Oxidized LDL activates phospholipase A_2 to supply fatty acids required for cholesterol esterification

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Abstract **We examined the roles of phospholipase A_2 (PLA_2) in oxidized LDL (oxLDL)-induced cholesteryl ester formation in macrophages. In [^{3}H]oleic acid-labeled RAW264.7 cells and mouse peritoneal macrophages, oxLDL induced [^{3}H]cholesteryl oleate formation with an increase in free [^{3}H]oleic acid and a decrease in [^{3}H]phosphatidylcholine. The changes in these lipids were suppressed by methyl arachidonyl fluorophosphonate (MAFP), a cytosolic PLA_2 ($cPLA_2$) inhibitor. However, MAFP had no effect on the ACAT activity or the binding and/or uptake of oxLDL. Stimulation with oxLDL in the presence of [^{3}H]cholesterol increased [^{3}H]cholesteryl ester bearing fatty acyl chains derived from cellular and/or exogenous (oxLDL) lipids. The formation of cholesteryl ester under this condition was also inhibited by MAFP, and the inhibitory effect was reversed by adding oleic acid. While oxLDL did not affect the activity or amounts of $cPLA_2$, preincubation with oxLDL enhanced the release of oleic acid and arachidonic acid induced by ionomycin in RAW264.7 cells. 13(*S*)-hydroxyoctadecadienoic acid, but not 7-ketocholesterol, also enhanced ionomycin-induced oleic acid release. These results suggest that oxLDL induces $cPLA_2$ activation, which contributes, at least in part, to the supply of fatty acids required for the cholesteryl esterification, probably through the acceleration by oxidized lipids of the catalytic action of $cPLA_2$ in macrophages.**—Akiba, S., Y. Yoneda, S. Ohno, M. Nemoto, and T. Sato. **Oxidized LDL activates phospholipase A_2 to supply fatty acids required for cholesterol esterification. *J. Lipid Res.* 2003.** 44: **1676–1685.**

Supplementary key words cholesteryl ester • cytosolic phospholipase A_2 • foam cell • hydroxyoctadecadienoic acid • macrophage

The oxidation of LDL within the artery wall is widely accepted to participate in atherogenesis (1–4). The oxidized LDL (oxLDL) stimulates the transmigration of monocytes into the arterial intima, their differentiation into macrophages, and the transformation of macrophages into lipid-laden foam cells (3–5). Similarly, oxLDL elicits the migration of smooth muscle cells from the media and their transformation into foam cells (3, 4, 6). The accumulation of foam cells, resulting from these biological activities of oxLDL, leads to the formation of fatty streak lesions, which is a critical event in the early stages of atherosclerosis (3, 4). In the advanced stages of atherosclerosis, the death and necrosis of foam cells facilitates the development of vulnerable atherosclerotic plaques with large lipid cores and very thin fibrous caps, the rupture of which leads to thrombus formation followed by clinical manifestations of coronary heart disease, such as myocardial infraction (3, 4). Thus, the foam cell formation by oxLDL represents a key event in the development and progression of atherosclerosis.

The formation of macrophage-derived foam cells is associated with the uptake of oxLDL particles by macrophages via several types of scavenger receptors, including two novel receptors (LOX-1 and SR-PSOX) identified recently (5, 7–9). The endocytosed oxLDL is delivered to lysosomes, and then cholesteryl esters in the oxLDL are hydrolyzed by lysosomal acid cholesteryl ester hydrolase to liberate free cholesterol, which moves to the cholesterol pool in the plasma membrane. The excessive free cholesterol is reesterified with fatty acyl-CoA by ACAT at the endoplasmic reticulum to form cholesteryl ester, which accumulates as cytoplasmic lipid droplets, thus leading to foam cell formation (10–12). In the process of accumulation of cholesteryl ester, although ACAT utilizes free cholesterol supplied from the cholesterol pool as a substrate, little is known about the pathways involved in the supply of fatty acids required for the ACAT-catalyzed cholesterol esterification. A previous study showed that while human LDL contains cholesteryl linoleate more than cholesteryl oleate, foam cells in the fatty streak lesions contain predominantly cholesteryl oleate (13). Furthermore, stimulation of mouse peritoneal macrophages with acetylated LDL

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Abbreviations: $cPLA_2$, cytosolic PLA_2; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine; 13-HODE, 13(*S*)-hydroxyoctadecadienoic acid; $iPLA_2$, Ca^{2+}-independent cytosolic PLA_2; MAFP, methyl arachidonyl fluorophosphonate; oxLDL, oxidized LDL; PLA_2, phospholipase A_2; TBARS, thiobarbituric acid-reactive substances.

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containing cholesteryl linoleate results in hydrolysis of the ester with an increase in cholesteryl oleate (14). These findings suggest that free fatty acids utilized for the reesterification are supplied from lipids other than cholesteryl esters derived from oxLDL. It has been reported that in [^{14}C]oleic acid-labeled mouse and [^{3}H]arachidonic acid-labeled rat peritoneal macrophages, uptake of acetylated LDL or liposomes containing cholesterol induces an increase in cholesteryl ester bearing radioactive fatty acyl chains with a decrease in radioactive phosphatidylcholine (15, 16), suggesting that fatty acids derived from the endogenous phospholipids are utilized, at least in part, for the cholesterol esterification. However, the mechanism by which the uptake of the lipid particles facilitates hydrolysis of phospholipids is yet to be elucidated.

Phospholipase A_2 (PLA_2) is an enzyme that catalyzes the hydrolysis of membrane glycerophospholipids to liberate free fatty acid. It has been shown that oxLDL stimulates the release of arachidonic acid and the production of prostaglandins in mouse peritoneal macrophages and $P388D_1$ macrophages (17–19). Among numerous types of PLA_2s identified in mammalian cells and tissues (20–22), Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$), but not Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$), participates in the oxLDL-induced arachidonic acid liberation in mouse peritoneal macrophages (19). Recently, we demonstrated that the oxLDL-induced formation of [^{3}H]cholesteryl oleate is suppressed by methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of $cPLA_2$, in RAW264.7 macrophages (23). These observations led us to assume that $cPLA_2$ plays an important role in the release of fatty acids utilized for cholesterol esterification in response to oxLDL. However, our results (23) also suggest the possible involvement of $iPLA_2$ in the esterification, because MAFP is shown to inhibit the activity of $iPLA_2$ in addition to that of $cPLA_2$ (24). Furthermore, because the uptake by mouse peritoneal macrophages of liposomes containing cholesterol and phosphatidylcholine bearing a radioactive fatty acyl chain induces an increase in radioactive cholesteryl ester (15), it is possible that fatty acids derived from phospholipids in oxLDL particles may be also utilized for the cholesterol esterification. Therefore, the present study was undertaken to further examine the contribution of $cPLA_2$ to the cholesteryl ester formation and the regulation of $cPLA_2$ activation associated with the uptake of oxLDL in RAW264.7 macrophages and mouse peritoneal macrophages.

MATERIALS AND METHODS

Materials

[^{3}H]oleic acid (7 Ci/mmol), [^{3}H]arachidonic acid (209 Ci/mmol), and [^{14}C]oleoyl-CoA (56 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). [^{3}H]cholesterol (51.2 Ci/mmol), 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine (172 Ci/mmol), and 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phosphocholine (159 mCi/mmol) were from PerkinElmer Life Science (Boston, MA). 13(*S*)-hydroxyoctadecadienoic acid (13-HODE), 7-ketocholesterol, FBS, bromoenol lactone, and 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindocarbocyanine (DiI) perchlorate were from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Human LDL (BT-903) was from Biomedical Technologies Inc. (Stoughton, MI), MAFP was from Cayman Chemical (Ann Arbor, MI), ionomycin was from Calbiochem (La Jolla, CA), triacsin C was from Kyowa Medex (Tokyo, Japan), and the anti-$cPLA_2$ antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

RAW264.7 cells (Dainippon Pharmaceutical Co., Ltd.) were maintained in DMEM (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under humidified air containing 5% CO_2. Cells were plated in 35 mm culture dishes at 6×10^5 cells or in 100 mm culture dishes at 6×10^6 cells in DMEM containing 0.01% BSA for the following experiments. Resident peritoneal macrophages were isolated from female ddY mice (25–30 g, Japan SLC, Inc.). Peritoneal cells were suspended in DMEM containing 0.01% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin, and plated in 35 mm culture dishes at 1×10^6 cells or in 100 mm culture dishes at 6×10^6 cells. The cells were incubated at 37°C for 2 h under humidified air containing 5% CO_2, and then washed with DMEM containing 0.01% BSA. The attached cells were placed in DMEM containing 0.01% BSA, and used as mouse peritoneal macrophages.

Preparation of oxLDL

Commercial human LDL, prepared from plasma from multiple donors, was dialyzed against PBS at 4°C. The LDL (2.5 mg protein/ml) was oxidized with 10 μM $CuSO_4$ at 37°C for 3 h. The degree of oxidation of the oxLDL was evaluated by a thiobarbituric acid-reactive substances (TBARS) assay according to the method of Yagi (25). The oxLDL contained 10–15 nmol TBARS/mg protein, while native LDL (before oxidation) contained 0.6–1.2 nmol TBARS/mg protein. The oxLDL was further dialyzed against PBS containing 200 μM EDTA at 4°C, and used within 2 weeks.

Measurement of cholesteryl ester, free fatty acid, and phosphatidylcholine in [^{3}H]oleic acid- and [^{3}H]arachidonic acid-labeled cells

For the labeling with [^{3}H]oleic acid, RAW264.7 cells or mouse peritoneal macrophages (35 mm dishes) were incubated with [^{3}H]oleic acid (0.5 μCi/ml) at 37°C for 12 h or 18 h, respectively. After being washed three times with PBS containing 0.01% BSA, the labeled RAW264.7 cells or mouse peritoneal macrophages were further cultured in DMEM containing 0.01% BSA for 12 h or 6 h, respectively. The labeled cells were placed in fresh DMEM containing 0.01% BSA. For the labeling with [^{3}H]arachidonic acid, RAW264.7 cells (35 mm dishes) were incubated with [^{3}H]arachidonic acid (1 μCi/ml) at 37°C for 12 h. After being washed, the cells were placed in DMEM containing 0.01% BSA. The labeled cells were treated with various reagents, and then stimulated with oxLDL as described in the figure legends. When cells were stimulated with ionomycin after incubation with oxLDL, the oxLDL-stimulated cells were washed three times with DMEM containing 0.01% BSA, and then ionomycin was added. Lipids in the medium and cells were extracted and separated by TLC on a silica gel G plate with the following development systems: for the analysis of cholesteryl ester and free fatty acid, petroleum ether/diethyl ether/formic acid (100:25:2.5; v/v/v); and for the analysis of phosphatidylcholine, the combination of chloroform/methanol/7 M NH_4OH (65:25:5.6; v/v/v) for the first dimension and chloroform/methanol/acetic acid/H_2O

(60:30:8:5; v/v/v/v) for the second. The area corresponding to cholesteryl ester, free fatty acid, phosphatidylcholine, or other lipids was scraped off, and the radioactivity was measured by liquid scintillation counting. The total radioactivity of the fractions recovered from the plate was usually in the range of 2.8×10^5 to 3.3×10^5 dpm in RAW264.7 cells and of 1.8×10^5 to 2.1×10^5 dpm in mouse peritoneal macrophages. The radioactivity of each lipid was corrected by adjusting the total radioactivity to 3.0×10^5 dpm in RAW264.7 cells and to 2.0×10^5 dpm in mouse peritoneal macrophages. These experimental conditions did not affect cell viability (more than 90%), as estimated by trypan blue dye exclusion.

Incorporation of [^{3}H]oleic acid into phosphatidylcholine

RAW264.7 cells (35 mm dishes) were treated with MAFP or triacsin C, and then incubated with [^{3}H]oleic acid (0.5 μCi/ml). After the cells were washed three times with PBS containing 0.01% BSA, lipids in the cells were extracted and separated by TLC as described above. The area corresponding to phosphatidylcholine was scraped off, and the radioactivity was measured by liquid scintillation counting.

Determination of cholesteryl ester formation with [^{3}H]cholesterol

RAW264.7 cells or mouse peritoneal macrophages (35 mm dishes) were treated with MAFP and then stimulated with oxLDL in the presence of [^{3}H]cholesterol (1 μCi/ml) and unlabeled cholesterol (10 μM) as described in the figure legends. After the cells were washed three times with PBS containing 0.01% BSA, lipids in the cells were extracted and separated by TLC on a silica gel G plate with petroleum ether/diethyl ether/formic acid (100:25:2.5; v/v/v) as the developing system. The area corresponding to cholesteryl ester was scraped off, and the radioactivity was measured by liquid scintillation counting.

Assay for activity of cPLA$_2$ and iPLA$_2$

For the cPLA$_2$ assay, RAW264.7 cells (100 mm dishes) were treated with MAFP or stimulated with oxLDL. After being washed, the cells were collected and sonicated in buffer A [100 mM NaCl, 2 mM EGTA, 100 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 10 mM Tris-HCl, pH 7.4]. The lysate was centrifuged at 100,000 *g* for 30 min at 4°C. The cPLA$_2$ activity in the resultant supernatant (cytosol fraction) was determined as described previously (26). Briefly, the supernatant was treated with 5 mM dithiothreitol at 37°C for 15 min. The reaction mixture (30 μg protein) was incubated with a mixture of 1-stearoyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine and the unlabeled compound (125 Ci/mol, 2 μM) at 37°C for 15 min in the presence of 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 7.4) in a final volume of 200 μl. For the iPLA$_2$ assay (27), RAW264.7 cells (100 mm dishes) were treated with MAFP or bromoenol lactone and washed. The cells were collected and sonicated in a buffer [340 mM sucrose, 2 mM EGTA, 100 μM leupeptin, 100 μM *p*-(amidinophenyl)methanesulfonyl fluoride, 0.05% Triton X-100, and 10 mM HEPES, pH 7.4]. The lysate (50 μg protein) was treated with 5 mM dithiothreitol at 37°C for 15 min and then incubated with a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]phosphocholine and the unlabeled compound (10 Ci/mol, 50 μM) at 37°C for 1 h in the presence of 5 mM EDTA, 0.03% Triton X-100 and 50 mM HEPES (pH 7.4) in a final volume of 200 μl. After lipids were extracted and separated by TLC, the radioactivity of the [^{3}H]arachidonic acid liberated and [^{14}C]lysophosphatidylcholine generated were determined as described previously (26, 27), and the enzymatic activity was calculated.

Immunoblot analysis for cPLA$_2$

RAW264.7 cells (100 mm dishes) were stimulated with oxLDL and washed. The cells were collected and sonicated in buffer A. The lysate (20 μg protein) was solubilized and subjected to SDS-PAGE on a 7.5% gel. The proteins were transferred to a nitrocellulose membrane, and then antibodies against cPLA$_2$ were applied. The bound antibodies were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

Assay for ACAT activity

RAW264.7 cells or mouse peritoneal macrophages (100 mm dishes) were incubated with cholesterol (10 μM) at 37°C for 12 h. After being washed, the cells were collected and sonicated in a buffer (10 mM EGTA and Tris-HCl, pH 7.7). The lysate was centrifuged at 100,000 *g* for 30 min at 4°C. The resultant pellet (membrane fraction) was resuspended in the buffer, and the protein concentrations in the sample were adjusted to 1 mg/ml. The sample (60 μl) was treated with MAFP at 37°C for 10 min, and then the ACAT activity in the mixture was determined by incubation with [^{14}C]oleoyl-CoA (40 μM) at 37°C for 10 min in the presence of 1% BSA in a final volume of 100 μl. Lipids were extracted and separated by TLC on a silica gel G plate with petroleum ether/diethyl ether/formic acid (100:25:2.5; v/v/v) as the developing system. The radioactivity of the [^{14}C]cholesteryl oleate formed was measured, and the enzymatic activity was calculated.

Assay for binding and/or uptake of oxLDL

The incubation of oxLDL (1 mg protein/ml) with DiI (30 μg DiI/mg protein of oxLDL, at 37°C for 3 h) and measurement of its fluorescence were performed as described elsewhere (28–30). RAW264.7 cells or mouse peritoneal macrophages (1×10^6/35 mm dish) were treated with MAFP for 1 h, and then stimulated with DiI-labeled oxLDL (20 or 50 μg/ml) or unlabeled oxLDL (50 μg/ml) as described in the figure legends. After being washed three times with PBS, cells were lysed with 0.1 M NaOH (29), and the solution was neutralized with 0.1 M HCl. The mixture was sonicated, and the protein concentrations in the sample were adjusted to 80 μg/ml (RAW264.7 cells) or 25 μg/ml (peritoneal macrophages). Fluorescence intensity in the sample was measured with a spectrofluorometer (F-2000, Hitachi), with excitation at 524 nm and emission at 567 nm (30).

Statistical analysis

Values are expressed as the mean ± SEM of 3–5 separate experiments. Student's *t*-test was used to analyze the significance of differences between two conditions. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of PLA$_2$ inhibitors on oxLDL-induced lipid metabolism

As shown in **Fig. 1A**, stimulation of [^{3}H]oleic acid-labeled RAW264.7 macrophages with oxLDL (50 μg/ml) induced the formation of [^{3}H]cholesteryl oleate time dependently. During the course of the formation, free [^{3}H]oleic acid increased, with a maximum observed 3 h after the stimulation (Fig. 1B), and [^{3}H]oleoyl phosphatidylcholine decreased (Fig. 1C). These findings seem to indicate that the oxLDL-induced production of cholesteryl oleate occurs in parallel with the release of oleic acid resulting

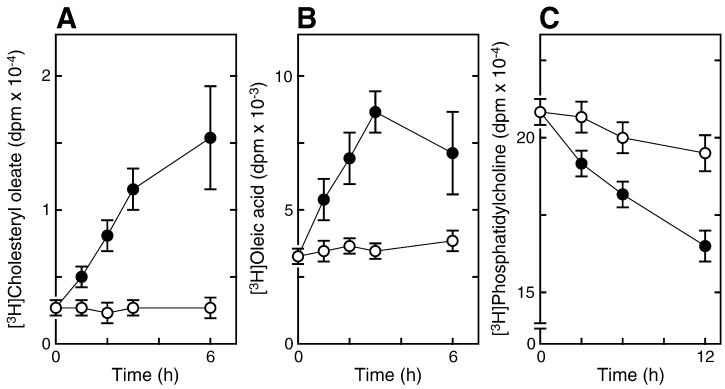

Fig. 1. Changes in cholesteryl oleate (A), free oleic acid (B), and phosphatidylcholine (C) in oxidized LDL (oxLDL)-stimulated RAW264.7 macrophages. [^{3}H]oleic acid-labeled cells were stimulated with (closed symbols) or without (open symbols) oxLDL (50 μg/ml) for the indicated periods. After lipid extraction, the radioactivity of cholesteryl oleate (A), free oleic acid (B), and phosphatidylcholine (C) was measured. Each point shown in C represents the mean ± ranges of two separate experiments.

from PLA_2-mediated hydrolysis of phosphatidylcholine. Therefore, to examine the possible involvement of PLA_2 in the cholesteryl oleate formation, the effects of MAFP, a $cPLA_2$ inhibitor, were tested as shown in **Fig. 2**. Pretreatment with MAFP (2–10 μM) suppressed oxLDL-induced cholesteryl oleate formation as well as oleic acid release in a dose-dependent manner (Fig. 2A, B). Under the conditions, $cPLA_2$ activity in the MAFP-pretreated cells was inhibited dose dependently (Fig. 2C). When [^{3}H]arachidonic acid-labeled RAW264.7 cells were stimulated with oxLDL (50 μg/ml), an increase in cholesteryl arachidonate (3,783 dpm; basal level, 714 dpm; the mean of two separate experiments) was observed and suppressed by MAFP (10 μM) (1,366 dpm; basal level, 634 dpm). Because MAFP is known to inhibit the activity of $iPLA_2$ in addition to that of $cPLA_2$ (24), we tested this possibility. As shown in **Fig. 3C**, $iPLA_2$ activity in MAFP- (10 μM) pretreated RAW264.7 cells was also inhibited. Therefore, to clarify whether the suppression by MAFP of oxLDL-induced oleic acid release and cholesteryl oleate formation results from inhibition of $iPLA_2$ activity, the effects of bromoenol lactone, an $iPLA_2$ inhibitor, were also examined. However, Fig. 3 showed that bromoenol lactone (2–10 μM) did not affect oxLDL-induced cholesteryl oleate formation or oleic acid release despite inhibiting $iPLA_2$ activity. Furthermore, under conditions where [^{3}H]oleic acid-labeled RAW264.7 cells were treated with manoalide (2 or 5 μM), an inhibitor for secretory PLA_2, the oxLDL- (50 μg/ml) induced formation of cholesteryl oleate (9,625 dpm; basal level, 2,473 dpm; the mean of two separate experiments) was not affected by 2 μM (11,282 dpm; basal level, 2,640 dpm) or 5 μM (9,071 dpm; basal level, 2,877 dpm) manoalide.

In [^{3}H]oleic acid-labeled mouse peritoneal macrophages, stimulation with oxLDL (50 μg/ml, 3–12 h) induced time-dependent increases in [^{3}H]cholesteryl oleate and free [^{3}H]oleic acid and a decrease in [^{3}H]oleoyl phosphatidylcholine, with marked changes observed 12 h after the stimulation (data not shown). Using mouse peritoneal macrophages, we examined the effects of MAFP on the oxLDL-induced lipid metabolism. As shown in **Fig. 4A** and B, pretreatment with MAFP (2-10 μM) dose dependently suppressed the oxLDL- (50 μg/ml) induced cholesteryl oleate formation and oleic acid release. Furthermore, MAFP (10 μM) also inhibited the decrease in oleoyl phosphatidylcholine induced by oxLDL (Fig. 4C).

MAFP has no effect on ACAT activity or binding and/or uptake of oxLDL

Acyl-CoA synthetase catalyzes the conversion of free fatty acids to fatty acyl-CoA, which is, in turn, utilized for ACAT-catalyzed cholesterol esterification. Therefore, it is possible that the inhibition by MAFP of oxLDL-induced cholesteryl oleate formation might be due to inhibitory effects on the activity of acyl-CoA synthetase or ACAT. However, MAFP is shown to have no effect on acyl-CoA synthetase activity (24). We also showed (**Fig. 5A**) that MAFP (10 μM) did not affect the incorporation of [^{3}H]oleic acid into phosphatidylcholine, whereas triacsin C (10 μM), an acyl-CoA synthetase inhibitor (31), as a control,

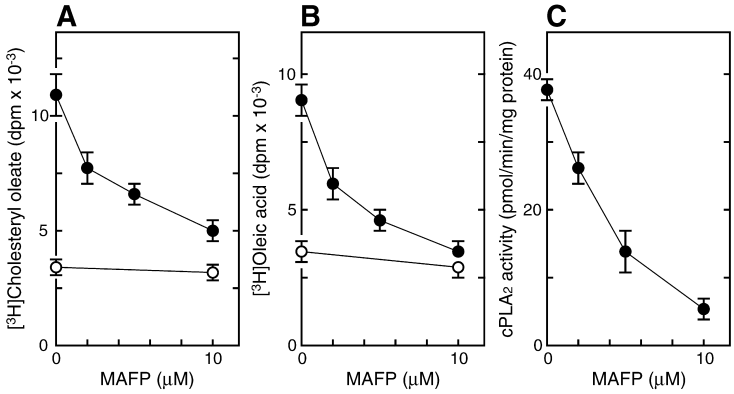

Fig. 2. Effects of methyl arachidonyl fluorophosphonate (MAFP) on cholesteryl oleate formation (A) and oleic acid release (B) in response to oxLDL, and on cytosolic PLA_2 ($cPLA_2$) activity (C) in RAW264.7 macrophages. A, B: [^{3}H]oleic acid-labeled cells were treated with various concentrations of MAFP for 1 h, and then stimulated with (closed symbols) or without (open symbols) oxLDL (50 μg/ml) for 3 h. After lipid extraction, the radioactivity of cholesteryl oleate (A) and free oleic acid (B) was measured. C: Cells were treated with various concentrations of MAFP for 1 h. The activity of $cPLA_2$ in the cytosol fraction of the cells was measured. Each point shown in (C) represents the mean ± ranges of two separate experiments.

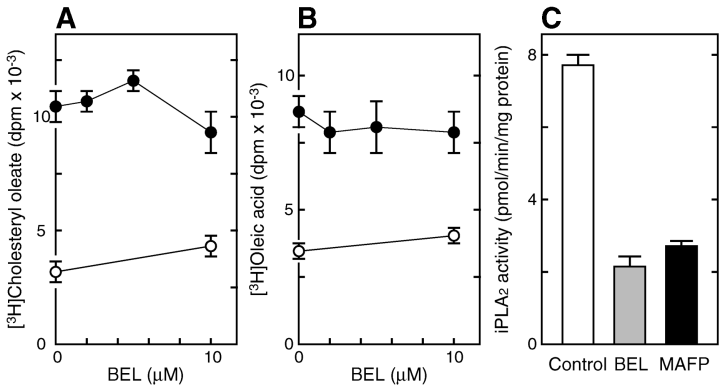

Fig. 3. Effects of bromoenol lactone on cholesteryl oleate formation (A) and oleic acid release (B) in response to oxLDL, and on Ca^{2+}-independent cytosolic PLA_2 ($iPLA_2$) activity (C) in RAW264.7 macrophages. A, B: [^{3}H]oleic acid-labeled cells were treated with various concentrations of bromoenol lactone (BEL) for 1 h, and then stimulated with (closed symbols) or without (open symbols) oxLDL (50 μg/ml) for 3 h. After lipid extraction, the radioactivity of cholesteryl oleate (A) and free oleic acid (B) was measured. C: Cells were treated with or without (Control) BEL (10 μM) or MAFP (10 μM) for 1 h. The activity of $iPLA_2$ in lysate of the cells was measured. Each value shown in (C) represents the mean ± ranges of two separate experiments.

prevented the incorporation, indicating that MAFP has no effect on enzymes, including acyl-CoA synthetase, which are responsible for the incorporation. Furthermore, no effect of MAFP (5 or 10 μM) on ACAT activity was observed in RAW264.7 cells and mouse peritoneal macrophages (Fig. 5B).

A recent report showed that nordihydroguaiaretic acid, known as an antioxidant and used as a PLA_2 inhibitor in the report, inhibits the binding and/or uptake of acetylated LDL in mouse peritoneal macrophages (30). We also examined effects of MAFP on the binding and/or uptake of oxLDL using DiI-labeled oxLDL. As shown in Fig. 5C, stimulation of RAW264.7 cells or mouse peritoneal macrophages with DiI-labeled oxLDL (20 or 50 μg/ml) resulted in a marked increase in fluorescence intensity in the cell lysate, while autofluorescence of the sample, prepared from cells stimulated with unlabeled oxLDL (50 μg/ml), was less than 10 units. Under the conditions, however, significant inhibitory effects of MAFP (10 μM) on the increase in fluorescence intensity were not observed (Fig. 5C). We confirmed that fucoidan, as a control experiment, prevented the increase in fluorescence intensity induced by DiI-labeled oxLDL (data not shown).

Inhibitory effect of MAFP on cholesteryl ester formation induced by oxLDL in the presence of [^{3}H]cholesterol

The present results, shown in Figs. 2–4, suggest a role for $cPLA_2$ in the oxLDL-induced formation of cholesteryl ester bearing a fatty acyl moiety derived from cellular phospholipids. It has been reported that the uptake by mouse peritoneal macrophages of liposomes containing cholesterol and phosphatidylcholine bearing a radioactive fatty acyl chain induces an increase in radioactive cholesteryl ester (15). Thus, it is likely that fatty acids derived from cellular and exogenous phospholipids are utilized for the cholesteryl ester formation. Therefore, to estimate the contribution of $cPLA_2$ to the supply of fatty acids utilized for cholesterol esterification in response to oxLDL, we determined the effects of MAFP on the formation of [^{3}H]cholesteryl ester induced by the combination of oxLDL with [^{3}H]cholesterol. As shown in **Fig. 6A**, stimulation of RAW264.7 cells with oxLDL (50 μg/ml) in the presence of [^{3}H]cholesterol (10 μM) increased [^{3}H]cholesteryl ester bearing fatty acyl chains derived from cellular and/or exogenous (oxLDL) lipids. We found that pretreatment with MAFP (2–10 μM) suppressed the increase in [^{3}H]cholesteryl ester dose dependently (Fig. 6A). Under conditions in which oleic acid (5 or 10 μM) was further added to the oxLDL-stimulated cells after treatment with MAFP (10 μM), addition of oleic acid overcame the inhibitory effect of MAFP on oxLDL-induced [^{3}H]cholesteryl ester formation (Fig. 6B). Similar results were also observed in mouse peritoneal macrophages (Fig. 6C).

Acceleration of $cPLA_2$-mediated fatty acid release by oxLDL and 13-HODE

To examine the mechanisms by which oxLDL stimulates $cPLA_2$-mediated fatty acid release, the effects of oxLDL on the activity and amounts of $cPLA_2$ were determined (**Fig. 7A**, **B**). However, stimulation with oxLDL (50

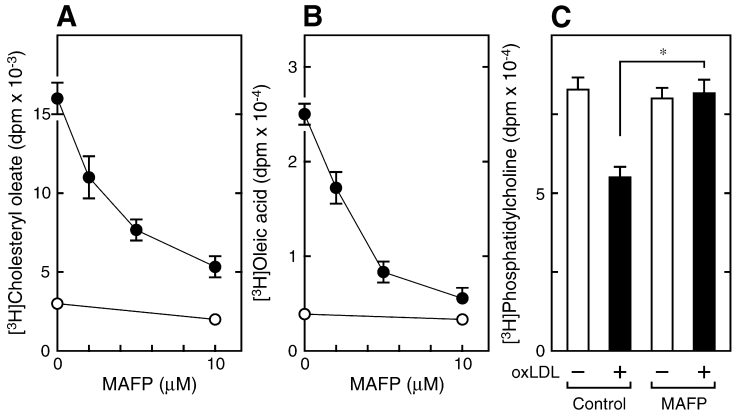

Fig. 4. Effects of MAFP on cholesteryl oleate formation (A), oleic acid release (B), and hydrolysis of phosphatidylcholine (C) in oxLDL-stimulated mouse peritoneal macrophages. A, B: [^{3}H]oleic acid-labeled cells were treated with various concentrations of MAFP for 1 h, and then stimulated with (closed symbols) or without (open symbols) oxLDL (50 μg/ml) for 12 h. C: [^{3}H]oleic acid-labeled cells were treated with or without (Control) MAFP (10 μM) for 1 h, and then stimulated with or without oxLDL (50 μg/ml) for 12 h. After lipid extraction, the radioactivity of cholesteryl oleate (A), free oleic acid (B), and phosphatidylcholine (C) was measured. * $P < 0.01$.

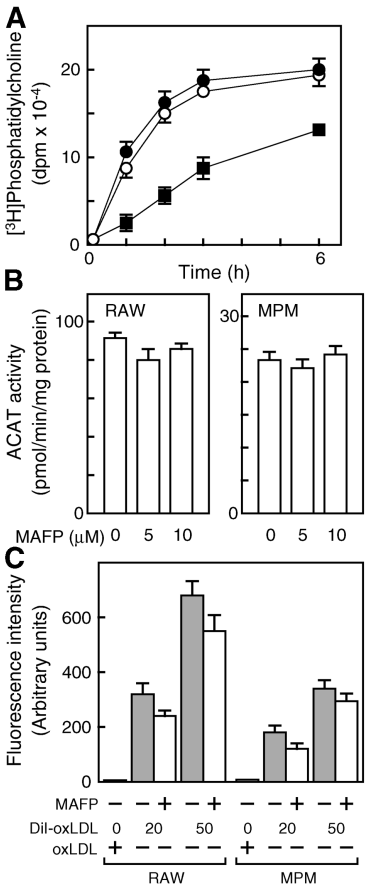

Fig. 5. Effects of MAFP on incorporation of [^{3}H]oleic acid into phosphatidylcholine (A), ACAT activity (B), and binding and/or uptake of DiI-labeled oxLDL. A: RAW264.7 macrophages were treated with or without (open circles) MAFP (10 μM, closed circles) or triacsin C (10 μM, closed squares) for 1 h, and then incubated with [^{3}H]oleic acid (0.5 μCi/ml) for the indicated periods. After lipid extraction, the radioactivity of phosphatidylcholine was measured. Each point shown in (A) represents the mean ± ranges of two separate experiments. B: RAW264.7 macrophages (RAW) or mouse peritoneal macrophages (MPM) were incubated with cholesterol (10 μM) for 12 h. After the cells were washed, the membrane fraction of the cells was treated with or without MAFP (5 or 10 μM) for 10 min. The activity of ACAT in the mixture was measured. C: RAW264.7 macrophages (RAW) or mouse peritoneal macrophages (MPM) were treated with or without MAFP (10 μM) for 1 h, and then stimulated with or without DiI-labeled oxLDL (20 or 50 μg/ml) or unlabeled oxLDL (50 μg/ml) for 3 h (RAW) or 12 h (MPM). After the cells were washed, fluorescence intensity in lysate of the cells was measured.

μg/ml) did not affect the activity or amounts. Therefore, to examine whether oxLDL facilitates the hydrolytic action of cPLA$_2$ toward membrane phospholipids, we determined the effects of oxLDL on fatty acid release induced by ionomycin, a Ca^{2+} ionophore (Fig. 7C, D). Stimulation with ionomycin (2 μM) alone induced the release of oleic acid (Fig. 7C) and arachidonic acid (Fig. 7D). When cells were preincubated with oxLDL (50 μg/ml) and washed before stimulation with ionomycin (2 μM), oxLDL enhanced the ionomycin-induced release of oleic acid and arachidonic acid. In contrast, preincubation with LDL (50 μg/ml) did not affect the ionomycin-induced release of oleic acid (control, 2,348 ± 163 dpm; LDL alone, 2,471 ± 194 dpm; ionomycin alone, 5,858 ± 433 dpm; LDL + ionomycin, 5,935 ± 578 dpm; n = 3). It has been suggested that the Ca^{2+} ionophore (A23187)-induced release of arachidonic acid or oleic acid is mediated by iPLA$_2$ in A-10 smooth muscle cells (32) or by secretory PLA$_2$ in mouse epidermal keratinocytes (33). However, we confirmed that the ionomycin-induced release of oleic acid and arachidonic acid was almost completely suppressed by MAFP but not bromoenol lactone (data not shown). These results suggest that oxLDL accelerates the hydrolytic action of cPLA$_2$. Previously, we showed that an increase in oxidized lipids in platelet membranes potentiates cPLA$_2$-catalyzed arachidonic acid release in response to ionomycin with no influence on cPLA$_2$ activity (34). Therefore, it is possible that oxidized lipids in oxLDL are involved in the stimulatory effect of oxLDL on ionomycin-induced fatty acid release. To examine this possibility, the effects of 13-HODE (35, 36) and 7-ketocholesterol (37, 38), oxidized lipids in oxLDL, were determined. As shown in **Fig. 8**, when cells were preincubated with each oxidized lipid before stimulation with ionomycin (2 μM), 13-HODE (2–10 μM) accelerated the ionomycin-induced release of oleic acid dose dependently, whereas 7-ketocholesterol did not exhibit a significant stimulatory effect, even at 50 μM. Furthermore, 13-HODE (10 μM) alone induced a slight but significant oleic acid release.

DISCUSSION

The transformation of oxLDL-stimulated macrophages into foam cells is associated with the accumulation of cytoplasmic lipid droplets consisting of cholesteryl ester, which is formed by the ACAT-catalyzed reesterification of free cholesterol derived from the endocytosed oxLDL particles. However, little is known about the mechanisms responsible for the supply of fatty acids, precursors for fatty acyl-CoA required for the cholesterol esterification. The present study was undertaken to investigate the possible involvement of cPLA$_2$ in the supply of fatty acids utilized for cholesteryl ester formation and the regulation of cPLA$_2$ activation in oxLDL-stimulated macrophages.

Involvement of cPLA$_2$ in the oxLDL-stimulated cholesteryl ester formation

It has been shown that oxLDL stimulates arachidonic acid release, which is catalyzed by cPLA$_2$ but not iPLA$_2$ in mouse peritoneal macrophages (19), and that acetylated LDL induces an increase in cholesteryl arachidonate with a decrease in arachidonoyl phosphatidylcholine in rat macrophages (16). We showed here that oxLDL induced cholesteryl arachidonate formation, which was suppressed by MAFP, a cPLA$_2$ inhibitor, in [^{3}H]arachidonic acid-labeled RAW264.7 macrophages. These findings suggest that the hydrolysis of membrane phospholipids by cPLA$_2$ is facilitated, and the released arachidonic acid is utilized for the cholesterol esterification in the oxLDL-stimulated macrophages.

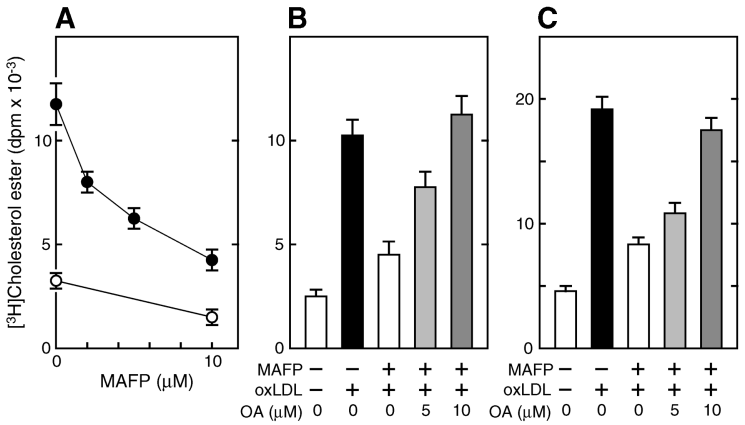

Fig. 6. Effects of MAFP on cholesteryl ester formation in response to oxLDL in the presence of [^{3}H]cholesterol. A: RAW264.7 macrophages were treated with various concentrations of MAFP for 1 h, and then stimulated with (closed symbols) or without (open symbols) oxLDL (50 μg/ml) for 3 h in the presence of [^{3}H]cholesterol (10 μM). B: RAW264.7 macrophages were treated with or without MAFP (10 μM) for 1 h, and then stimulated with or without oxLDL (50 μg/ml) for 3 h in the presence of oleic acid (OA, 5 or 10 μM) and [^{3}H]cholesterol (10 μM). C: Mouse peritoneal macrophages were treated with MAFP and stimulated with oxLDL for 12 h in the presence of oleic acid (OA) and [^{3}H]cholesterol as in (B). After lipid extraction, the radioactivity of cholesteryl ester was measured.

A previous study showed that foam cells in the fatty streak lesions contain cholesteryl oleate (50%) more than cholesteryl linoleate (15%) or cholesteryl arachidonate (3%), while cholesteryl linoleate (50%) is a major cholesteryl ester (cholesteryl oleate, 25%; cholesteryl arachidonate, 5%) in human LDL (13). This finding suggests that free oleic acid is mainly utilized for the reesterification of free cholesterol associated with the uptake of oxLDL in macrophages. Therefore, [^{3}H]oleic acid-labeled macrophages were used in the present experiments to determine cholesteryl ester formation. In this study, a time-dependent increase in cholesteryl oleate and decrease in phosphatidylcholine were observed in [^{3}H]oleic acid-labeled RAW264.7 cells in response to oxLDL (Fig. 1). The oxLDL-induced changes in these lipids are consistent with previous results with mouse and rat peritoneal macrophages in response to acetylated LDL or liposomes containing cholesterol (15, 16). We further demonstrated here that oxLDL stimulated oleic acid release in parallel with the changes in cholesteryl oleate and phosphatidylcholine (Fig. 1), suggesting that oleic acid liberated from phosphatidylcholine is utilized for the formation of cholesteryl oleate.

It is widely accepted that cPLA$_2$ preferentially hydrolyzes phospholipids bearing an arachidonoyl residue at the *sn*-2 position (20). Considering the substrate specificity, it seems probable that while the acyl moiety of cholesteryl arachidonate is supplied by cPLA$_2$-catalyzed hydrolysis of arachidonoyl phospholipids, other pathways might be involved in the supply of oleic acid utilized for the formation of cholesteryl oleate. However, cPLA$_2$ purified from human monocytic THP-1 cells has been shown to equally hydrolyze arachidonic acid- and linoleic acid-containing phospholipids when membrane phospholipids are used as a substrate (39), suggesting that cPLA$_2$ contributes somewhat to the release of fatty acids other than arachidonic acid in cells. Recently, we reported that MAFP suppresses the formation of cholesteryl oleate induced by ox-

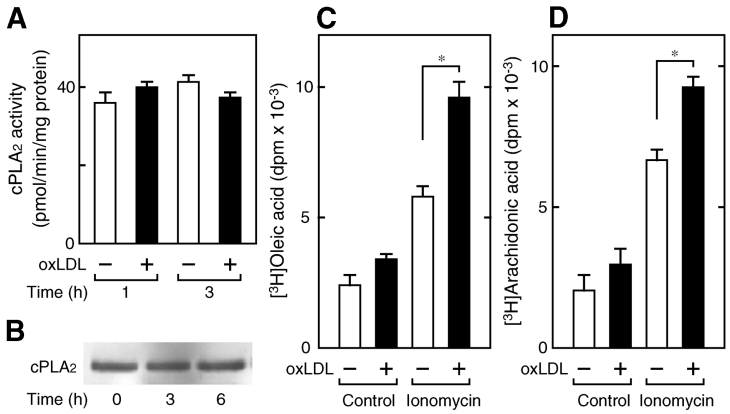

Fig. 7. Effects of oxLDL on the activity (A) and amounts (B) of cPLA$_2$, and on ionomycin-induced release of oleic acid (C) and arachidonic acid (D) in RAW264.7 macrophages. A: Cells were stimulated with or without oxLDL (50 μg/ml) for 1 or 3 h. The activity of cPLA$_2$ in the cytosol fraction of the cells was measured. Each value shown in (A) represents the mean ± ranges of two separate experiments. B: Cells were stimulated with oxLDL (50 μg/ml) for 3 or 6 h. Proteins of cPLA$_2$ in the cells were analyzed by immunoblotting. C: [^{3}H]oleic acid-labeled cells were stimulated with or without oxLDL (50 μg/ml) for 3 h. After being washed, the cells were stimulated with or without (Control) ionomycin (2 μM) for 30 min. The radioactivity of free oleic acid was measured. D: [^{3}H]arachidonic acid-labeled cells were stimulated with oxLDL as in (C). The cells were further stimulated with ionomycin for 10 min. The radioactivity of free arachidonic acid was measured. * $P < 0.01$.

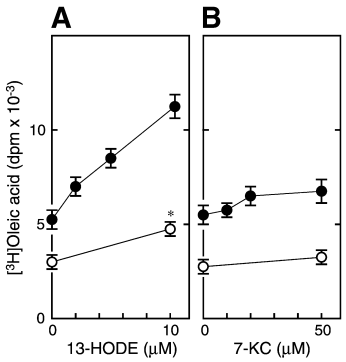

Fig. 8. Effects of 13(*S*)-hydroxyoctadecadienoic acid (13-HODE) (A) and 7-ketocholesterol (B) on ionomycin-induced oleic acid release in RAW264.7 macrophages. [^{3}H]oleic acid-labeled cells were treated with various concentrations of 13-HODE (A) or 7-ketocholesterol (B, 7-KC) for 3 h, and then stimulated with (closed symbols) or without (open symbols) ionomycin (2 μM) for 30 min. After lipid extraction, the radioactivity of free oleic acid was measured. * $P <$ 0.01, relative to the response of untreated cells.

LDL in RAW264.7 macrophages (23). The present study further showed that the inhibition by MAFP of oxLDL-induced cholesteryl oleate formation occurred in parallel with the suppression of oleic acid release and of cPLA$_2$ activity (Fig. 2). Furthermore, we showed with mouse peritoneal macrophages that MAFP exhibited similar inhibitory effects on the oxLDL-induced cholesteryl oleate formation, oleic acid release, and hydrolysis of phosphatidylcholine (Fig. 4). Under the conditions, iPLA$_2$ activity in the MAFP-treated RAW264.7 cells was also inhibited (Fig. 3C), as reported previously (24). However, bromoenol lactone, an iPLA$_2$ inhibitor, did not affect oxLDL-induced cholesteryl oleate formation or oleic acid release despite inhibiting iPLA$_2$ activity (Fig. 3). Moreover, MAFP had no influence on the acyl-CoA synthetase activity (Fig. 5A) (24), the ACAT activity (Fig. 5B), or the binding and/or uptake of DiI-labeled oxLDL (Fig. 5C). These results indicate that the inhibitory effect of MAFP on the oxLDL-induced cholesteryl oleate formation depends on the suppression of oleic acid release, hydrolysis of phosphatidylcholine, and cPLA$_2$ activity. It has been reported that group IIA and group X secretory PLA$_2$s are markedly expressed in the arterial intima or media of human and mouse atherosclerotic lesions (40–42), and contribute to the facilitation of uptake of LDL by macrophages to form foam cells through the modification (hydrolysis) of LDL (42, 43). Furthermore, oxLDL has been shown to accelerate the expression of group IIA secretory PLA$_2$ in human monocyte-derived macrophages (44). In the present study, we showed that the oxLDL-induced formation of cholesteryl oleate was insensitive to manoalide, a secretory PLA$_2$ inhibitor, in RAW264.7 macrophages. Thus, secretory PLA$_2$ plays a role in the modification of LDL, but is not implicated in the formation of cholesteryl oleate under our experimental conditions. It has been shown that LDL contains lipoprotein-associated PLA$_2$, also known as platelet-activating factor acetylhydrolase, which participates in the modification of oxLDL through the hydrolysis of oxidized phospholipids (45). Because the PLA$_2$ hydrolyzes oxidized, but not nonoxidized, phospholipids (46, 47), it seems unlikely that this enzyme directly hydrolyzes cellular [^{3}H]oleoyl phosphatidylcholine to liberate free [^{3}H]oleic acid. Considering these findings, therefore, we suggest that fatty acids released by cPLA$_2$-catalyzed hydrolysis of cellular phospholipids are utilized, at least in part, for the cholesterol esterification in response to oxLDL.

It has been reported that the uptake by mouse peritoneal macrophages of liposomes containing cholesterol and phosphatidylcholine bearing a radioactive fatty acyl chain induces an increase in radioactive cholesteryl ester (15), indicating that fatty acids derived from exogenous phospholipids are also utilized for cholesteryl ester formation. The present study showed that stimulation with oxLDL in the presence of [^{3}H]cholesterol increased [^{3}H]cholesteryl ester bearing fatty acyl chains derived from cellular and/or exogenous (oxLDL) lipids (Fig. 6). Under the conditions, MAFP was found to significantly inhibit the oxLDL-induced [^{3}H]cholesteryl ester formation, and the inhibitory effect of MAFP was reversed by addition of oleic acid (Fig. 6). This observation also suggests that fatty acyl chains of cholesteryl esters formed in response to oxLDL are supplied from cellular phospholipids by the hydrolytic action of cPLA$_2$ in macrophages.

Activation of cPLA$_2$ by oxLDL

A previous report demonstrated that an increase in cPLA$_2$ is observed in human atherosclerotic lesions (40). However, oxLDL fails to stimulate the expression of cPLA$_2$ mRNA in human monocyte-derived macrophages (44). This inability of oxLDL is consistent with the present result that oxLDL did not affect amounts of cPLA$_2$ proteins (Fig. 7B). On the other hand, it has been reported that stimulation with oxLDL for 24 h increases cPLA$_2$ activity in bovine retinal pericytes (48) and rat mesangial cells (49). In contrast, under our experimental conditions, in which stimulation of RAW264.7 macrophages with oxLDL for 1–3 h induced oleic acid release, no change in cPLA$_2$ activity was observed (Figs. 1B, 7A). Furthermore, our results showed that oxLDL enhanced the ionomycin-induced release of oleic acid and arachidonic acid (Fig. 7C, D). On the basis of the present results, we suggest that oxLDL accelerates the hydrolytic action of cPLA$_2$ toward membrane phospholipids, thus inducing the release of oleic acid.

With regard to the mechanisms responsible for the acceleration by oxLDL of the hydrolytic action of cPLA$_2$, in a recent report we proposed the possible involvement of ceramide (23). In the report, we showed that oxLDL induces an increase in ceramide, which is suppressed by fumonisin B$_1$, an inhibitor of the de novo synthesis of ceramide, with the formation of cholesteryl oleate in RAW264.7 macrophages. However, while MAFP inhibits almost completely the formation of cholesteryl oleate, the inhibitory effect of fumonisin B$_1$ on the formation is partial (about 40% inhibition) (23), suggesting that factors other than ceramide are also involved in the acceleration by oxLDL of the hydrolytic action of cPLA$_2$. In this study,

we showed that the ionomycin-induced oleic acid release was enhanced by preincubation with 13-HODE, an oxidized lipid in oxLDL particles (Fig. 8A). It was reported that oxidized fatty acids including 13-HODE are formed during the oxidation of LDL (35, 36), probably by the hydrolysis of oxidized phospholipids by lipoprotein-associated PLA_2 (45). Furthermore, we confirmed that preincubation with native LDL did not enhance the ionomycin-induced release of oleic acid. Therefore, we speculate that oxidized lipids in oxLDL particles, such as 13-HODE, are involved in the acceleration by oxLDL of the hydrolytic action of $cPLA_2$, although further study is needed to clarify the mechanisms responsible for the stimulatory effect of 13-HODE.

In summary, we demonstrated in the present study that the accumulation of cholesteryl esters associated with the uptake of oxLDL in macrophages depends on the activation of $cPLA_2$, which contributes, at least in part, to the supply of fatty acids required for the cholesterol esterification. Furthermore, the activation of $cPLA_2$ by oxLDL may be ascribed to the acceleration of the hydrolytic action of $cPLA_2$ by oxidized lipids in oxLDL particles.

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