions and low concentration of phenol red, and thus suitable to support cell-mediated oxidation of LDL.

Several observations suggest a role for thiol compounds in cellular oxidation of LDL in medium containing transition metal ions [8–10,22,24,25]. Heinecke et al. [8] first showed that oxidative modification of LDL by arterial smooth muscle cells was dependent on L-cystine in the medium, and they proposed that cellular oxidation of LDL depended on cellular production of thiols. A very similar proposal has been done by Sparrow and Olszewski [9] in LDL oxidation by aortic endothelial cells and by peritoneal macrophages. They identified the major thiol compound appearing in the medium as L-cysteine and suggested that L-cystine in the medium is its source.

Thiols are traditionally considered antioxidants. However, they can act as pro-oxidants, especially in the presence of transition metal ions [26]. Actually the addition of millimolar concentrations of thiols to F-10, which contained Fe^{2+}, caused LDL oxidation without cells [20]. The exact mechanism of thiol-dependent oxidation of LDL remains obscure. However, metal ions in the reduced state are important mediators of LDL oxidation [7]. Thiols most likely aid the propagation of oxidative modification of LDL by generating the reduced metal ions from the oxidized form, particularly Fe^{3+}, which is not reduced by LDL alone [27].

The mechanism by which cells generate thiols has been extensively studied in cultured human fibroblasts [11,12,28]. They take up L-cystine in the culture medium, the L-cystine is immediately reduced to L-cysteine in the cells, and the L-cysteine thus formed is released from the cells into the medium. Since L-cysteine in the medium is sensitive to autoxidation and readily changes back to L-cystine, we can see a redox cycle of L-cystine and L-cysteine across the plasma membrane. In human fibroblasts L-cystine is transported into the cells almost exclusively through the cystine-glutamate exchange system, or System x_c^-. The driving force of this exchange seems to be a steep concentration gradient of L-glutamate (inner high), and the excess amount of L-glutamate outside the cell competitively inhibits the uptake of L-cystine and also blocks the generation of thiols [12,28]. It has been reported that aortic endothelial cells can generate thiols, identified as those of L-cysteine, and oxidatively modify LDL in the presence of metal ions [9]. The excess L-glutamate potently inhibited these processes, suggesting that the endothelial cells have an activity of System x_c^- which is involved in the thiol generation. System x_c^- occurs in most mammalian cell types in culture and is the major transport system for L-cystine [29]. However, the activity of System x_c^- was not found in many cell types in vivo, including macrophages, endothelial cells and hepatocytes, although it was induced soon after culture [29]. Supposing that cellular oxidation of LDL is accounted for exclusively by the cell-dependent production of thiols in the media, all cell types so far found to modify LDL would have System x_c^- activity. Oxidation of LDL has been shown to be mediated in vitro by various cell types including macrophages [9,10], smooth muscle cells [8] and endothelial cells [9,30]. These experiments were performed with cultured cell lines or cells isolated from animals and cultured for about half a day or longer. Therefore the above assumption may be reasonable because System x_c^- activity is ubiquitous in cultured cell lines and is sufficiently induced in the latter cells.

As shown here the induction of System x_c^- activity upon culture is rather weak in peritoneal macrophages but profoundly enhanced in the presence of LPS. As a result of the induction supply of cystine to the cells was enhanced and the intracellular concentration of GSH increased [15]. We measured GSH/GSSG status in these cells and found that almost all glutathione occurred as GSH and the content of the oxidized form GSSG was negligibly low in both LPS-pretreated and non-treated macrophages [15]. Thus pretreatment of macrophages with LPS does not alter GSH/GSSG status significantly, and L-cystine taken up by the cells is thought to be rapidly reduced to L-cysteine in the cells by GSH and its regeneration system.

One of the important points in LDL oxidation by macrophages is the uptake of the modified LDL by the macrophages

and what will happen in these macrophages. Leake and Rankin [23] clearly demonstrated that LDL oxidatively modified by macrophages can be taken up by other macrophages via scavenger receptors. The macrophages that modified the LDL were obviously damaged, probably due to cytotoxic effect of the oxidized LDL taken up. These macrophages were incubated in a serum-free medium in order to modify LDL, and this seemed to enhance the cytotoxicity because in the presence of serum macrophages did not appear to be damaged by such LDL, resulting in large-scale cholesterol ester accumulation.

An important and unsolved question is whether macrophages can oxidatively modify LDL in vivo. The present experiments suggest that resting macrophages are unable to do this, but once activated by endotoxins or by TNF-α as an autocrine regulator they are competent to modify LDL. Macrophage-mediated oxidation of LDL shown here was carried out in the presence of 1 μM $FeSO_4$. Because physiological concentration of free iron is considered much lower, such oxidation may not occur in vivo. However, in some cases, i.e. in erythrophagocytosis by tissue macrophages, concentrations of iron in these cells may be increased by lysosomal degradation of phagocytosed heme proteins and the excretion of iron from the cells has been suggested [31]. Intracellular status of iron is a critically important factor for the oxidation of LDL by phagocytes and remains to be investigated.

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