Components of the protein fraction of oxidized low density lipoprotein stimulate interleukin-1α production by rabbit arterial macrophage-derived foam cells

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Abstract Oxidized low density lipoproteins (oxLDL) (0.5–50 μg/ml) generated from both rabbit and human LDL stimulated the production of interleukin-1α (IL-1α) by as much as 2- and 6-fold, respectively, as compared to native LDL after a 2-h incubation with macrophage-derived foam cells isolated from the balloon-injured arteries of cholesterol-fed rabbits. Northern blot analyses confirmed that there was also an increase in the mRNA for IL-1α and IL-β in response to oxLDL in the isolated foam cells. The stimulation of IL-1 expression and production was not due to the contamination of the oxLDL preparations with endotoxin as neither the amount of endotoxin found to be associated with the lipoproteins nor amounts up to 1 ng/ml stimulated IL-1α production to the same degree as oxLDL. Neither oxidized β-very low density lipoprotein (VLDL) nor oxidized high density lipoprotein (HDL) stimulated IL-1α production by the foam cells. Furthermore, acetyl-LDL had a very limited stimulatory effect, but other known ligands of the scavenger receptor such as maleylated-BSA, polyinosinic acid, and fucoidin elicited maximal IL-1α responses. Fractionation of the oxLDL into lipid- and protein-soluble fractions showed that there was some stimulatory activity in the lipid phase but that known products of lipid peroxidation such as 9- and 13-HODE had no effect when added independently of lipoproteins. When added in combination with native LDL, only 13-HODE stimulated IL-1α production. The delipidated apolipoprotein fragments of oxLDL that had been solubilized in β-octylglucoside stimulated the production of IL-1α by the foam cells to a greater degree than the lipid extract, while reductively methylated oxLDL did not. These data suggest that interactions of components of both the lipid- and protein-soluble fractions of oxLDL with scavenger receptors or potentially with surface proteins that bind oxLDL may induce production of IL-1 by arterial macrophages.–**Lipton, B. A., S. Parthasarathy, V. A. Ord, S. K. Clinton, P. Libby, and M. E. Rosenfeld.** Components of the protein fraction of oxidized low density lipoprotein stimulate interleukin-1α production by rabbit arterial macrophage-derived foam cells. *J. Lipid Res.* 1995. **36:** 2232–2242.

Supplementary key words atherosclerosis • cholesterol-fed rabbit

Macrophages are considered to be one of the major cell types involved in both the initiation and progression of atherosclerotic lesions (1). Morphologic studies have demonstrated that circulating monocytes become attached to specific regions of the endothelium with subsequent migration into the subendothelial space. Once there, monocytes differentiate into macrophages and accumulate large amounts of lipid becoming further transformed into foam cells. Perhaps as a result of the accumulation of this lipid, most likely in the form of modified lipoproteins, macrophages may be activated to release a variety of growth regulatory molecules such as cytokines, proteolytic enzymes, and growth factors.

Recent studies have shown that arterial macrophages within atherosclerotic lesions may be capable of secret-

Abbreviations: oxLDL, oxidized low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IL-1, interleukin-1; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; NZW, New Zealand White; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; moLDL, mildly oxidized LDL; RM, reductively methylated; mal-BSA, maleylated-bovine serum albumin; poly I, polyinosinic acid; PS, phosphatidylserine; LPS, lipopolysaccharide; RIA, radioimmunoassay.

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ing inflammatory mediators such as monocyte chemotactic protein-1 (MCP-1) (2), platelet-derived growth factor (3), macrophage-colony stimulating factor (4, 5), and lipoprotein lipase (6). The local production of such factors may play a role in the atherogenic process by regulating cellular proliferation, differentiation, and lipoprotein metabolism of vascular cells and infiltrating monocytes.

There is also evidence that suggests that local production of cytokines by cells within the vessel wall could contribute to the pathogenesis of atherosclerosis (7). In this regard, interleukin-1α and β (IL-1α and IL-1β) are cytokines produced by vascular cells including macrophages, in response to various stimuli associated with immune and inflammatory events. Studies conducted in vitro have shown that IL-1 acts to modulate processes such as low density lipoprotein (LDL) metabolism (8–10), vascular smooth muscle cell proliferation (11–13), and leukocyte adherence to the endothelium (14–16), all of which may be involved in the pathogenesis of atherosclerosis.

The stimulatory triggers for IL-1α production by arterial macrophages remain unclear. In the setting of atherosclerosis, lipoproteins represent one possible modulator of local cytokine production. Recent studies have shown that oxidized LDL (oxLDL) increases IL-1β secretion in human peripheral blood monocytes (17) and in zymosan-primed mouse peritoneal macrophages (18). In contrast, other in vitro studies indicate that oxidized LDL inhibits the lipopolysaccharide-induced expression of IL-1β (19) and tumor necrosis factor-α (TNF-α) (20) mRNA in stimulated mouse peritoneal macrophages. As the study of peritoneal macrophages and monocytes may not reflect responses by macrophage-derived foam cells present in the vessel wall during atherogenesis, we have developed techniques for isolating large numbers of purified, macrophage-derived foam cells from balloon-injured, cholesterol-fed rabbits (21). Previous studies have shown that these cells can produce large amounts of IL-1α, especially after stimulation with lipopolysaccharide (B. A. Lipton, S. Parthasarathy, V. A. Ord, S. K. Clinton, P. Libby, and M. E. Rosenfeld, unpublished observations). These in vitro studies showing inducible IL-1α expression in rabbit foam cells are further supported by in vivo studies in rabbits documenting significantly enhanced induction of IL-1 gene expression in fatty streaks compared to normal aortic tissue after lipopolysaccharide administration (22, 23). To further understand the events surrounding cytokine production by macrophages in the artery wall, we have examined the effect of lipoproteins on IL-1α production by rabbit arterial foam cells.

TABLE 1. Effects of OxLDL on IL-1 production by rabbit macrophage-derived foam cells

Addition	IL-1α (pg/2.5 × 10^5 cells/24 h)
No addition	466.5 ± 55.7
Rabbit LDL	438.2 ± 60.3
Rabbit oxidized LDL	958.2 ± 200.9
Human LDL	474.7 ± 41.9
Human oxidized LDL	2750.0 ± 265.7

Native or oxidized rabbit or human LDL (50 μg/ml) was incubated with cultured cells for 2 h. The cultures were refed with fresh medium and incubated for an additional 22 h. The data shown represent the total production of IL-1 (i.e., both cell-associated and secreted) during the observation period. Values are means ± SEM from triplicates of four separate experiments.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and Opti-MEM I medium were purchased from Gibco Laboratories (Grand Island, NY). Ham's F-10 medium, *E. coli* lipopolysaccharide (LPS; serotype 026.B6) and n-octyl β-D-glucopyranoside (β-octyl glucoside) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum (FCS) was obtained from Irvine Scientific (Santa Ana, CA) and was heat inactivated before use (56°C for 30 min). The linoleate oxidation product, 9-hydroxyoctadecadienoic acid (9-HODE), was purchased from Cayman Chemical (Ann Arbor, MI). The product, 13-HODE, was generated by the oxidation of linoleic acid with soybean lipoxygenase and was verified by high performance liquid chromatography (24).

Cells

Rabbit arterial macrophage-derived foam cells were isolated as previously described (21). Briefly, atherosclerotic lesions were induced by balloon de-endothelialization of the entire aorta and iliac arteries of New Zealand White (NZW) rabbits with a Fogerty embolectomy catheter (4F) followed by feeding a 2% cholesterol (w/w) chow diet for 10–11 weeks. Foam cells were released from finely minced arterial tissue using collagenase and elastase digestions and purified using discontinuous metrizamide gradient centrifugations. After isolation, the cells were placed in culture with Opti-MEM I medium (Gibco) containing 0.5% heat-inactivated fetal calf serum and 20 μg/ml gentamicin at a density of 0.25 × 10^6 cells/well. The purity of the macrophage-derived foam cells was verified immunocytochemically. All solutions and buffers used in these experiments were evaluated for endotoxin with the Limulus amoebocyte lysate assay (Whittaker, MA) and contained less than 50 pg/ml.

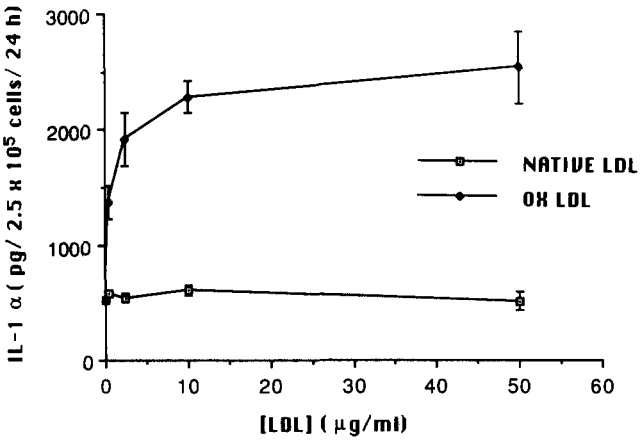

Fig. 1. Response of arterial macrophage-derived foam cells to increasing concentrations of human native and oxidized LDL. The cells were stimulated for 2 h with the lipoproteins, washed, and refed with fresh medium and the total IL-1α (both cell-associated and secreted) was measured 22 h later. Values shown are the means plus and minus the standard error of the mean of duplicates from three separate experiments.

Lipoproteins

LDL (d 1.019–1.063 g/ml) was isolated by preparative ultracentrifugation from fresh human and rabbit plasma collected in EDTA (1 mg/ml). Beta-VLDL was obtained from the plasma of hypercholesterolemic rabbits by centrifugation for 15 h at 50,000 rpm in a Ti 60 rotor. All lipoprotein samples were dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/9.5 mM phosphate, pH 7.4) containing 0.01% EDTA at 4°C. Residual EDTA was removed by dialysis against PBS without EDTA before modification. All lipoprotein concentrations added to the cells were determined on the basis of protein content of the lipoproteins.

LDL oxidation

LDL (100 μg/ml) was incubated with 10 μM copper acetate in Ham's F-10 medium (2 ml) at 37°C for 18 h in 60-mm plastic dishes (26, 27). The medium containing the modified LDL was harvested and an aliquot was used to measure lipid peroxidation as described below. Lipids from the remaining medium were extracted by the method of Bligh and Dyer (28). The protein component was dissolved in 6 mg/ml β-octylglucoside solution as described (29). The recovery of solubilized protein was calculated using ^{125}I-labeled oxLDL in parallel under identical conditions. The recovery varied between 20–40% of the initial oxLDL protein used. The extent of lipid peroxidation was determined by quantitation of thiobarbituric acid-reactive substances (TBARS) (30). Tetramethoxypropane was used as standard and results are expressed as nmol equivalents of malondialdehyde (MDA). The average amount of TBARS generated for the five different human oxidized LDL preparations used in this study was 69 nmol MDA equivalents/mg LDL protein and 31 nmol MDA equivalents/mg protein for the three rabbit oxidized LDL preparations. This difference in the degree of oxidation of the rabbit and human LDL may in part reflect differences in the fatty acid composition of the LDL. Normal NZW rabbit LDL has a fairly high percentage of oleic acid in the cholesteryl esters and phospholipids (about 25%) which may limit the oxidizability of these particles (31). Mildly oxidized LDL (moLDL) was prepared with the human LDL by stopping the reaction with 50 μM BHT after only 30–45 min of incubation. The TBARS generated from the moLDL was less than 5 nmol MDA equivalents/mg protein.

Acetylated LDL was prepared as described by Basu et al. (32). The modification of LDL was examined by measuring the electrophoretic mobility of the lipoproteins on agarose gels. LDL was reductively methylated as described by Weisgraber, Innerarity, and Mahley (33).

Measurement of IL-1α production by cultured cells

IL-1α production in response to stimuli was evaluated in cultured rabbit arterial foam cells. The focus on IL-1α production by the foam cells as opposed to IL-1α and IL-1β is based on previous observations that rabbit cells (foam cells as well as alveolar macrophages and monocytes) have more than a 10-fold greater content of IL-1α than IL-1β (data not shown). [In contrast, IL-1β is the predominant form found associated with human monocytes as measured using a radioimmunoassays specific for human IL-1α and IL-1β (Collaborative Research Inc., Bedford, MA)]. Cultures were treated for 2 h at 37°C with the stimulatory agents added directly to the culture medium. After incubation, media were collected and the cells were provided with fresh medium and incubated for up to an additional 22 h. At designated times, both conditioned media and cell monolayers were collected for assay of IL-1α. Cells were collected by scraping with a rubber policeman in 1 ml of PBS containing 2 mM phenylmethylsulfonyl fluoride and 0.67 inhibitory units/ml of aprotinin as protease inhibitors to prevent enzymatic degradation of IL-1 and stored at -70°C until assayed. A radioimmunoassay (RIA) specific for rabbit IL-1α (Cytokine Sciences, Inc., Boston, MA) was used to quantitate IL-1 levels. For quantitating intracellular IL-1, cell samples were subjected to three freeze–thaw cycles and clarified by centrifugation prior to assay. Fresh preparations of foam cells and oxLDL were utilized for each set of different experiments included in these studies. This may account for the variation obtained in the absolute amounts of IL-1 produced in the different sets of experiments.

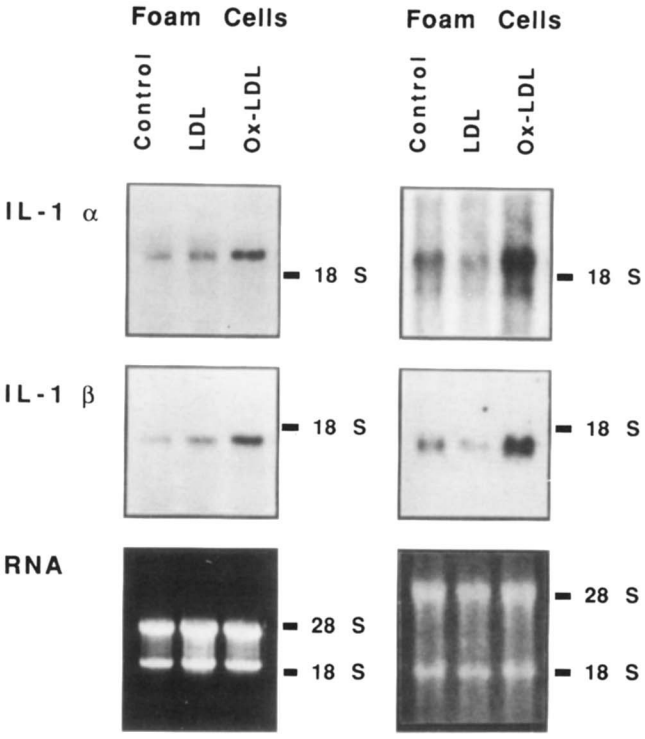

Fig. 2. Northern blot analyses of mRNA isolated from two separate preparations of the arterial macrophage-derived foam cells 3 h after incubation with 25 μg/ml of either native or oxidized human LDL. The blots were hybridized with cDNA probes specific for rabbit IL-1α and IL-1β as described in Methods. Bottom panels, ethidium bromide staining.

Northern blot analysis of IL-1 gene expression

Cultures of macrophage-derived foam cells were treated for 3 h at 37°C with native or oxidized human LDL (25 μg/ml) or with no addition. After the incubation, total cytoplasmic RNA was isolated as described by Chomczynski and Sacchi (34). Northern blotting used 20 μg of total cellular RNA applied to each lane, separated on a 1.2% agarose–formaldehyde gel and transferred to a nylon membrane (Amersham Hybond-N, Arlington Heights, IL). The rabbit IL-1α and β cDNA probes were used as previously described (35). Hybridization and autoradiography used standard techniques (36).

Other procedures

Protein was measured by the method of Lowry et al. (37) using albumin as a standard. In efforts to assess the functional integrity and cell viability of macrophage-derived foam cells after exposure to oxidized LDL, adherent cells were assayed for their ability to phagocytose latex beads (1 μm diameter, Sigma Chemical Co., St. Louis, MO) as well as assaying the conditioned culture medium for lactate dehydrogenase (Sigma Chemical Co.) according to the method of Wroblewski and LaDue (38).

RESULTS

Previous studies in our laboratory have shown that freshly isolated rabbit arterial macrophage-derived foam cells from ballooned, cholesterol-fed animals produce IL-1α and IL-1β mRNA and protein that is enhanced by LPS stimulation. Within the first few hours post stimulation with LPS, the majority of the IL-1 is cell associated, but by 24 h post stimulation greater than 80% is secreted into the culture medium (B. A. Lipton, S. Parthasarathy, V. A. Ord, S. K. Clinton, P. Libby, and M. E. Rosenfeld, unpublished observations). Because vascular cells including macrophages may oxidatively modify plasma lipoproteins that infiltrate the vessel wall (21), additional studies were designed to determine whether oxidized lipoproteins influence IL-1 production in the arterial foam cells in a fashion analogous to LPS. **Table 1** shows that both oxidized rabbit and oxidized human LDL stimulated production of immunoreactive IL-1α in macrophage-derived foam cells by 2- and nearly 6-fold, respectively, whereas neither native rabbit nor native human LDL had any significant effect. These data represent the total IL-1α content, i.e., both cell-associated and secreted IL-1α. The response to rabbit oxidized LDL was less than that to human oxidized LDL which may be due to the lesser extent of oxidation of the former, as indicated by the lower TBARS value (31 nmol MDA/mg LDL protein versus 69 nmol MDA/mg LDL protein). Similar increases in IL-1α production by the foam cells were observed with endothelial cell-modi-

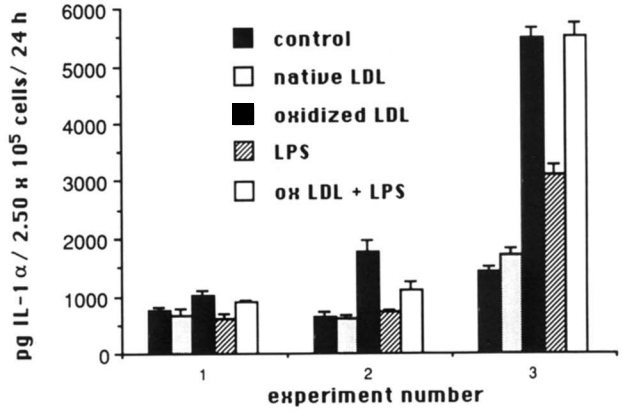

Fig. 3. Comparison of the effects of LDL, oxLDL, and endotoxin (LPS) on total IL-1α production by three separate preparations of the arterial macrophage-derived foam cells. The cells were incubated with either non-conditioned culture medium (Opti-MEM), the lipoproteins (50 μg/ml), the amount of endogenous LPS measured in each lipoprotein preparation (experiment 1, 10 pg/ml; experiment 2, 200 pg/ml), 1 ng/ml which was previously shown to elicit a maximal LPS stimulation of IL-1 production by the foam cells, (experiment 3), or a combination of the lipoproteins plus LPS for 2 h. The cultures were washed and refed with fresh medium and both cell-associated and secreted IL-1α were measured 22 h later. Values shown are the means plus and minus the standard error of the mean of triplicates for each condition.

TABLE 2. Effects of β-VLDL, HDL, and mildly oxidized-LDL on IL-1α production by rabbit arterial foam cells

Addition	Concentration	IL-1α[a]
	μg/ml	
None		263 ± 14
β-VLDL	10	234 ± 14
	25	237 ± 12
Oxidized β-VLDL	10	277 ± 2
	25	304 ± 4
HDL	10	237 ± 3
	25	252 ± 3
Oxidized HDL	10	260 ± 4
	25	254 ± 5
LDL	10	237 ± 12
	25	214 ± 17
Mildly oxidized LDL	2.5	159 ± 15
	25	149 ± 24
Oxidized LDL	10	547 ± 18[b]
	25	657 ± 7[b]

Cells were incubated for 2 h at 37°C with the indicated additions. The medium was then removed and the cultures were refed with fresh medium for an additional 22 h. The values represent the mean and standard error of triplicates from two separate experiments.

[a]Values for IL-1α are pg/2.5 × 10^5 cells/24 h.

[b]$P < 0.05$ as compared to the respective concentration of LDL alone.

fied rabbit LDL (data not shown). The concentration dependency of the increased IL-1α response of the rabbit foam cells to human oxLDL was examined. **Figure 1** shows that arterial foam cells produce greater amounts of IL-1α in response to increasing concentrations of human oxidized LDL. As observed in our studies with LPS, the initial increase in IL-1α is cell-associated followed by slow release during the ensuing 24 h (by 24 h the average cell-associated content of IL-1α was 60% of the total with a range between 40 and 83% in five different preparations of the foam cells). The results also show that incubation with concentrations as low as 0.5 μg/ml oxLDL for 2 h results in over a 2-fold increase in IL-1α production by the foam cells as compared to native LDL or no addition. Higher concentrations of oxLDL (up to 50 μg/ml) increased IL-1α production in the same cell preparation by nearly 5-fold without affecting cell viability (there was no increase in the release of lactate dehydrogenase activity; data not shown). In support of the above observations, Northern blot analyses of the mRNA isolated from foam cells treated with oxidized LDL demonstrated an increased expression of IL-1α and IL-1β in these cells within 3 h post-stimulation as compared to control or native LDL-treated cells (**Fig. 2**).

As lipoproteins can bind lipopolysaccharides (LPS) (39, 40), we investigated the possibility that the observed IL-1α response to oxidized LDL might be due to endotoxin contamination. In a series of experiments, LPS concentrations equivalent to that determined in each of the lipoprotein preparations used in these studies or 1 ng/ml, which was previously shown in dose-response studies to induce a maximal response (B. A. Lipton, unpublished observations) were added alone or in combination with native or oxidized LDL. The results shown in **Fig. 3** indicate that the IL-1α produced in response to oxidized LDL was much greater than that induced by concentrations of endotoxin found in the lipoprotein preparations, suggesting that any low level of LPS contamination in the LDL was not responsible for the majority of IL-1α produced in response to oxidized LDL. Furthermore, the response to oxidized LDL was always greater than that to LPS, even at concentrations of 1 ng/ml, and no significant synergistic effect on IL-1α production was observed when oxidized LDL and LPS were added in combination (even at 1 ng/ml LPS). In all experiments, care was taken in the preparation of the LDL to minimize any LPS contamination, and the same preparations of LDL used as the controls were also used to make oxidized LDL, acetyl-LDL, and reductively methylated oxidized LDL, showing that the conditions utilized to modify the LDL did not introduce endotoxin into the system.

Further studies examined whether other plasma lipo-

TABLE 3. Effects of scavenger receptor ligands on IL-1α production by rabbit arterial foam cells

Addition	Concentration	IL-1α[a]
	μg/ml	
None		355 ± 3
Acetyl-LDL	0.5	380 ± 13
Acetyl-LDL	5	421 ± 30
Acetyl-LDL	20	429 ± 15
Maleylated BSA	10	666 ± 41[b]
Polyinosinic acid	25	1076 ± 86[b]
Polyguanylic acid	25	375 ± 100
Polyadenylic acid	25	309 ± 147
Polycytidylic acid	25	604 ± 253
Fucoidin	25	932 ± 219[b]
Oxidized LDL	0.5	384 ± 12
Oxidized LDL	5	455 ± 41
Oxidized LDL	20	705 ± 14[b]

Cells were incubated for 2 h at 37°C with the indicated additions. The medium was then removed and the cultures were refed with fresh medium for an additional 22 h. The values represent the mean and standard error of duplicates from at least two separate experiments.

[a]Values for IL-1α are pg/2.5 × 10^5 cells/24 h.

[b]$P < 0.05$ as compared to no addition.

TABLE 4. Effects of lipid extract and peroxidation products of oxidized LDL on IL-1α production by rabbit arterial foam cells

Addition	Concentration	IL-1α[a]
	μg/ml	
None		469 ± 32
9-HODE alone	10	554 ± 66
13-HODE alone	10	469 ± 35
Linoleic acid (18:2)	1	386 ± 38
Lipid extract of oxLDL	10	567 ± 42
Lipid extract of oxLDL	50	755 ± 29[b]
Oxidized LDL	50	1520 ± 55
Separate set of experiments		
None		210 ± 51
Native LDL	50	162 ± 16
9-HODE + LDL	10/50	229 ± 54
13-HODE + LDL	10/50	406 ± 150
9-HODE alone	10	123
13-HODE alone	10	106

Cells were incubated as previously described. Rabbit oxidized LDL was used in these studies. The concentration used for linoleic acid and 9- and 13-HODE is equivalent to 33 μM. The total lipid extract used in these studies was that obtained from 10 and 50 μg protein of oxidized LDL, respectively. The values represent the mean and standard error of triplicates from three separate experiments.

[a]Values for IL-1α are pg/2.5 × 10^5 cells/24 h.

[b]$P < 0.05$ as compared to no addition.

proteins affected IL-1α production by the arterial foam cells. The results in **Table 2** indicate that exposure of the foam cells to freshly isolated rabbit β-VLDL, the predominant lipoprotein fraction of cholesterol-fed rabbits, or human HDL did not affect IL-1α production by these cells as compared to that of no addition. Furthermore, the addition of mildly oxidized human LDL (moLDL) (TBARS < 5 nmol MDA equivalents/mg protein) also had no stimulatory effect and may have actually inhibited IL-1α production by the foam cells (Table 2). However, because BHT was used to stop the reaction and prevent additional oxidation of the moLDL, it is not clear whether there is an independent inhibitory effect of the BHT. After subjecting the β-VLDL and HDL fractions to copper-ion modification, oxidized β-VLDL (94.3 nmol MDA/mg protein) increased IL-1α production in the foam cells only 10–20% versus native β-VLDL (6 nmol MDA/mg protein) at both concentrations examined while oxidized HDL (31 nmol MDA/mg protein) had no effect. In contrast, 10 and 25 μg/ml concentrations of oxLDL increased IL-1α production by 2.3- and 3-fold versus native LDL, respectively, in the same preparation of foam cells.

Additional studies showed that incubation of the foam cells with increasing concentrations of acetyl-LDL (0.5–20 μg/ml) only slightly stimulated (20%) IL-1α production while similar concentrations of oxidized LDL, prepared from the same human LDL, again resulted in a 2-fold increase in IL-1α production in the foam cells (**Table 3**). In contrast to acetyl-LDL, maleylated-BSA (10 μg/ml), polyinosinic acid (25 μg/ml), and fucoidin (25 μg/ml), all classical ligands of the scavenger receptor (41), stimulated IL-1α production by up to 4-fold in the foam cells. Interestingly, polyguanylic acid, which is also a known ligand of the scavenger receptor, did not stimulate IL-1α production by the foam cells. As the acetyl-LDL was prepared from the same human LDL stock solution used to prepare oxLDL, these results again suggest that the effect of oxLDL on IL-1α production is not due to endotoxin contamination but rather is a response that is specific to the oxidized LDL particle.

Localization of the stimulatory component of oxLDL

Subsequent studies sought to determine which component(s) of oxidized LDL account for the IL-1α response observed in these studies. Our initial efforts focused on the lipid component of the lipoprotein as substantial amounts of lipid peroxidation products are generated during copper-ion modification of LDL, some of which dissociate from the LDL particle (26). In this regard, we examined the effect of linoleic acid (18:2) on IL-1α production by the foam cells, and of two of its peroxidation products, 9- and 13-HODE, as well as the total lipid fraction extracted from some of the oxidized LDL preparations. The results shown in **Table 4** indicate that linoleic acid and 9- and 13-HODE, when added

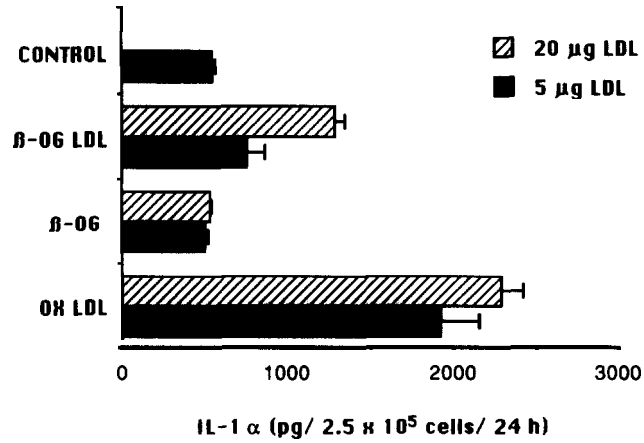

Fig. 4. Effects of the protein components of oxLDL on total IL-1α production by the arterial macrophage-derived foam cells. Delipidated oxLDL was solubilized in 6 mg/ml of β-octylglucoside (β-OG LDL). The concentrations of β-OG LDL were estimated from recovery studies as described in Methods. The cells were incubated for 2 h with the indicated concentrations of oxLDL, β-OG LDL, or β-OG alone followed by washing and refeeding with fresh medium. Both the cell-associated and secreted IL-1α were measured 22 h later. Values shown are the means plus and minus the standard error of the mean of triplicates from two separate experiments.

independently, had little or no effect on IL-1α production in the foam cells. However, when 9- and 13-HODE were added to the foam cells in combination with native LDL, there was some stimulation by the 13-HODE/LDL combination but not by the 9-HODE/LDL. In addition, the total lipid fractions of human oxidized LDL preparations increased foam cell IL-1α production up to approximately 60% at the highest levels examined.

The possibility that the stimulatory activity of oxLDL also resides in the protein-soluble fraction was investigated. After delipidation, the apolipoprotein component of oxLDL from several different preparations was solubilized in β-octylglucoside as described in Methods. The results of these studies indicate that incubation of the foam cells with the solubilized apolipoprotein of oxLDL alone significantly increases IL-1α production in these cells (**Fig. 4**). This effect is not due to the β-octylglucoside itself as incubation of the cells with comparable volumes of the detergent had no effect on IL-1α levels.

Further investigation of the above observations was conducted using reductively methylated (RM) human LDL which was then oxidized. Reductive methylation derivatizes 90% of lysine residues, preventing much of the lipid–protein conjugation that normally occurs, but allowing lipid oxidation to proceed (39) (control LDL, 59 nmol MDA/mg protein; RM-LDL, 46 nmol MDA/mg protein, average of two experiments). The results in **Fig. 5** show that oxidized, reductively methylated LDL increased IL-1α production in the foam cells but by only 30–60% versus control. Oxidized LDL at similar concentrations again stimulated IL-1α production by 2- to 2.5-fold in the same foam cell preparation. These data further support our observations that part of the stimulatory activity of oxLDL involved in the IL-1α response of the foam cells lies in components associated with the apoprotein fraction of the LDL particle.

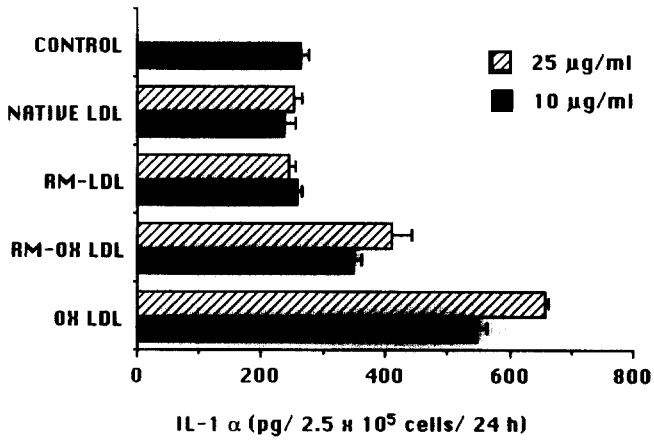

Fig. 5. Effects of reductive methylation of LDL and oxLDL on the production of total IL-1α by the arterial macrophage-derived foam cells. LDL was reductively methylated (RM-LDL) as described (32) and, after oxidation (RM-OX LDL), was added to the cells in the concentrations indicated above for 2 h. The cells were washed, refed with fresh medium, and both the cell-associated and secreted IL-1α were measured 22 h later. Values shown are the means plus and minus the standard error of the mean of triplicates from two separate experiments.

DISCUSSION

The oxidative modification of LDL may play an important role in the pathogenesis of atherosclerosis (40–45). One property of oxidized LDL is its capacity to interact with scavenger receptors on macrophages which may lead to unregulated cholesteryl ester deposition and the formation of foam cells. Oxidized LDL has also recently been shown to effect various other aspects of macrophage function such as chemotaxis (46), cholesterol esterification (47), and secretion of vasoactive substances (48). Thus, one might postulate that oxLDL may also influence IL-1 expression by foam cells. The present studies show that oxLDL stimulates both IL-1α mRNA and protein expression in arterial macrophage-derived foam cells isolated from balloon-injured, cholesterol-fed rabbits. Several previous in vitro studies have provided contradictory evidence regarding the effects of oxLDL on IL-1β production by macrophages. Fong, Fong, and Cooper (19) have shown that oxLDL inhibits the expression of IL-1β in LPS-stimulated mouse peritoneal macrophages while Ku and Jackson (17) and Ku et al. (18) have observed an increase in IL-1β secretion in human blood monocytes and mouse peritoneal macrophages in response to oxLDL. The disparate effect of oxidized LDL on IL-1 expression by macrophages may be due to a number of factors. For example, the source and properties of the macrophages used in the current and cited studies differ (rabbit arterial, mouse peritoneal, human blood monocyte-macrophages), and clearly, the foam cells used in the present studies contain massive intracellular lipid stores (21) as compared to the non-lipid loaded cells used in the other studies. It is also possible that arterial foam cells may express components (receptors, proteins) that are not present in resident peritoneal, monocyte-derived, or cultured macrophages. Arterial foam cells may have undergone alternate pathways of differentiation or activation (49), perhaps due to exposure to particular signals found in the microenvironment of the artery wall. More importantly, the extent of oxidation of LDL used in the studies may also differ. This later possibility has been nicely demonstrated by Thomas et al. (50) where LDL oxidation was controlled by varying the ratio of the copper to LDL concentrations. LDL of increasing degrees of oxi-

dation were more stimulatory of IL-1β release by human mononuclear cells.

The present studies also focused on determining whether the stimulatory effects of oxLDL in increasing IL-1α production in the foam cells were due entirely to the oxidized lipid components of the oxLDL particles. In this regard, our data suggest that the extractable lipid components of oxLDL are only partially responsible for eliciting the observed effects on IL-1α production by the foam cells. For example, 9- and 13-HODE, lipid peroxidation products generated during oxidative modification (26), have been shown to affect IL-1β expression in a stimulatory fashion (50, 51). In isolated arterial macrophage-derived foam cells, 9- and 13-HODE themselves do not greatly increase IL-1α production. This may be due to the higher endogenous levels of these products present in the foam cells (S. Parthasarathy, unpublished results) or due to poor uptake of HODEs by these cells. This latter possibility is supported by our observation that addition of 13-HODE to native LDL causes a modest stimulation of IL-1α production. In most of the current experiments, extensively oxidized LDL (TBARS ~70 nmol/mg LDL protein) was used. It is possible that in these oxidized LDL preparations, the HODEs have undergone further decomposition into carbonyl compounds (52), thus providing below threshold amounts of HODEs to the foam cells. This, however, seems unlikely as authentic 9- and 13-HODE also did not induce any IL-1 secretion. It is also possible that the HODE products simply diffused into a storage pool of lipids in the foam cells and were unable to exert any cellular effect. A similar lack of effect of HODEs on IL-1 production has also been noted in acetyl-LDL-loaded mouse peritoneal macrophages (C. E. Thomas, personal communication). However, it is important to note that total lipids extracted from oxLDL also had a modest stimulatory effect on IL-1α production by the foam cells.

The present data with reductively methylated oxidized LDL and oxidized β-VLDL provide additional evidence that rules against oxidized lipids that are not covalently bound to protein as being solely responsible for stimulating IL-1α expression in the foam cells. However, additional experimentation such as dialysis of the oxLDL or addition of oxidation products to purified apolipoprotein B is required to help delineate between effects of free aldehydes and covalently bound products of oxidation. This type of experiment was used by Thomas et al. (50) to convincingly demonstrate that most of the stimulatory activity for IL-1β release from human mononuclear cells resides in free aldehydes that can be removed by dialysis.

Despite having comparable degrees of oxidation as oxLDL, neither reductively methylated oxLDL nor oxidized β-VLDL had a large effect on foam cell IL-1α production. Previous studies have shown that despite considerable oxidation, the degree of degradation of oxidized β-VLDL by macrophages is at best doubled (53). Therefore, it is not surprising that oxidized β-VLDL is not as effective in stimulating IL-1α production in the foam cells as is oxidized LDL.

To our knowledge, this is the first report that indicates that components of the protein-soluble fraction of oxLDL can stimulate a biological response. This conclusion is based on the observation that octyl-glucoside-solubilized protein from oxidized LDL alone increased IL-1α production. This material has previously been shown to be recognized by the scavenger receptor of macrophages (29) and suggests that the observed IL-1α response may be a result of ligand–receptor interaction. Furthermore, oxidized, reductively methylated LDL had a reduced effect on IL-1α production, while maleylated-BSA (mal-BSA), polyinosinic acid (poly I), and fucoidin, known ligands of the scavenger receptor (41), greatly stimulated IL-1α production by the foam cells. A similar finding was reported by Palkama (54) that interleukin-1 production was induced by ligands such as polyinosinic acid binding to the scavenger receptor in human monocytes. Together, these observations further suggest that some component(s) of the protein-soluble fraction of oxidized LDL may be important in mediating the IL-1α response. To date, it is still unclear whether the effect is due to binding of peptides derived from apolipoprotein B or is due to covalently linked lipid-derived aldehydes that remain after delipidation of the oxLDL.

Curiously, these studies also show that the IL-1α response appears to discriminate between oxLDL and acetyl-LDL, as acetyl-LDL stimulated only a very modest IL-1α response in the foam cells. The acetyl-LDL preparations used in these studies showed an increase in electrophoretic mobility on agarose gels and were effective competitors for the degradation of ^{125}I-labeled acetyl-LDL by cultured macrophages, validating the preparation of the material. One possibility for the difference between oxidized LDL and acetyl-LDL may be that there is "differential signalling" induced by binding of these different ligands to different locations on the scavenger receptors. Alternatively, the IL-1α response may be elicited by binding of the stimulatory ligands to a separate oxLDL receptor (55). Sambrano, Parthasarathy, and Steinberg (56) and Sambrano and Steinberg (57) have recently shown that this oxLDL binding protein also recognizes oxidized red blood cells and has a high affinity for binding phosphatidylserine (PS) liposomes and red blood cell membranes enriched with PS. Ottnad et al. (55) have demonstrated via ligand blotting the presence of both a classic acetyl-LDL receptor (scavenger receptor) and a putative "oxidized LDL" binding protein in the rabbit arterial foam cells. In fact, the

isolated foam cells are, to date, one of the richest sources of this oxLDL binding protein (55). It is possible therefore, that ligands that primarily interact with the oxLDL binding protein (mol wt 94 kD) may elicit a stimulation in IL-1α production in the foam cells while the classic acetyl-LDL receptor may be incapable of stimulating this biological response. Lipopolysaccharide, poly I, and maleylated-BSA have been shown to be effective competitors of both oxLDL (58) and acetyl-LDL (41) degradation by macrophages. In addition, recent studies by Hampton, Golenbock, and Raetz (59) have shown with ligand blot analysis that labeled LPS binds to a 95 kD protein on macrophages as compared to the classical 210 kD trimer that binds acetyl-LDL. Thus, these studies suggest that there may be a family of macrophage receptors that recognize different types of modified lipoproteins, some of which may be capable of stimulating different biological responses. Future studies of the properties and functions of these receptors await their purification and cloning.

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