Inhibition of Cytosolic Phospholipase A₂ Suppresses Production of Cholesteryl Ester through the Reesterification of Free Cholesterol but not Formation of Foam Cells in Oxidized LDL-Stimulated Macrophages

Hiromi Ii, Mayuko Oka, Atsushi Yamashta, Keizo Waku, Naonori Uozumi, Takao Shimizu, Takashi Sato, and Satoshi Akiba

Department of Pathological Biochemistry, Kyoto Pharmaceutical University; Misasagi, Yamashina-ku, Kyoto 607-8414, Japan; Faculty of Pharmaceutical Sciences, Teikyo University; Sagamiko, Kanagawa 229–0195, Japan; and Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo; Bunkyo-ku, Tokyo 113–0033, Japan.

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Macrophage-derived foam cells are formed as a result of the accumulation of cholesteryl ester (CE) not only in cytoplasm where CE is produced by the reesterification of free cholesterol derived from oxidized low density lipoprotein (OxLDL) undergoing hydrolysis, but also in lysosomes where the remaining CE of OxLDL is deposited. We examined the possible involvement of cytosolic phospholipase A₂ (cPLA₂) isoforms in the production of CE through the reesterification and in the formation of foam cells. In [³H]oleic acid-labeled human acute monocytic leukemia (THP-1) cell-derived macrophages (THP-M) and mouse peritoneal macrophages (MPM), which possessed at least cPLA₂α and cPLA₂γ, stimulation with OxLDL induced the production of [³H]cholesteryl oleate ([³H]CE). The production was suppressed by an inhibitor of cPLA₂. However, the inhibitor tended to slightly decrease total intracellular levels of CE, and did not affect the formation of foam cells, as estimated by staining with Oil Red O. In cPLA₂γ-knockout MPM, OxLDL-induced increases in [³H]CE and total CE did not differ from those in wild-type MPM. Our results suggest that cPLA₂ other than cPLA₂α contribute to the supply of fatty acids, which are utilized for the production of CE through the reesterification, in OxLDL-stimulated macrophages. However, the formation of foam cells could not be inhibited only by the suppression of cPLA₂-mediated CE production.

Key words: foam cell; oxidized low density lipoprotein; phospholipase A₂; cholesteryl ester; macrophage

Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of the sn-2 ester bond in glycerophospholipids to generate nes-terified fatty acids and lysophospholipids. These products are known to act as second messengers for cellular signaling and as biologically active lipid mediators involved in homeostasis or various pathological conditions, mainly inflammatory diseases including atherosclerosis. Among them, secretory PLA₂ type II (sPLA₂-II), a secretory PLA₂ isoform, and cytosolic PLA₂ (cPLA₂, also known as PLA₂ type IVA), a cytosolic PLA₂ (cPLA₂) isoform, have been shown to increase in carotid atherosclerotic lesions. sPLA₂-II hydrolyzes phospholipids of apolipoprotein B-100-containing lipoproteins, such as low density lipoprotein (LDL), resulting in the accumulation of the modified lipoproteins within the intima. The sPLA₂-II-modified lipoproteins are more susceptible to further enzymatic and oxidative modifications and to uptake by macrophages. Thus, sPLA₂-II plays a critical role in the formation of atherosclerotic lesions. In contrast, roles of cPLA₂ in atherogenesis are not yet completely elucidated.

In atherosclerotic lesions, the deposition of macrophage-derived lipid-laden foam cells is associated with the accumulation of intracellular free cholesterol (FC) and cholesteryl ester (CE) resulting from the uptake of modified LDL, especially oxidized LDL (OxLDL), by macrophages. Normally, modified LDL is delivered to lysosomes for the hydrolysis of CE to FC and free fatty acid, which can be transported out of the lysosomes. Excess extralysosomal FC, because of its poor efflux, is reesterified to an acyl-CoA by the catalytic action of acyl-CoA:cholesterol acyltransferase (ACAT) and stored as CE in cytoplasm. Recently, we demonstrated that in [³H]oleic acid-labeled mouse peritoneal macrophages (MPM) and RAW264.7 macrophages, methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of cPLA₂, suppressed the production of [³H]cholesteryl oleate and release of free [³H]oleic acids induced by OxLDL. Furthermore, we and other investigators showed that among cPLA₂ isoforms, OxLDL promoted the hydrolytic action of cPLA₂α in MPM. These findings suggest that cPLA₂ including cPLA₂α are involved in the supply of free fatty acids, precursors for acyl-CoA required for the reesterification of FC. However, the roles of cPLA₂ in the formation of foam cells remain unclear.

The formation of foam cells has been reported to be induced by LDLs undergoing modification including acetylation, oxidation, and aggregation in in vitro experiments. Acetylated LDL (AcLDL) can induce the formation of foam cells in association with the accumulation of CE. Much CE was produced through the reesterification of FC induced by AcLDL than that induced by OxLDL. However, only OxLDL has been indicated to exist in atherosclerotic lesions and plasma, with OxLDL levels found to be higher in patients with coronary heart disease than in healthy subjects. Furthermore, there are differences in the mechanisms of the formation of foam cells between OxLDL and AcLDL. OxLDL but not other modified LDLs including AcLDL induces lysosomal dysfunction through the inactivation of lysosomal proteases, and thereby the accumulation of CE in cytoplasm was not as great for OxLDL as for AcLDL. These findings indicate that not only the production of CE via the reesterification of FC, but also the dep-
osition of CE caused by lysosomal dysfunction contributes to the formation of foam cells upon stimulation with OxLDL. Considering this notion, it is unclear whether cPLA₂-mediated production of CE through reesterification is involved in the formation of foam cells induced by OxLDL. In the present study, therefore, we examined the contribution of cPLA₂s including cPLA₂α to the production of CE and to the formation of foam cells in response to OxLDL using human acute monocytic leukemia (THP-1) cell-derived macrophages (THP-M) and MPM derived from cPLA₂α-knockout (cPLA₂α-KO) mice.

MATERIALS AND METHODS

Materials [³H]Oleic acid (7 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA, U.S.A.). Human native LDL (BT-903) was from Biomedical Technologies Inc. (Stoughton, MI, U.S.A.). MAFP was from Cayman Chemical (Ann Arbor, MI, U.S.A.). Antibodies against cPLA₂α and CD11b were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) and R&D systems (Minneapolis, MN, U.S.A.), respectively. The affinity-purified rabbit polyclonal antibody against a peptide of cPLA₂γ was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan.

Cell Culture THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml glucose, 2 mM GlutaMax, 1 mM sodium pyruvate, and 10 mM HEPES (RPMI-medium) at 37 °C under humidified air containing 5% CO₂. THP-1 cells were plated in 24-well culture plates at 2 × 10⁵ cells and cultured for 5 d in RPMI-medium containing 40 nM phorbol 12-myristate 13-acetate (PMA) to induce the differentiation into macrophages. The medium was changed every 2 d, and used throughout the experiments. The PMA-treated cells, used as THP-M, were treated with MAFP and stimulated with OxLDL or AcLDL as described in figure legends. For the preparation of MPM, 10% peptone was injected into the peritoneal cavity of wild-type or cPLA₂α-KO mice in a C57BL/6 background. After 3 d, peritoneal cells were suspended in DMEM (Nissui Pharmaceutical Co., Ltd., Osaka, Japan). The resulting reaction mixture was subcloned into the BglII–EcoRI sites of pIRE2-EGFP (a gift from Dr. E. Hirayama, Kyoto Pharmaceutical University) to yield a plasmid carrying a sequence corresponding to the native cPLA₂γ. HEK293 cells were transfected with pIRE2-EGFP (negative control) or pIRE2-EGFP/cPLA₂γ (positive control of cPLA₂γ), using lipofectamine 2000 (Invitrogen) and colonies of EGFP-expressing cells were chosen. Proteins of cPLA₂γ in the membrane fraction were detected by immunoblotting.

Immunoblot Analysis For the detection of cPLA₂α, cPLA₂γ, and CD11b, cell lysate was solubilized with cell collection reagent (150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% sodium deoxycholate (w/v), 0.5% sodium dodecyl sulfate (w/v), protease inhibitors, and 50 mM Heps, pH 7.4) as described elsewhere, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel. Proteins were transferred to a nitrocellulose membrane, and then antibodies against cPLA₂α (×200), cPLA₂γ (×200), or CD11b (×500) were applied. The bound antibodies were visualized using a peroxidase-conjugated secondary antibody and Western blotting detection reagents (GE Healthcare BioScience).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The mRNA expression of cPLA₂α and cPLA₂γ in MPM was analyzed by RT-PCR. Total RNA was extracted from MPM with ISOGEN (NIPPON GENE Co., Ltd., Tokyo, Japan). First-strand cDNA was generated from the total RNA in a reaction mixture containing random ninemers, dNTP, RNase inhibitor, and ReverTra Ace (TOYOBO, Co., Ltd.). The resulting reaction mixture was subjected to PCR with AmpliTaq Gold using 5'-CTC TG TGT GAT GAA GGC ACT GTA TGC TGT GG-3' (sense) and 5'-ACG GCA GGT TAA ATG TGA GC-3' (antisense) for mouse cPLA₂α (Gen Bank accession no. NM_008869), and 5'-AAG GAG CAG TTT CTC GAC CA-3' (sense) and 5'-TTG GCA GTC TGC TGT GG-3' (antisense) for mouse cPLA₂γ (BC054740). Expression of β-actin was used as the internal standard. The PCR conditions were as follows: 94 °C for 10 min; followed by 30 cycles (for β-actin) or 35 cycles (for cPLA₂α and cPLA₂γ) of 95 °C for 60 s, 60 °C for 60 s, and 72 °C for 150 s. Products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

Determination of CE Content MPM or THP-M were treated with OxLDL for 12 h or 24 h, respectively, and washed with PBS. Lipids were extracted from the cells with hexane-2-propanol (3:2, v/v). CE was measured according to the method of Mizoguchi et al. Measurement of [³H]Cholesteryl Oleate ([³H]CE) Formation, [³H]Phosphatidylcholine Reduction, and [³H]Oleic acid Libration THP-1 cells were differentiated...
in PMA (40 nm)-containing RPMI-medium for 3 d. The cells were labeled with [3H]oleic acid (0.5 μCi/ml) for 18 h in the same fresh medium. MPM were incubated with [3H]oleic acid (0.5 μCi/ml) for 12 h. After being washed, the labeled THP-M and MPM were treated and stimulated as described in the figure legends. Lipids in the medium and cells were extracted and applied onto a Silica Gel G plate. The lipids of THP-M were separated by TLC with a developing solvent (petroleum ether/diethyl ether/acidic acid, 90 : 10 : 1, v/v/v). For the analysis of lipids of MPM by TLC, a solvent (chloroform/methanol/acidic acid, 65 : 25 : 10, v/v/v) was used and allowed to run one-third of the length of the plate. After drying, the plate was developed again in the same direction with another solvent (petroleum ether/diethyl ether/acidic acid, 80 : 20 : 1, v/v/v). This solvent was allowed to run to the top of the plate. The area corresponding to CE, free fatty acid, phosphatidylcholine, or other lipids was scraped off, and the radioactivity was measured by liquid scintillation counting.

Oil Red O Staining Cells were fixed with 3.7% formaldehyde in PBS for 30 min, then washed twice with PBS. The cells were stained with 0.5% Oil Red O in 50% 2-propanol for 30 min, and then washed sequentially with 70% ethanol and PBS. The stained cells were photographed using a microscope (Olympus IX71) with a digital camera. Statistical Analysis Values are expressed as the mean±S.E.M. Data were analyzed by Student’s t-test. p<0.05 was considered statistically significant.

RESULTS

Characterization of PMA-Differentiated THP-1 Macrophages and Modified LDL. CD11b is a subunit of Mac-1, which has been identified as a specific marker of the differentiation of monocytes into macrophages, and increases with the differentiation. Mac-1 is one of integrin family adhesion receptors that interact with ICAM-1 on endothelial cells, and is involved in the transendothelial migration of monocytes. We confirmed that when THP-1 cells were treated with 40 nm PMA for 6 d, the expression of CD11b increased time-dependently with a maximal increase 4—5 d after the treatment (Fig. 1A). In this study, therefore, THP-1 cells treated with PMA for 5 d were used as THP-M. Under the conditions, while cPLA2α was detected in THP-1 cells, a transient increase in this isoform was observed during the treatment with PMA (Fig. 1A). In contrast, we found that cPLA2γ, known as cytosolic (intracellular) PLA2 type IVC, was time-dependently increased by the treatment with PMA (Fig. 1A). Thus, the expression of cPLA2α and cPLA2γ was confirmed in THP-M.

The charge modification of OxLDL and AcLDL was monitored by agarose gel electrophoresis. The relative electrophoretic mobility of OxLDL and AcLDL was 2.3—2.6 and 3—3.5, respectively (Fig. 1B).

Production of CE and Formation of Foam Cells Induced by OxLDL and AcLDL in THP-M and MPM As shown in Fig. 2, stimulation of THP-M (Fig. 2A) or MPM (Fig. 2B) with OxLDL (50 μg/ml) or AcLDL (50 μg/ml) increased the mass of CE time-dependently with a maximal increase 24 h or 12 h after the stimulation. Under the conditions, intracellular lipids in THP-M apparently increased upon stimulation with OxLDL (50 μg/ml, for 24 h) (Figs. 3A-e, -d) and AcLDL (50 μg/ml, for 24 h) (Figs. 3A-i, -j) or AcLDL (Figs. 3A-k, -l), while it had no effect on intracellular lipids in unstimulated cells (Figs. 3A-a, -b). Furthermore, it is notable that the Oil Red O-staining patterns of intracellular lipids in OxLDL-stimulated THP-M differed from those in AcLDL-stimulated THP-M. The stained lipids in
OxLDL-stimulated cells were relatively small and present around the nucleus, while the lipids in AcLDL-stimulated cells were relatively large and formed droplets in the cytoplasm. Such differences were more clearly observed in MPM stimulated with OxLDL (Figs. 3B-b, -e) and AcLDL (Figs. 3B-c, -f). A few previous reports showed similar differences between OxLDL and AcLDL in macrophages.\(^{14,19}\)

**Effects of MAFP on the Production of CE and \(^{3}H\)CE Induced by OxLDL and AcLDL in THP-M**

To examine the possible involvement of cPLA\(_2\)s in the production of CE induced by OxLDL or AcLDL, the effects of MAFP were tested. The results shown in Fig. 4A demonstrated that pretreatment with MAFP (30 \(\mu\)g/ml) tended to slightly reduce the increase in CE induced by OxLDL, while it significantly suppressed the AcLDL (50 \(\mu\)g/ml)-induced accumulation of CE (Fig. 4A). To examine the effects of MAFP on production of CE through the reesterification of FC, \(^{3}H\)oleic acid-labeled THP-M were used. MAFP markedly inhibited an increase in \(^{3}H\)CE induced by OxLDL and AcLDL to the levels of unstimulated cells, although MAFP had no effect on the basal level (Fig. 4B). These findings suggest that MAFP-sensitive cPLA\(_2\)s are involved in the formation of CE that is produced through the reesterification of FC in response to OxLDL and AcLDL, but inhibition of cPLA\(_2\)s has no effect on the increase in total intracellular levels of CE in OxLDL-stimulated cells. Furthermore, it is possible that the main source of CE accumulated in OxLDL-stimulated THP-M is not the reesterification of FC.

**Effects of MAFP on the Production of CE and \(^{3}H\)CE Induced by OxLDL in MPM**

We further examine the
Roles of cPLA₂s in the production of CE induced by OxLDL in MPM. As shown in Figs. 5A and B, stimulation of MPM with OxLDL (50 μg/ml) for 12 h increased the mass of CE as well as [³H]CE. Pretreatment with MAFP (30 μM) significantly suppressed [³H]CE (Fig. 5B), but CE mass tended to slightly decrease (Fig. 5A). Furthermore, stimulation with OxLDL induced a decrease in [³H]oleoyl phosphatidylcholine and an increase in free [³H]oleic acid, as shown in Figs. 5C and D. MAFP also suppressed the OxLDL-induced hydrolysis of [³H]oleoyl-phosphatidylcholine and release of [³H]oleic acid. Based on these findings, we assumed that the OxLDL-induced production of [³H]CE through the reesterification of phospholipids. MAFP is known to inhibit the activity of calcium-independent PLA₂s (iPLA₂s) in addition to cPLA₂ and cPLA₂γ. However, bromoeanol lactone, an inhibitor of iPLA₂s, did not affect the OxLDL-induced release of [³H]oleic acid and production of [³H]CE (data not shown). Consequently, the present results suggest that fatty acids utilized for acyl chains of CE are supplied by the hydrolytic action of cPLA₂α and/or cPLA₂γ in OxLDL-stimulated MPM (Fig. 5) and THP-M (Fig. 4).

Production of CE and [³H]CE Induced by OxLDL in cPLA₂α-KO MPM To examine the possible involvement of cPLA₂α in the production of CE, MPM derived from cPLA₂α-KO mice were used. We found, however, that OxLDL (50 μg/ml) increased the mass of CE and [³H]CE in cPLA₂α-KO MPM, as shown in Fig. 6A and B. The degree of augmentation of CE did not differ between the MPM derived from cPLA₂α-KO and wild-type mice, while the production of [³H]CE was much greater in cPLA₂α-KO MPM than in wild-type MPM. Furthermore, the Oil Red O-staining patterns of intracellular lipids in cPLA₂α-KO MPM stimulated with OxLDL did not differ from those in wild-type MPM (Fig. 6C). In cPLA₂α-KO MPM, no expression of cPLA₂α protein and mRNA was detected (Figs. 6D, E). These findings indicate that cPLA₂α does not contribute to the supply of fatty acids utilized for the reesterification of FC. MAFP exhibited inhibitory effects on the OxLDL-induced increases in [³H]CE and free [³H]oleic acid even in cPLA₂α-KO MPM (data not shown). Since MAFP inhibits cPLA₂γ as well as cPLA₂α, it is possible that cPLA₂γ is involved in the supply of fatty acids utilized for the production of CE. To confirm the expression of cPLA₂γ in MPM, cPLA₂γ mRNA was examined by RT-PCR. The result indicates that cPLA₂γ mRNA was expressed in MPM derived from cPLA₂α-KO mice as well as wild-type mice (Fig. 6E).

DISCUSSION

The earliest atherosclerotic lesion is the fatty streak, which consists of an aggregation of lipid-rich macrophage-derived foam cells and T-lymphocytes within the innermost layer of the arterial wall. Fatty streaks are high in CE and high in cholesteryl oleate compared with their cholesteryl linolate content, while plasma LDL contains more cholesteryl linolate than cholesteryl oleate. It is conceivable that oleic acid, as an acyl chain of cholesteryl oleate produced in foam cells, is derived from cellular lipids, so it is effective at preventing the development of fatty streaks to inhibit the production of CE by suppressing the supply of fatty acids, precursors for acyl-CoA, from cellular lipids. In fact, triacsin C, an inhibitor of acyl-CoA synthetase, has been shown to inhibit the formation of foam cells induced by FC-rich liposomes in vitro. Previously, we and other investigators re-
[46x278]ment (N), total RNA extracted from wild-type macrophages was subjected to reverse
[46x318]cPLA2
[46x342]wild-type (Wt) and cPLA2
[46x50]OxLDL-induced production of CE, it is possible that free fatty acids utilized as acyl chains of CE are supplied, at least in part, through the hydrolysis of cellular phospholipids catalyzed by cPLA2\(\alpha\) and/or cPLA2\(\gamma\). The activation of cPLA2\(\alpha\) is regulated by phosphorylation and by a calcium-dependent translocation from the cytosol to the membranes.31) In cPLA2\(\alpha\)-overexpressing CHO cells, 25-hydroxycholesterol, an oxysterol present in OxLDL particles, induces the release of arachidonic acid and the translocation of the enzyme to the membranes.12) Recently, we also reported that cPLA2\(\alpha\) translocates to the membranes in MPM upon stimulation with OxLDL.11) Based on these findings indicating that OxLDL activates cPLA2\(\alpha\), it is possible that this enzyme participates in the production of CE. However, we found here that the production of \([^{3}H]\)CE and accumulation of total CE mass induced by OxLDL did not differ between the MPM derived from cPLA2\(\alpha\)-KO and wild-type mice (Fig. 6). Our results suggest that cPLA2\(\alpha\) does not contribute to the supply of free fatty acids utilized as acyl chains of CE. In the atherosclerotic lesions, foam cells contain more cholesteryl olate (50%) than cholesteryl arachidonate (3%).30) This also suggests a lack of contribution of cPLA2\(\alpha\) to the production of CE, because cPLA2\(\alpha\) preferentially hydrolyzes glycerophospholipids bearing an arachidonoyl residue at the sn-2 position. Other PLA2\(s\) including cPLA2\(\gamma\) do not exhibit such a preference. While cPLA2\(\alpha\) was expressed ubiquitously in the body, cPLA2\(\gamma\) was expressed in heart, skeletal muscle, brain,34) and monocytes from peripheral blood.35) We also showed here that cPLA2\(\gamma\) was expressed in MPM as well as THP-M (Figs. 1, 6). Furthermore, in contrast to cPLA2\(\alpha\), cPLA2\(\gamma\) is located in membrane fractions,25) especially in the endoplasmic reticulum,36) in which ACAT is also located.37) Considering these findings and our results that MAFP inhibited OxLDL-induced production of \([^{3}H]\)CE in not only THP-M and wild-type MPM (Figs. 3, 5) but also cPLA2\(\alpha\)-KO MPM (data not shown), all of which expressed cPLA2\(\gamma\) (Figs. 1, 6), it could be assumed that cPLA2\(\gamma\) is involved in the MAFP-sensitive production of \([^{3}H]\)CE. To clarify this possibility, further studies with cPLA2\(\gamma\)-knockdown THP-M are needed.

The CE within OxLDL taken up by macrophages is digested to FC and free fatty acid in lysosomes. The FC is reesterified by the catalytic action of ACAT to form CE, which is accumulated in the cytoplasm to protect cells from the toxicity of FC. However, the excessive uptake of modified LDL, especially OxLDL, leads to the formation of foam cells with dysfunctional lysosomes and the deposition of CE in lysosomes38) or induces apoptosis through damage of the endoplasmic reticulum with the accumulation of FC.39) Regarding targets to suppress the formation of foam cells, ACAT is considered one. Although an inhibitor of ACAT actually suppressed the reesterification of FC,40) suppression of ACAT results in the accumulation of intracellular FC, as shown in ACAT-deficient macrophages upon stimulation with AcLDL.41) In fact, an ACAT inhibitor did not inhibit the formation of foam cells induced by FC-rich liposomes.31) Similarly, hypercholesterolemic LDL receptor-knockout mice reconstituted with ACAT-deficient macrophages developed larger atherosclerotic lesions than control LDL receptor-knockout mice.42) Thus, ACAT contributes to the reesterification of FC, but its suppression causes the accumulation of FC followed by the formation of foam cells and/or FC-in-

![Fig. 6. Production of CE, Formation of Foam Cells, and Expression of cPLA2\(\alpha\) and cPLA2\(\gamma\) in cPLA2\(\alpha\)-KO MPM in Response to OxLDL](image-url)

\[^{3}H\]Oleic acid-labeled (B) or unlabeled (A, C—E) macrophages were prepared from wild-type (Wt) and cPLA2\(\alpha\)-KO mice. (A—C) Cells were stimulated with (+) or without (−) OxLDL (50 μg/ml) for 12 h. The mass of CE (A) and the radioactivity of \[^{3}H\]CE (B) were determined. Bright field micrographs of wild-type (Wt, C-a) and cPLA2\(\alpha\)-KO (C-b) macrophages stimulated with OxLDL are shown. (D) cPLA2\(\alpha\) proteins prepared from unstimulated Wt or cPLA2\(\alpha\)-KO macrophages were analyzed by immunoblotting. The expected sizes of products of RT-PCR are indicated. As a negative control experiment (N), total RNA extracted from wild-type macrophages was subjected to reverse transcription in the absence of reverse transcriptase followed by PCR. The resulting reaction mixture was also resolved on agarose gels.

The present study showed that OxLDL promoted the production of \[^{3}H\]CE in both THP-M and MPM, and the production was significantly suppressed by MAFP, an inhibitor of cPLA2\(\alpha\), cPLA2\(\gamma\), and iPLA2\(\alpha\) (Figs. 4, 5). Furthermore, MAFP inhibited a decrease in \[^{3}H\]phosphatidylcholine as well as the liberation of \[^{3}H\]oleic acid induced by OxLDL in MPM (Fig. 5). These findings are consistent with the results of our previous study with OxLDL-stimulated RAW264.7 macrophages and mouse resident peritoneal macrophages.10) Considering that an inhibitor of iPLA2\(\alpha\) had no effect on OxLDL-induced production of CE,10) it is possible that free fatty acids utilized as acyl chains of CE are supplied, at least in part, through the hydrolysis of cellular phospholipids catalyzed by cPLA2\(\alpha\) and/or cPLA2\(\gamma\). The activation of cPLA2\(\alpha\) is regulated by phosphorylation and by a calcium-dependent translocation from the cytosol to the membranes.31) In cPLA2\(\alpha\)-overexpressing CHO cells, 25-hydroxycholesterol, an oxysterol present in OxLDL particles, induces the release of arachidonic acid and the translocation of the enzyme to the membranes.12) Recently, we also reported that cPLA2\(\alpha\) translocates to the membranes in MPM upon stimulation with OxLDL.11) Based on these findings indicating that OxLDL activates cPLA2\(\alpha\), it is possible that this enzyme participates in the production of CE. However, we found here that the production of \[^{3}H\]CE and accumulation of total CE mass induced by OxLDL did not differ between the MPM derived from cPLA2\(\alpha\)-KO and wild-type mice (Fig. 6). Our results suggest that cPLA2\(\alpha\) does not contribute to the supply of free fatty acids utilized as acyl chains of CE. In the atherosclerotic lesions, foam cells contain more cholesteryl olate (50%) than cholesteryl arachidonate (3%).30) This also suggests a lack of contribution of cPLA2\(\alpha\) to the production of CE, because cPLA2\(\alpha\) preferentially hydrolyzes glycerophospholipids bearing an arachidonoyl residue at the sn-2 position. Other PLA2\(s\) including cPLA2\(\gamma\) do not exhibit such a preference. While cPLA2\(\alpha\) was expressed ubiquitously in the body, cPLA2\(\gamma\) was expressed in heart, skeletal muscle, brain,34) and monocytes from peripheral blood.35) We also showed here that cPLA2\(\gamma\) was expressed in MPM as well as THP-M (Figs. 1, 6). Furthermore, in contrast to cPLA2\(\alpha\), cPLA2\(\gamma\) is located in membrane fractions,25) especially in the endoplasmic reticulum,36) in which ACAT is also located.37) Considering these findings and our results that MAFP inhibited OxLDL-induced production of \[^{3}H\]CE in not only THP-M and wild-type MPM (Figs. 3, 5) but also cPLA2\(\alpha\)-KO MPM (data not shown), all of which expressed cPLA2\(\gamma\) (Figs. 1, 6), it could be assumed that cPLA2\(\gamma\) is involved in the MAFP-sensitive production of \[^{3}H\]CE. To clarify this possibility, further studies with cPLA2\(\gamma\)-knockdown THP-M are needed.

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duced cytotoxicity. Consistent with the lack of an effect of ACAT inhibition on the formation of foam cells, the present study showed that MAFP did not inhibit the formation of foam cells or the increase in intracellular total CE induced by OxLDL, despite a significant suppression of the increase in [3H]CE, which was produced through the reesterification of FC, in THP-M and MPM (Figs. 3—5). The lack of inhibition may be explained by the following: (1) Acyl-CoA used to form CE is supplied not only from cPLA2-mediated release of free fatty acids but also from other pathways. (2) The formation of foam cells with an increase in CE content upon the OXL stimulation is predominantly mediated by the deposition of CE derived from OXL in lysosomes rather than the accumulation of CE through the reesterification of FC in cytoplasm. In other words, the contribution of CE produced through the reesterification to the deposition of intracellular CE is not major upon stimulation with OXL. This latter notion is supported by the previous finding that the lysosomal CE and FC derived from OXL are not as easily cleared as those derived from AcLDL, thus being accumulated within lysosomes in THP-1-derived macrophages.15) The difference between OXL and AcLDL is consistent with the present results that Oil Red O-stained lipids in OXL-stimulated THP-M and MPM were relatively small and present around the nucleus, while the lipids in AcLDL-stimulated cells were relatively large and formed droplets in cytoplasm (Fig. 3). Consequently, it is conceivable that the reduction in the amounts of CE and FC deposited in lysosomes is important to prevent the formation of foam cells in response to OXL. However, considering that the amount of FC deposited intracellularly upon stimulation with OXL can be reduced in the presence of a certain cholesterol acceptor in the extraluminal medium,15) inhibition of CE production through the reesterification of FC might be effective in preventing the formation of foam cells when the pathways of FC efflux function efficiently.

In conclusion, on the basis of the present results, we suggest that MAFP-sensitive cPLA2s, probably cPLA2γ but not cPLA2δ, are involved, in part, in the supply of fatty acids utilized for the reesterification of FC to produce CE in OXL-stimulated THP-M and MPM. Furthermore, with regard to the formation of foam cells induced by OXL, the contribution of CE produced through reesterification is not as great as that of CE deposited in lysosomes. Taking this into account, to evaluate the roles of cPLA2 γ in the formation of foam cells, further experiments under conditions where cPLA2 γ-knockdown cells are stimulated in the presence of cholesterol acceptors, such as apolipoprotein A-I and high density lipoprotein, are underway in our laboratory.

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