Stimulated Arachidonate Metabolism during Foam Cell Transformation of Mouse Peritoneal Macrophages with Oxidized Low Density Lipoprotein

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Abstract

Changes in arachidonate metabolism were examined in mouse peritoneal macrophages incubated with various types of lipoproteins. Oxidized low density lipoprotein (LDL) was incorporated by macrophages and stimulated macrophage prostaglandin E_2 (PGE_2) and leukotriene C_4 syntheses, respectively, 10.8- and 10.7-fold higher than by the control. Production of 6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin, was also stimulated. No stimulation was found with native LDL, which was minimally incorporated by the cells. Acetylated LDL and beta-migrating very low density lipoprotein (β-VLDL), though incorporated more efficiently than oxidized LDL, also had no stimulatory effect. When oxidized LDL was separated into the lipoprotein–lipid peroxide complex and free lipid peroxides, most of the stimulatory activity was found in the former fraction, indicating that stimulation of arachidonate metabolism in the cell is associated with uptake of the lipoprotein–lipid peroxide complex. These results suggest that peroxidative modification of LDL could contribute to the progression of atheroma by stimulating arachidonate metabolism during incorporation into macrophages.

Introduction

One of the initial events of atherosclerosis shown in animal experiments is the migration of circulating monocytes into the subendothelial space and their development into macrophages (1, 2). The intimal macrophages incorporate lipids into the cell body and eventually get converted into lipid-laden foam cells, which are characteristically found in the early stage of atheroma (2, 3). However, matured macrophages have only a limited number of classical low density lipoprotein (LDL) receptor, and take up native LDL at a very low rate, which is insufficient to cause foam cell transformation in vitro (1, 4, 5). Therefore investigators have been attracted to other mechanisms of incorporation and metabolism of lipoproteins in macrophages. These cells have been reported to efficiently incorporate beta-migrating very low density lipoprotein (β-VLDL)[1] (6) and VLDL from the Watanabe heritable hyperlipidemic rabbits, an animal model for familial hypercholesterolemia (7–9) via a specific receptor, the β-VLDL receptor. In addition, macrophages were also demonstrated to incorporate certain types of chemically modified LDL. This pathway was first shown for acetylated LDL (acetyl-LDL) and the receptor is called the acetyl-LDL receptor (4, 5). Although the discovery of this pathway appeared to explain the paradox of macrophages in atherogenesis, a naturally occurring modified form of LDL has not yet been identified in the body. Recently oxidized LDL, in which acyl chains are peroxidized, has been reported to be efficiently taken up by macrophages at least in part by way of acetyl-LDL receptor (10–12). Because lipid peroxidation has been suggested to be involved in atherogenesis by pathological or epidemiological investigation (13, 14), oxidized LDL is considered as a candidate for an example of naturally occurring, modified LDL.

While foam cell transformation of macrophages has been indicated to be one of the key events of atheroma formation, it has not been clarified yet whether it is only a process of accumulation of the lipid-laden cells or has some pathophysiological effects on the progression of atheroma. Macrophages release various biologically active substances when they are activated by phagocytic particles such as zymosan A or inflammatory stimuli such as lipopolysaccharides (15). Among those substances, the products derived from arachidonate metabolism have attracted increasing attention, since recent studies have indicated their close relation to atherogenesis (16–19). However, little is known yet about arachidonate metabolism during the process of receptor-mediated incorporation of lipoproteins by macrophages and foam cell transformation. Investigation on this line, therefore, would be of some value, inasmuch as it might give us a clue for determining the functional role of the foam cells as to whether they act as a promotor for atheromatous formation (20).

In this study we incubated mouse peritoneal macrophages with lipoproteins and examined production of arachidonate metabolites with gas chromatography-mass spectrometry (GC/MS) and radioimmunoassay (RIA). We report that oxidized LDL stimulates arachidonate metabolism in macrophages during its incorporation into the cells.

Methods

Materials

[5,6,8,11,12,14,15-^{3}H(N)]prostaglandin E_2 (PGE_2) (100–200 Ci/mmol), [14,15-^{3}H(N)]leukotriene C4 (LTC_4) (20–60 Ci/mmol),

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1. *Abbreviations used in this paper:* acetyl-LDL, acetylated LDL; β-VLDL, beta-migrating VLDL; GC/MS, gas chromatography-mass spectrometry; ir-PGE_2, immunoreactive PGE_2; MDA, malodialdehyde; TBARS, thiobarbituric acid-reactive substances.

[1-^{14}C]arachidonic acid (40–60 mCi/mmol), Na^{125}I (17.4 Ci/mg), and an LTC_4 ^{3}H-RIA kit were obtained from New England Nuclear (Boston, MA). Balb/3T3 clone A31 cells (catalog no. 03-415) were obtained from Flow Laboratories (Mclean, VA). Fetal calf serum (FCS) (catalog no. 14-5018) and calf serum (catalog no. 382-02) obtained from M. A. Bioproducts (Walkersville, MD) and the Research Foundation for Microbial Disease of Osaka University (Osaka, Japan), respectively, were heat-inactivated at 56°C for 30 min before use. Dulbecco's modified Eagle's medium (DME) and Dulbecco's phosphate-buffered saline (PBS) were purchased from Nissui Seiyaku (Tokyo, Japan). L-Glutamine and penicillin-streptomycin were obtained from Flow Laboratories (North Ryde, New South Wales, Australia) and Gibco Laboratories (Chagrin Falls, OH), respectively. Fucoidin, zymosan A, and cytochalasin B were obtained from Sigma Chemical Co. (St. Louis, MO). $CuSO_4$ and EDTA were obtained from Nakarai Chemicals (Kyoto, Japan). Standard PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and antiserum to PGE_2 were kind gifts from Ono Pharmaceutical (Osaka, Japan). Plastic petri dishes were obtained from Nunc (Roskilde, Denmark). A Lipoperoxide test kit was obtained from Wako Chemicals (Osaka, Japan). All other chemicals used were of reagent grade.

Cells

Macrophages. Peritoneal cells were harvested from unstimulated female DDY mice (25–30 g) in PBS as described by Edelson et al. (21) and Kita et al. (7). The peritoneal perfusates from 20–40 mice were pooled and the cells, 3–6 × 10^6 cells per mouse, were collected by centrifugation at 400 *g* at 4°C for 10 min. After being washed once with 30 ml of PBS, the cells were resuspended in the culture medium, DME containing 10% (vol/vol) FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml), at a final density of 3 × 10^6 cells/ml. Aliquots (1 ml) were dispersed on to plastic petri dishes (35 × 10 mm) and the cells were cultured in humidified air containing 5% CO_2 at 37°C. After 2 h, each dish was washed twice with 2 ml of DME without serum to remove nonadherent cells. After the cells were cultured for another 18 h at 37°C in 1 ml of the culture medium, they were washed with 1 ml of DME without FCS and used for the experiment. The experiment was initiated by adding various amounts of lipoproteins or other reagents in 1 ml of the culture medium, and incubation was carried out under conditions described above for the indicated time periods. Zymosan (80 mg) was boiled for 30 min in PBS and then incubated with 4 ml of mouse serum for 30 min at 37°C. After being washed twice with PBS, zymosan was finally resuspended in 4 ml of PBS and added to the culture medium to a final concentration of 50 μg/ml. Cytochalasin B, prepared as a stock solution of 1.0 × 10^{-2} M in dimethylsulfoxide (DMSO), was added to the culture medium to a final concentration of 1 × 10^{-4} M with lipoproteins. After the medium was removed from each dish at the end of incubation for determination of arachidonate metabolites, the cells were washed with 2 ml of PBS twice and received 1.0 ml of 0.2 N NaOH. After overnight incubation, 50-μl aliquots were used for measurement of protein contents with bovine serum albumin as a standard according to the methods of Lowry et al. (22).

3T3 cells. 3T3 cells were plated at a density of 9.6 × 10^4 cells/ml in 1 ml of DME containing 10% (vol/vol) calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) on plastic petri dishes (35 × 10 mm) and maintained in humidified air containing 5% CO_2 at 37°C. On day 4 of culture the cells were washed with 1 ml of DME without serum, and various amounts of lipoproteins in the culture medium were added. Measurements of arachidonate metabolites and cellular protein contents were performed as described for macrophages.

Lipoproteins

Plasma was obtained from 14-d fasting Japanese white rabbits (3.0–3.5 kg) prepared with EDTA as an anticoagulant (7, 9, 23, 24), and LDL (*d* = 1.019–1.063 g/ml) was isolated by ultracentrifugation (25) and dialyzed against two changes of at least 500 vol of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. β-VLDL was isolated from Japanese white rabbits fed a 2% cholesterol-10% corn oil diet for 14 d (7, 9). Acetyl-LDL was prepared from LDL of healthy human subjects by the method described by Basu et al. (26). For preparation of oxidized LDL, 5.0 mg of protein from rabbit LDL was suspended in 2 ml of PBS containing 5 μM $CuSO_4$ and was incubated at 37°C for 40 h in a CO_2 incubator. LDL within 14 d of isolation was used for oxidation.

For all experiments native LDL, acetyl-LDL, β-VLDL, and oxidized LDL were filtered through a 0.45-μm filter (Millipore Japan, Tokyo, Japan). The recovery of protein and lipid peroxides determined as thiobarbituric acid-reactive substances (TBARS) was 70–80% during filtration.

HPLC and electrophoresis of lipoproteins

Lipoproteins (50–100 μg of protein) were applied to the gel permeation columns (TSK GEL, G5000PW + G3000SW; Toyo Soda, Tokyo, Japan) following the method described by Okazaki et al. (27). The experimental conditions were as follows: temperature of the separation columns, 4°C; eluent, 150 mM Tris Cl, pH 7.4; flow rate, 0.20 ml/min. Detection of peaks was carried out by measuring cholesterol in the eluate from the column by the bioreactor-type detection system with cholesterol esterase and cholesterol oxidase, which were immobilized on TSK GEL G6000PW (Toyo Soda) (28). To the inlet of the bioreactor column was introduced the staining fluid composed of peroxidase, 4-aminoantipyrine, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-m-toluidine, and Triton X-100. Using these reagents, cholesterol can be measured by the absorbance at 550 nm of the quinone diimine dye. The experimental conditions of the enzymatic reactions were as follows: temperature of the reactor, 40°C; flow rate of the staining fluid; 0.1 ml/min. Recovery for lipoproteins as determined by cholesterol content was more than 95% after passage through the HPLC system.

Agarose gel electrophoresis of lipoproteins was carried out as described by Nobel (29).

Electron microscopy of lipoproteins

Electron microscopy of negatively stained lipoproteins was performed as described by Forte et al. (30). In brief, LDL filtered through a 0.45-μm filter was dialyzed against 500 vol of 3 mM sodium phosphate buffer, pH 7.4, and then mixed with the same volume of 2% sodium phosphotungstate, pH 7.2, to make the final concentration of 250 μg of protein/ml. A small aliquot of the mixture was overlayed on a Formvar-carbon-coated grid and was examined under a electron microscope (Hitachi H-7000).

Assay for lipid peroxides

Lipid peroxide formation was estimated as TBARS using a Lipoperoxide test kit according to the methods of Heinecke et al. (12) and Yagi (31) with a slight modification. LDL (50 μg of protein) was suspended in 1.5 ml of 150 mM NaCl, and the suspension was mixed with 0.5 ml of 20% trichloroacetic acid (TCA) and 0.5 ml of TBA reagent (0.67% TBA aqueous solution/glacial acetic acid, 1:1, vol/vol) and boiled at 95°C for 60 min. The mixture was cooled with water and shaken vigorously with 2.0 ml of *n*-butanol for at least 15 s. After centrifugation at 4,000 *g* for 10 min, the *n*-butanol layer was removed, and the fluorescence was measured on a Hitachi fluorescence spectrophotometer (No. 650-60, Hitachi, Tokyo, Japan) with excitation at 515 nm and emission at 550 nm. Tetramethoxypropane was used as a standard and results were expressed as nmol of malondialdehyde (MDA) equivalents.

Extraction and radioimmunoassay of PGE_2 and LTC_4

At the end of the incubation, the medium was removed and applied to extraction. After [^{3}H]PGE_2 or [^{3}H]LTC_4, 3,000–4,000 cpm, was added, the samples were applied to a SEP-PAK C18 cartridge (Waters Associates, Milford, MA) as described by Powell (32) with a slight modification. The PGE_2- and LTC_4-like immunoreactivities were eluted from the cartridges with ethyl acetate and methanol, respectively. The ethyl acetate fraction was evaporated and the dried residue was dissolved in 1 ml of 50 mM Tris-Cl, pH 7.4, and used for RIA (33). The recovery of the PGE_2 internal standard was 60–70%. Methanol

fraction was also evaporated, and the dried residue was used for RIA with an LTC_4 ^3H-RIA kit (34). The recovery of the LTC_4 internal standard in the final sample was 75–80%.

For thin-layer chromatography of PGE_2 immunoreactivity extracted from culture medium, aliquots of the extract dissolved in ethyl acetate were applied to 20 × 20-cm silica gel plates, and the plates were developed with the solvent, ethyl acetate/acetic acid (98:2, vol/vol). After development the plate was air-dried and the silica gel between the front and the origin was evenly divided into 14 fractions. PGE_2 immunoreactivity was extracted from each fraction with ethyl acetate. The extracts were evaporated with N_2 and then used for RIA of PGE_2. Authentic PGE_2, $PGF_{2\alpha}$ and PGD_2 were applied to the silica gel plates on a separate lane as standard markers.

Study on macrophages prelabeled with [1-^{14}C]arachidonic acid

A macrophage monolayer (3×10^6 cells) was incubated with 0.85 μCi of [1-^{14}C]arachidonic acid in 1 ml of DME containing 10% FCS. After 18 h, the cells were washed twice with 1 ml of DME and incubated with 1 ml of DME containing various additions. After 6 h, the medium was collected and applied to a SEP-PAK C18 cartridge as described above. PGs were eluted with 10 ml of ethyl acetate. 8 ml of the ethyl acetate fraction was evaporated and the residue dissolved in 100 μl of ethyl acetate was applied to the silica gel thin layer as described above. After development the plate was air-dried and the radioactivity at the PGE_2 position was measured. The rest (2 ml) of the ethyl acetate fraction was evaporated and the dried residue was used for RIA for PGE_2. The specific radioactivity for [^{14}C]PGE_2 was determined and corrected with [^{3}H]PGE_2 added as an internal standard.

Assays of cholesterol reacylation and the degradation of ^{125}I-LDL

Reacylation of cholesterol was measured as an incorporation of [^{14}C]-oleate (5 mCi/mmol) into cellular cholesteryl oleate by cell monolayers as described by Brown et al. (5) except that culture was performed without FCS (7). The degradation of ^{125}I-labeled lipoproteins by macrophage monolayer was measured as described by Goldstein et al. (4) and Kita et al. (7).

Derivatization and GC/MS

[$^2H_4$]PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 (40 ng each) were added to a medium as internal standards. Extraction was performed as described above. After evaporation of ethyl acetate, methylation was carried out by treatment of a sample with diazomethane in methanol. After purification by SEP-PAK C18 cartridge, methyl esters were treated with a saturated solution of methoxamine hydrochloride in pyridine at 60°C for 1 h. Dimethylisopropylsilyl imidazole was then added to a reaction mixture and the solution was allowed to stand at room temperature for another hour. The reaction mixture was applied on a Sephadex LH-20 Column (5 × 0.8 cm) and the final derivative was eluted with 2.8 ml of $CHCl_3$/*n*-hexane/methanol, 10:10:1 (vol/vol/vol). After evaporation the residue was dissolved in *n*-hexane containing 1% (vol/vol) pyridine and used for GC/MS as described by Miyazaki et al. (35).

GC/MS was carried out with a JEOL DX 300 GC/mass spectrometer (JEOL Ltd., Tokyo) with a data processing system. The column employed was a thermostable cross-linked OV-1 fused silica capillary column (25 m × 0.31 mm i.d., Hewlett Packard Co., Palo Alto, CA), and helium was used as a carrier gas with an inlet pressure of 0.4 kg/cm^2. The column temperature was maintained isothermal at 265°C and samples were applied via a Van den Berg-type solventless injector. The ionization energy and accelerating voltage were 70 eV and 2.5 kV, respectively. PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 were quantified by peaks at m/Z 552, 552, 625, 670, 670, respectively. Quantifications of these PGs were corrected on the basis of recovery of the internal standards, which were monitored at m/Z 556, 556, 629, 674, and 674 for [$^2H_4$]PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2, respectively.

Gel filtration of oxidized LDL

A PD-10 column (Pharmacia Fine Chemicals, Uppsala Sweden) was equilibrated with PBS at 4°C, and 2.5 mg of protein from oxidized LDL suspended in 1 ml of PBS was loaded and eluted with 20 ml of PBS. Fractions (1.0 ml) were collected and 10- and 25-μl aliquots were used for measurement of protein contents and for TBARS assay, respectively.

Analysis of data

The values shown represent mean±SE and statistical significance was determined by Student's *t* test.

Results

Physicochemical properties of oxidized LDL. We first examined the gel permeation behavior of our preparations of oxidized LDL by using HPLC. As shown in Fig. 1, a major peak was found at the volume of 28.5 ml, which was a position identical with that of native LDL. In addition, a small shoulder was found at 24.4 ml in the uprising of this major peak. Calibration of the column showed that this shoulder represents particle(s) larger than VLDL but smaller than β-VLDL. We next examined the morphological profile of native and oxidized LDL particles under electron microscopy. As shown in Fig. 2 *A*, native LDL particles were uniform in size and shape. The diameter was 22±4 nm. On the other hand, as shown in Fig. 2 *B*, the oxidized LDL preparation contained particles of two different types. One particle type had almost the same size as that of native LDL and the other was enlarged and of uneven shape. The diameter of the enlarged particles was 91±17 nm, almost equivalent to the minimal size of chylomicron

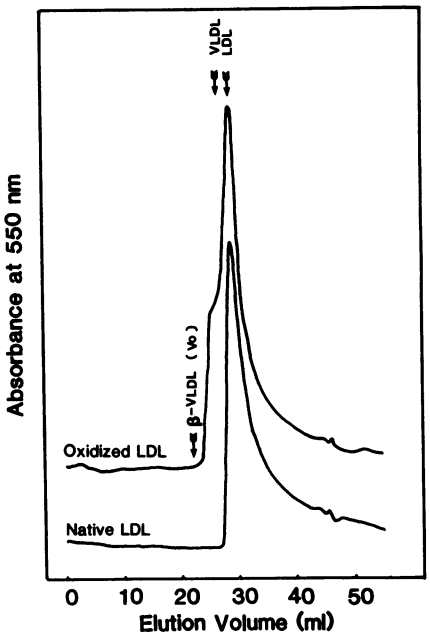

Figure 1. Gel permeation chromatography of native and oxidized LDL. Oxidized LDL was obtained by incubating rabbit LDL with Cu^{2+} at 37°C for 40 h. Oxidized LDL and native LDL (50–100 μg of protein) were applied to TSK GEL columns (G5000PW+G3000SW). Chromatography was performed at 4°C at a constant flow of 0.20 ml/min. Cholesterol content was measured by determination of the absorbance at 550 nm. β-VLDL was eluted in the void volume (Vo). Other markers used are VLDL and LDL.

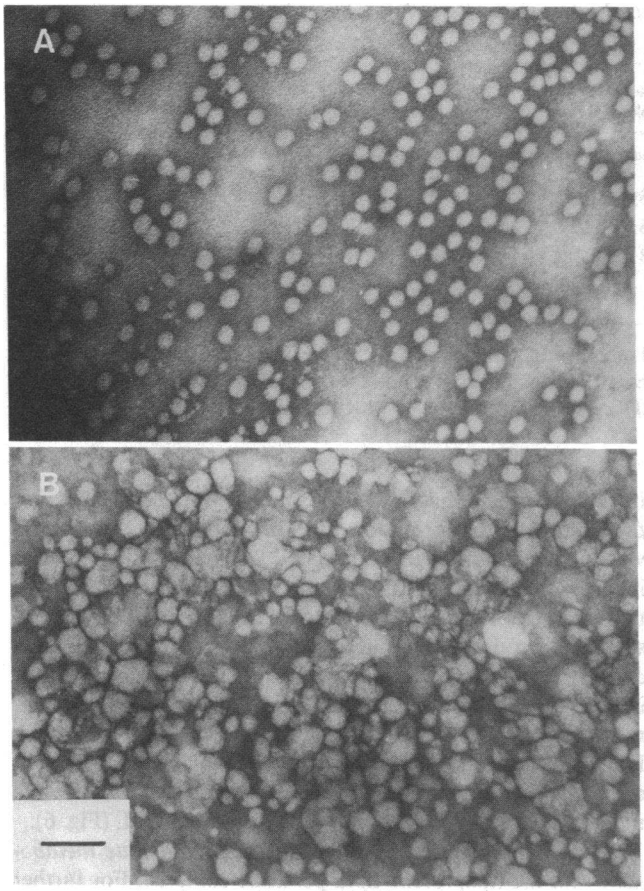

Figure 2. Transmission electron microscopy of (*A*) native LDL and (*B*) oxidized LDL. Lipoproteins were stained with 1% phosphotungstate, pH 7.2. Bar, 100 nm.

remnants, i.e., 90 nm (36). These results indicate that enlargement of particles could occur during the oxidative modification of LDL. To investigate whether both the enlarged and native-sized particles were modified, we applied our preparation of oxidized LDL to agarose gel electrophoresis. As shown in Fig. 3, almost all the protein in our oxidized LDL preparation migrated more negatively than native LDL, suggesting that not only the enlarged particle but also the particle with native-sized diameter underwent oxidative modification.

Stimulation of macrophage PGE_2 synthesis by oxidized LDL. As shown in Table I, TBARS content in the oxidized LDL preparation characterized above was 32.80±2.10 nmol MDA/mg protein, which was 13.7-fold higher than that in native LDL (2.41±0.32). Consistent with previous reports (11, 12), our preparation of oxidized LDL was incorporated by macrophages about 10-fold more efficiently than by native LDL. We incubated macrophages with these preparations of lipoproteins and investigated the changes in arachidonate metabolism in the cells. We measured immunoreactive PGE_2 (ir-PGE_2) biosynthesis by macrophages during incubation with native and oxidized LDL. When macrophages were incubated with native LDL (90 μg/ml), no increase in ir-PGE_2 production was detected; ir-PGE_2 formations with and without LDL were 78.2±5.3 and 75.1±8.4 ng/mg cellular protein per 6 h, respectively. In contrast, when macrophages were incubated with oxidized LDL, ir-PGE_2 biosynthesis was much elevated. The formation was 397.0±12.7 ng/mg cellular protein per 6 h, which was 5.3-fold higher than the control ($P < 0.01$). To exclude the possibility that Cu^{2+} in the oxidized LDL fraction stimulated arachidonate metabolism, we incubated macrophages with 2 μM Cu^{2+} instead of oxidized LDL. No enhanced production of ir-PGE_2 was observed, i.e., 76.2±4.5 ng/mg cellular protein per 6 h. When native LDL was preincubated in PBS without Cu^{2+}, no increase in TBARS formation nor stimulatory effect on ir-PGE_2 synthesis was detected. We also examined the effect of human LDL on macrophage ir-PGE_2 synthesis. Oxidized human LDL (90 μg/ml) also stimulated ir-PGE_2 production to an extent similar to that of oxidized rabbit LDL, although native human LDL had no effect (data not shown). In order to assess specificity of our RIA, we added indomethacin to the culture medium and followed ir-PGE_2 biosynthesis by macrophages. Formation of ir-PGE_2 was completely inhibited by 10 μg/ml indomethacin. We next subjected the medium extract to thin-layer chromatography as described in Methods. Immunoreactivity was observed only at the position identical with authentic PGE_2 (data not shown).

Elevation in PGE_2 biosynthesis in the cells incubated with oxidized LDL was also observed by the GC/MS analysis of cyclooxygenase metabolites, and PGE_2 contents measured were comparable with those obtained by RIA (Table II). Enhanced production was also detected in 6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin (PGI_2), although the extent was less than that of PGE_2. We also measured formation of 6-keto-$PGF_{1\alpha}$ with RIA and the value was comparable to that by GC/MS (data not shown). No significant stimulation was observed on synthesis of PGD_2, PGF_2, or thromboxane B_2 (TxB_2).

When macrophages were incubated with oxidized LDL, PGE_2 synthesis increased in a time-dependent curve (Fig. 4 *A*). PGE_2 synthesis increased almost linearly within a few hours after contact and continued to increase up to 12 h. This time course was similar to that of incorporation of oxidized LDL into the cells (data not shown). As shown in Fig. 4 *B*, this stimulatory effect was dose dependent on protein contents of oxidized LDL. PGE_2 synthesis was linear up to 180 μg of protein from oxidized LDL/ml; the PGE_2 biosynthesis at 180 μg of protein/ml was 813±123 ng/mg cellular protein per 6 h, 10.8-fold higher than the control.

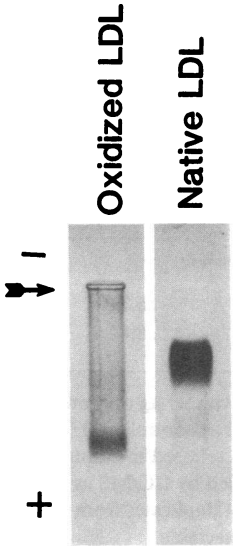

Figure 3. Agarose gel electrophoresis of native LDL and oxidized LDL. Lipoproteins (50–100 μg of protein) were applied to 0.5% agarose gel electrophoresis and stained with Fat Red 7B. The site of application is shown by the arrow.

Table I. Effects of Preincubation of LDL with Cu^{2+} on TBARS Contents, Incorporation Rates into Macrophages, and Stimulatory Effect on Macrophage PGE_2 Synthesis

	TBARS contents	Incorporation of [^{14}C]oleate into cholesteryl ester	Macrophage PGE_2 synthesis	Protein contents of remaining cells
	nmol MDA/mg protein	*nmol/mg cellular protein per 6 h*	*ng/mg cellular protein per 6 h*	*μg of protein per dish*
None (control)	—	0.15±0.05	75.1±8.4	80.6±7.7
Native LDL	2.41±0.32	0.96±0.58	78.2±5.3 NS	93.6±18.4 NS
Oxidized LDL	32.80±2.10*	9.33±0.80*	397.0±12.7‡	80.0±13.2 NS
LDL preincubated without Cu^{2+}	2.45±0.31 NS	—	75.7±6.5 NS	—

Macrophage monolayer, 6×10^6 cells, was incubated for 6 h with 90 μg/ml of native LDL, oxidized LDL, and LDL preincubated for 40 h without Cu^{2+}. TBARS contents, incorporation rates, and effect on macrophage PGE_2 synthesis of LDL were determined as described in Methods. Values represent mean±SE. * $P < 0.01$ compared with native LDL. ‡ $P < 0.01$ compared with control.

To examine whether the stimulation of oxidized LDL on macrophage PGE_2 production was correlated with cellular injury, the cells attached to the dish were dissolved with 0.2 N NaOH at the end of incubation and the protein contents of the extracts were measured. In contrast to the dramatic effect on PGE_2 biosynthesis, oxidized LDL caused no decrease in cellular protein contents at the end of a 6-h incubation compared with native LDL or control (80.0±13.2, 93.6±18.4, 80.6±7.7 μg per dish, respectively) (Table I). We also examined microscopically the appearance of the cells stained with trypan blue. Less than 1% of the total cells were stained and there was no difference between the ratio of the stained cells in culture with and without lipoproteins.

Stimulatory effect of oxidized LDL on macrophage LTC_4 synthesis. When incubated with oxidized LDL (90 μg of protein/ml), macrophages synthesized and released LTC_4 about three times more than the control culture (29.7±15.3 and 8.3±4.6 ng/mg cellular protein per 6 h, respectively). The stimulatory effect was dose dependent on protein contents of oxidized LDL; LTC_4 formation at the concentration of 180 μg of protein/ml was 10.7-fold higher than the control, 89.5±29.5 ng/mg cellular protein per 6 h. No stimulatory effect on LTC_4 synthesis was observed in native LDL (Fig. 5).

Specificity of lipoproteins on the stimulation of arachidonate metabolism. In order to investigate whether the stimulatory effect of oxidized LDL on arachidonate metabolism was correlated with endocytotic incorporation of the lipoproteins in general, macrophages were incubated with acetyl-LDL and β-VLDL, and the formation of PGE_2 and LTC_4 by the cells was followed. Although both acetyl-LDL and β-VLDL were incorporated by the cells more efficiently than oxidized LDL, neither of them had any stimulatory effect on PGE_2 biosynthesis; PGE_2 production by macrophage during incubation with acetyl-LDL or β-VLDL was significantly less than the control, i.e., 42.1±6.8 and 51.5±9.4 ng/mg cellular protein per 6 h, respectively ($P < 0.05$). Neither of them caused any increase in LTC_4 synthesis, 8.4 and 9.3 ng/mg cellular protein per 6 h, respectively. TBARS contents in acetyl-LDL or β-VLDL were not significantly different from those in native LDL (Fig. 6).

Association of stimulatory effect on arachidonate metabolism with the lipoprotein–lipid peroxide complex. For further elucidation of the stimulatory effect of oxidized LDL on arachidonate metabolism, oxidized LDL was applied to gel filtration as described in Methods, and elution of TBARS and protein contents were followed. As shown in Fig. 7, two peaks of

Table II. GC/MS Analysis of Various Prostaglandins Formed by Macrophages

	Prostaglandins formed by macrophages				
	PGE_2	PGD_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TxB_2
	ng per sample				
Oxidized LDL	29.9	ND	5.4	16.7	ND
Control	9.6	ND	6.7	8.8	ND

Macrophages, 3×10^6 cells, were incubated with 90 μg of protein/ml of oxidized LDL or without LDL (designated as control) at 37°C for 6 h. After incubation, various prostaglandins released in culture medium was extracted, derivatized, and quantified by GC/MS as described in Methods. Values are the average of duplicates from a representative of two experiments. ND, not detectable.

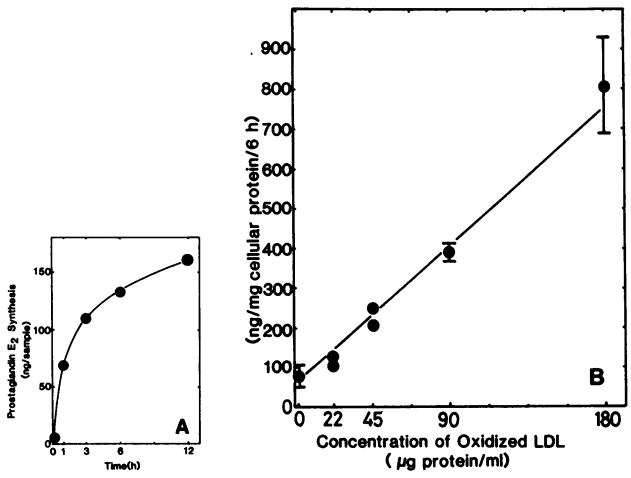

Figure 4. Time course and dose response of stimulation of macrophage PGE_2 synthesis by oxidized LDL. (*A*) Macrophage monolayer, 6×10^6 cells, was incubated with 90 μg of protein/ml of oxidized LDL. After incubation for indicated time, media were taken from dishes and the amount of PGE_2 was determined by RIA. Values are the average of duplicates from a representative of two experiments. (*B*) Macrophages, 6×10^6 cells, were incubated with indicated concentration of oxidized LDL at 37°C for 6 h. PGE_2 amounts were determined by RIA. Values represent mean±SE of four experiments.

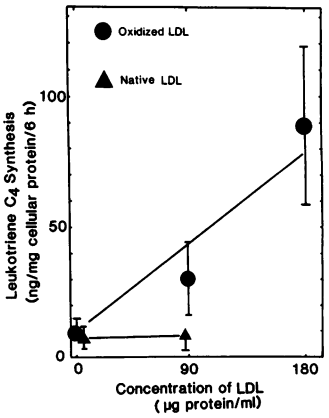

Figure 5. Stimulatory effect of oxidized LDL on macrophage LTC_4 biosynthesis. Macrophage monolayer, 6×10^6 cells, was incubated with various concentrations of oxidized LDL or native LDL at 37°C for 6 h. After incubation LTC_4 amounts in the medium were determined by RIA. Values are mean±SE of three experiments.

TBARS were detected, those of fraction A (nos. 2–4) and fraction B (nos. 7–9), and a protein peak was observed only in fraction A. Thus fractions A and B were considered as the lipoprotein–lipid peroxide complex and free lipid peroxides, respectively. The TBARS level in fraction A was 13.2±1.5 nmol MDA/mg protein, 40.2±4.6% of that in unfractionated oxidized LDL. We incubated macrophages with various concentrations of either fraction A or B to elucidate which fraction was more responsible for the stimulation of macrophage arachidonate metabolism. As shown in Fig. 8, fraction A stimulated PGE_2 synthesis in dose-dependent way. At the concentration of 2.4 nmol MDA/ml (180 μg protein/ml), the stimulatory effect of fraction A on PGE_2 synthesis was 724.5 ng/mg cellular protein per 6 h. This stimulation was ~ 88% of that caused by unfractionated LDL containing the same protein contents, i.e., 813 ng/mg cellular protein per 6 h. On the other hand, the stimulatory effect of fraction B was < 15% of that of fraction A, i.e., 194.3±26.5 ng/mg cellular protein per 6 h ($P < 0.05$ v.s. control). Stimulation by fraction A and B was not enhanced in the medium without FCS.

In order to determine whether the stimulatory action of fraction A was associated with incorporation by the cell, we incubated macrophages with cytochalasin B, an inhibitor of receptormediated incorporation of lipoproteins (37), and examined the changes in the rate of cholesterol reacylation and PGE_2 formation in macrophages. Cytochalasin B (1×10^{-4} M) almost completely inhibited incorporation of [^{14}C]oleate into cholesteryl oleate and reduced stimulation by fraction A on macrophage PGE_2 synthesis by 91%. When macrophages were incubated with 90 μg/ml fraction A, cholesterol reacylation rates with and without cytochalasin B were 0.75 and 12.5 nmol/mg cellular protein per 6 h, respectively, and increases in macrophage PGE_2 formations were 35.0 and 405.0 ng/mg cellular protein per 6 h, respectively. Cytochalasin B had no inhibitory effect on basal PGE_2 formation, i.e., 90.2 μg/mg cellular protein per 6 h. To test whether stimulatory effect of fraction A was associated with the acetyl-LDL receptor-mediated endocytosis, we examined the inhibitory effect of an excessive amount of acetyl-LDL on the degradation of fraction A by the macrophage monolayer. When the cells were incubated with 90 μg/ml ^{125}I-labeled fraction A and 900 μg/ml acetyl-LDL, the degradation rate was suppressed by 55%. The stimulatory effect of 90 μg/ml fraction A on PGE_2 synthesis was not significantly suppressed by the addition of 900 μg/ml acetyl-LDL; PGE_2 formations at the presence or absence of acetyl-LDL in these experiments were 256.5 and 267.0 ng/mg cellular protein per 6 h, respectively. However, we could not definitely exclude involvement of acetyl-LDL receptor pathway in stimulation of PGE_2 formation, since acetyl-LDL at this concentration caused increase in PGE_2 formation, i.e., 130.1 μg/mg cellular protein per 6 h, almost twice as the control level, i.e., 67.5 μg/mg cellular protein per 6 h. We also examined the effect of either 100 μg/ml fucoidin, a specific inhibitor of acetyl-LDL receptor, or 40 μM chloroquine, a lysosomal inhibitor (1, 4, 5), on the stimulation of arachidonate metabolism in the cells by fraction A. Both of the reagents, however, potently stimulated arachidonate metabolism by themselves.

Study on macrophages prelabeled with [^{14}C]arachidonate. In order to determine the source of arachidonate released on the stimulation by oxidized LDL, we labeled the cells with [1-^{14}C]arachidonate as described in Methods and then stimulated them with either fraction A or zymosan. When macrophages were stimulated with 90 μg/ml fraction A, the specific radioactivity of PGE_2 formed was not significantly decreased from the value in the control experiments with no additions, i.e. 1,032±299 or 1,268±425 dpm/ng PGE_2 (n = 4), respectively. Nor was it different from that found in macrophages stimulated by 50 μg/ml zymosan A, i.e., 1,346±299 dpm/ng PGE_2 (n = 4).

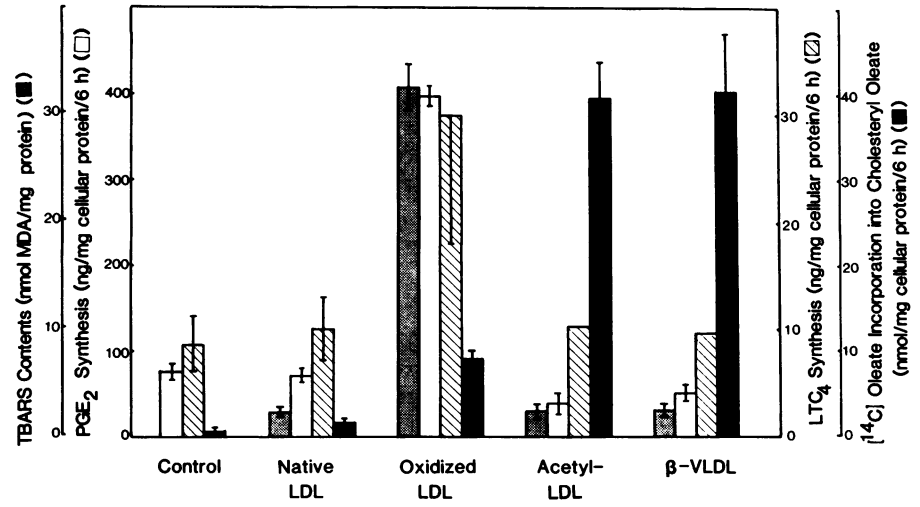

Figure 6. TBARS contents, effect on PGE_2 and LTC_4 synthesis, and incorporation rates into macrophages of various lipoproteins. Macrophage monolayer, 6×10^6 cells, was incubated with 90 μg/ml of oxidized LDL, native LDL, acetyl-LDL, and β-VLDL at 37°C for 6 h. Assay for TBARS contents was carried out as described in Methods. After incubation, amounts of PGE_2 and LTC_4 were measured by RIA. Control represents incubation without lipoprotein. Assessment of incorporation of [^{14}C]oleate into cholesteryl ester was carried out as described in Methods.

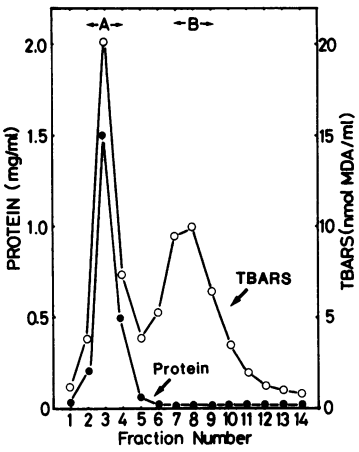

Figure 7. Gel filtration of oxidized LDL. 2.5 mg of oxidized LDL suspended in 1.0 ml of PBS was applied to a PD-10 column. The column was then washed with PBS and 1 ml fractions were collected. In each fraction 10- and 25-μl aliquots were used for determination of the contents of protein (●) and TBARS (○), respectively.

Specificity of cell species on the stimulatory effects of oxidized LDL on arachidonate metabolism. In order to examine the specificity of cell species sensitive to the stimulatory effect of oxidized LDL on arachidonate metabolism, the 3T3 cell monolayer was incubated with native and oxidized LDL. Oxidized LDL stimulated cholesteryl ester formation in 3T3 cells about twofold more than native LDL; the rates of cholesterol reacylation in the control and during incubation with 90 μg/ml native LDL and oxidized LDL were 10.3, 10.2, and 24.1 nmol/mg cellular protein per 6 h, respectively. However, oxidized LDL had no stimulatory effect on PGE_2 synthesis by these cells. Native LDL had no stimulatory effect either. The formation of PGE_2 was less than 10 ng/mg cellular protein per 6 h, whether the cells were incubated with or without lipoproteins.

Discussion

In this study we have shown that oxidized LDL was not only incorporated by macrophages but also stimulated arachidonate metabolism (Table I). The stimulatory effect of oxidized LDL on PGE_2 synthesis by macrophages occurred in both a dose- and time-dependent manner (Fig. 4, *A* and *B*). In addition to PGE_2, oxidized LDL stimulated macrophage LTC_4 synthesis (Fig. 5). Native LDL was taken up by macrophages at a very low rate and had no stimulatory effect on arachidonate metabolism (Table I, Fig. 5).

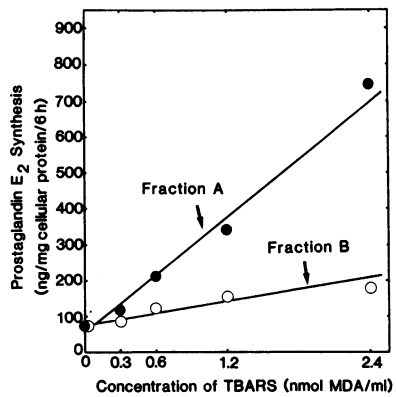

Figure 8. Dose response of macrophage PGE_2 biosynthesis by fractions A and B. After filtering through a 0.45-μm filter, indicated TBARS concentrations of fractions A (●) and B (○) were added to the macrophage monolayer (6×10^6 cells). After 6 h of incubation PGE_2 amounts were determined by RIA. The recovery for TBARS in fractions A and B after filtration were 78% and 95%, respectively. Values are the average of duplicates from a representative of four experiments.

Since the scavenger pathway in macrophages for modified LDL was revealed in vitro, LDL modification has been proposed to play an important role in atheroma formation in vivo. Oxidized LDL is now considered to be a candidate for naturally occurring modified LDL, as lipid peroxides have been suggested to be closely correlated with initiation and progression of atheroma (14). Peroxidized lipids have been demonstrated to accumulate in atherosclerotic lesions (13) and to cause injuries on the vascular tissues (38). Quinn et al. have reported that oxidized LDL has inhibitory effect on the endothelial cell-derived chemotactic activity for mouse peritoneal macrophages (39). In addition, Parthasarathy et al. (40) have also reported that probucol, a known inhibitor of both progression of atherosclerotic lesion and xanthoma formation (41), could block oxidative modification of LDL at the concentration lower than the plasma levels of the patients during ordinary treatment. Heinecke et al. (42) have shown that superoxide released from arterial smooth muscle cells enhances Cu^{2+}- or Fe^{2+}-catalyzed modification of LDL. Cathcart et al. (43) have also reported that incubation with monocytes and neutrophils leads to oxidation of LDL. If oxidized LDL could be produced in the body, it would be incorporated rapidly by macrophages and stimulate arachidonate metabolism thereby inducing enhanced release of PGE_2 and LTC_4.

Since oxidized LDL is reported by some authors to have a cytotoxic effect on certain cell types including endothelial cells and fibroblasts (10, 43–46), we examined whether the stimulation of arachidonate metabolism was correlated with cellular injury. In our study, however, no evidence of cellular injury on the macrophage monolayer was observed within 12 h, neither the decrease in cellular protein contents (Table I), nor that in viable cells, excluding trypan blue, was found.

In our study we have demonstrated that major stimulatory activity on arachidonate metabolism exists in the lipoprotein–lipid peroxide complex, and not in free lipid peroxides (Fig. 8). Steinberg and his collaborators reported that peroxidation of LDL is accompanied with degradation of phosphatidylcholine into lysophosphatidylcholine and suggested possible release of free fatty acids (11, 47). Since FCS is known to trap free fatty acids in the medium, we examined the stimulatory effect of fraction B in DME without FCS. Our results, however, showed that the stimulatory effect of fraction B on arachidonate metabolism was not enhanced under these conditions. Thus it is hardly possible that unsaturated fatty acids liberated from phospholipids of LDL during its peroxidation should participate as substrate for prostaglandin or leukotriene cascade.

In this study we have also shown that some of oxidized LDL particles could become enlarged severalfold in diameter (Figs. 1, 2, *A* and *B*, and 3). Previous studies suggest that oxidized LDL is recognized and incorporated via the acetyl-LDL receptor of macrophages (11, 48, 49). In our results, however, acetyl-LDL suppressed the degradation of ^{125}I-oxidized LDL by macrophages only by 55% and did not suppress oxidized LDL-induced PGE_2 formation completely. Our preliminary experiments showed that the binding of ^{125}I-oxidized LDL to the surface of macrophage and its degradation by the cell were almost completely suppressed by an excessive amount of unlabeled oxidized LDL, while unlabeled acetyl-LDL was less potent as a competitive inhibitor (unpublished observation). Thus, we could not exclude the existence of other receptors which are also responsible for this incorporation. It would be natural, therefore, that stimulation of PGE_2

synthesis could not be inhibited completely by an excessive amount of acetyl-LDL. At present it is not known whether such multiplicity of receptor for oxidized LDL corresponds to the heterogenous population of this modified LDL.

Macrophages are known to release arachidonate metabolites when activated by phagocytic stimuli (50–53). In this situation, cytochalasin B augments prostaglandin synthesis by macrophages stimulated by the particles, although it inhibits phagocytotic incorporation of phagocytosed particles (54, 55). These observations are contrary to our results. In our experiments, cytochalasin B suppressed both incorporation of oxidized LDL and stimulation of PGE_2 synthesis by the cell. Moreover, stimulation of macrophage arachidonate metabolism by phagocytosis reaches plateau within 1 h after the stimuli (56), while in our experiments release of arachidonate metabolite from the cell increased gradually and continued to increase up to 12 h after contact. This time course was similar to that of incorporation of oxidized LDL by the cells. These findings suggest that the process through which oxidized LDL stimulated macrophage arachidonate metabolism is distinct from that with phagocytosis and that receptor-mediated incorporation of lipoprotein particle could be prerequisite for the stimulatory effect.

What was noteworthy in our study was that acetyl-LDL and β-VLDL, both of which were incorporated by macrophages by receptor-mediated endocytosis more efficiently than oxidized LDL, had little stimulatory effect on arachidonate metabolism (Fig. 6). These results indicate that the receptor-mediated endocytosis of lipoprotein particle in general is not associated with activation of macrophage arachidonate metabolism. Inasmuch as acetyl-LDL and β-VLDL had no increased TBARS contents (Fig. 6), incorporation of the lipoprotein particle with peroxidative modification would play a primary role in stimulation of arachidonate metabolism in macrophages.

Pomerantz et al. (57) reported that HDL and LDL could stimulate arachidonate metabolism in vascular smooth muscle cells by providing arachidonate for the pools sensitive to cyclooxygenase. However, in our experiments, when macrophage was labeled with [^{14}C]arachidonate and then stimulated with unlabeled fraction A, the specific radioactivity of PGE_2 formed was not decreased significantly from that observed in the control experiments. These results suggest that the stimulatory effect of oxidized LDL on macrophage arachidonate metabolism is not induced by introduction of arachidonate into the cells but could be due to activation of phospholipase A_2 or other enzymes in the arachidonate cascade by the incorporated lipoprotein–lipid peroxide complex. Our suggestions are consistent with the report by Weglicki et al. (58), who described that activation of phospholipase A_2 was induced by free radicals derived from lipid peroxides. Our working hypothesis is depicted in Fig. 9. Such a mechanism appears to be restricted to macrophages, since oxidized LDL failed to stimulate 3T3 cells to synthesize PGE_2.

PGE_2, among the products of arachidonate metabolism, has been known to have the most active proinflammatory effects especially in the early stage, such as extensive vasodilation, enhancement of vasopermeability, and potentiation of pain caused by bradykinin or histamine (53). In contrast, LTC_4 is recognized as a potent vasoconstrictor for arteries and is known to induce an increase in vascular permeability, inducing plasma exudation (59). In addition to the effects on

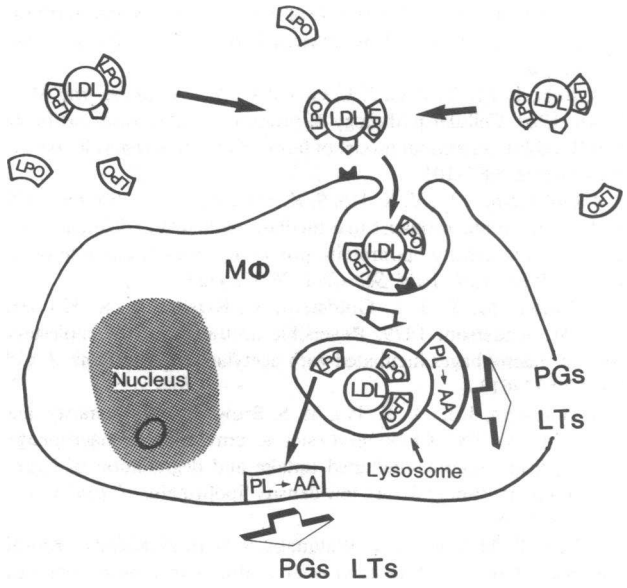

Figure 9. Proposed mechanism for stimulation of arachidonate metabolism in macrophage by oxidized LDL. AA, arachidonic acid; PL, phospholipid; LPO, lipid peroxides.

inflammatory response, a growing body of evidence indicates that lipoxygenase products play a role as a promotor of atheromatous formation (59–61). Once these events could take place in arterial walls, they would induce infiltration of more amounts of plasma LDL into the subendothelial space and facilitate the vascular injury, eventually leading further to vasospasm and progression of atheroma.

These findings indicate that oxidized LDL could induce progression of atheroma by stimulating arachidonate metabolism in macrophages. These events could occur in the body, since several lines of evidence indicate that oxidized LDL may be an analogue for the lipoproteins accumulated in the vessel walls. Our results would be of importance in elucidating the role of macrophages and the modification of LDL in atherogenesis.

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