J. Biochem. **125**, 319-327 (1999)

Complete Inhibition of Mouse Macrophage-Derived Foam Cell Formation by Triacsin C[1]

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Received July 14, 1998; accepted October 26, 1998

Primary mouse peritoneal macrophages effectively take up and metabolize phosphatidylcholine/cholesterol liposomes containing a small amount of phosphatidylserine, which results in the massive accumulation in the cytoplasm of oil red O positive lipid droplets consisting of cholesteryl ester (CE) and triacylglycerol (TG) [Nishikawa *et al.* (1990) *J. Biol. Chem.* 265, 5226-5231]. A number of inhibitors of CE synthesis have been reported, but their effects on the lipid droplet formation have not been fully examined. Furthermore, the contribution of TG synthesis to lipid droplet formation has been poorly investigated. We have investigated the relationship between CE and TG syntheses and cytosolic lipid droplet formation in macrophages cultured in the presence of inhibitors with different modes of action. When macrophages were cultured with liposomes and [^{14}C]oleic acid in the presence of triacsin C, a potent inhibitor of long chain acyl-CoA synthetase, both [^{14}C]CE and [^{14}C]TG syntheses were inhibited to similar extents with IC_{50} values of 0.19 and 0.10 μM, respectively. On the other hand, pregnenolone, a well-known inhibitor of cellular cholesterol transport, and CL-283,546, a potent inhibitor of acyl-CoA:cholesterol acyltransferase, inhibited [^{14}C]CE synthesis specifically with IC_{50} values of 5.0 and 0.038 μM, respectively. Microscopic observation revealed that the inhibitors of cholesterol metabolism produce only a partial decrease in cytosolic lipid droplets even at the highest doses which cause almost complete inhibition of [^{14}C]CE synthesis. However, the triacsin C-dose dependent inhibition of lipid droplet formation was almost complete at 0.59 μM, a concentration that inhibits [^{14}C]CE and [^{14}C]TG syntheses by about 90%. These results show that inhibiton of acyl-CoA synthetase by triacsin C causes a decrease in the cellular levels of acyl-CoA, the common substrate for CE and TG syntheses, leading to an inhibiton of neutral lipid synthesis and eventually to the complete disappearance of cytosolic lipid droplets from macrophages. These findings suggest that TG synthesis, as well as CE synthesis, is responsible for macrophage-derived foam cell formation, and is therefore a potential target for new antiatherosclerotic agents.

Key words: acyl-CoA synthetase inhibitor, cholesteryl ester, macrophage-derived foam cell formation, triacsin, triacylglycerol.

In the early stages of atherosclerogenesis, macrophages that have penetrated into the intima efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acids as neutral lipids in cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall (*1-4*). Recently, Ishii *et al.* (*5*) analyzed the lipid composition of lipid droplets from β-very low density lipoprotein (β-VLDL)-loaded macrophages and reported that the main components in the lipid droplets are cholesteryl ester (CE) (58 ± 4%, w/w) and triacylglycerol (TG) (27 ± 3%, w/w). Much attention has been paid to the molecular mechanism of intracellular cholesterol transport and cholesterol metabolism as potential targets for pharmaceutical intervention. Accordingly, several types of inhibitors have been reported, including sterol derivatives such as U18666A (*6*), progesterone, and pregnenolone (*7-9*), which inhibit cholesterol escape from lysosomes or inhibit the activity of

[1] This work was supported by grants from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF96I00304), from the Ministry of Education, Science, Sports and Culture of Japan, and from Japan Keirin Association.

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Abbreviations: ACAT, acyl-CoA: cholesterol acyltransferase; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's buffered salt solution; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IC_{50}, drug concentration required for 50% inhibition of an enzyme activity or biological reaction; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate buffered saline; PL, phospholipids; TG, triacylglycerol; VLDL, very low density lipoprotein.

multidrug resistant P-glycoproteins in the plasma membrane (*10, 11*), as well as a large number of inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT), the enzyme of cholesterol esterification in the endoplasmic reticulum. All these compounds have been shown to block CE formation in macrophages (*12, 13*), but the correlation between CE synthesis and foam cell formation from macrophages has not been fully examined. Furthermore, the contribution of TG, another main component of cytosolic lipid droplets (*5*), to the foam cell formation has been poorly investigated.

We previously reported a series of compounds, termed triacsins A to D, isolated from the culture filtrate of *Streptomyces* sp. SK-1894, which contain 11 carbon alkenyl chains with a common triazenol moiety at their termini (*14, 15*). Although all triacsins inhibit long chain acyl-CoA synthetases from a wide variety of sources, triacsins C and A were shown to be potent inhibitors in a large number of studies that utilized triacsins to investigate the function of acyl-CoA synthetase in lipid metabolism (*16-20*).

We have established a model of foam cell formation using mouse peritoneal macrophages (*21*). When the macrophages are cultured in the presence of negatively charged liposomes, they take up the liposomes *via* the scavenger receptors and metabolize their components, including phospholipids and cholesterol, to form lipid droplets in the cytosol. Taking advantage of this model in the present study, we investigated and compared the effects of triacsins, pregnenolone, and CL-283,546 on CE and TG syntheses using radiolabeled substrates and on lipid droplet formation. To our knowledge, this is the first demonstration of a good correlation between triacylglycerol synthesis and lipid droplet formation in macrophages. Finally, we found that inhibiton of the synthesis of both neutral lipids is effective in blocking cytosolic lipid droplet formation in mouse peritoneal macrophages.

EXPERIMENTAL PROCEDURES

Materials—Triacsins A and C were purified from the culture broth of *Streptomyces* sp. SK-1894 as reported previously (*14*). [1-^{14}C]Oleic acid (50 mCi/mmol) was purchased from DuPont NEN, and 1,2-di[1-^{14}C]palmitoylglycerophosphocholine (110 mCi/mmol) was from Amersham. Dulbecco's modified Eagle's medium (DMEM) and Hank's buffered salt solution (HBSS) were purchased from Nissui Pharmaceutical, GIT medium was from Nippon Seiyaku, and penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and glutamine (200 mM) solutions were from GIBCO. Phosphatidylcholine, phosphatidylserine, dicetylphosphate, cholesterol, 3β-hydroxy-5-pregnen-20-one (pregnenolone), and oil red O were all purchased from Sigma. CL-283,546, an ACAT inhibitor (*22*), and compactin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (*23*), were generous gifts from Dr. Hans-Jurgen Hess, Pfizer, Groton, CT, and Dr. Akira Endo, Tokyo Noko University, Tokyo, respectively. An HMG-CoA synthase inhibitor, 1233A (*24*), and a fatty acid synthase inhibitor, cerulenin (*25*), were isolated from the culture broth of *Scopulariopsis* sp. F-244 (*26*) and *Cephalosporium caerulens* KF-140 (*27*), respectively. Other reagents used were of commercially available analytical grade.

Cells—Female ICR mice (25-30 g) were obtained from Japan SLC. Mouse peritoneal macrophages were prepared as described previously (*21*). Peritoneal cells were harvested from unstimulated mice using HBSS and then suspended at 2×10^6 cells/ml in GIT medium. Aliquots (0.25 ml) were dispensed into a 48-well plastic microplate (Corning) or a tissue culture chamber (LAB-TEK 8-chamber, Nunc) and incubated in a humidified CO_2 (5% v/v) atmosphere at 37°C for 2 h, after which each plate was washed three times with 0.25 ml HBSS to remove the unattached cells. The medium was then replaced immediately with 0.25 ml DMEM containing 8% (v/v) lipoprotein-deficient serum (LPDS) (*28*), penicillin (100 units/ml), and streptomycin (100 μg/ml) (hereafter referred to as medium A).

Assay for Cell Viability—Macrophage viability in the presence of the inhibitors was measured using alamar Blue (Iwaki Glass).

Preparation of the Membrane Fraction from Macrophages—The membrane fraction of mouse peritoneal macrophages was prepared using a method similar to that previously reported for Raji and Sf 9 cells (*17, 20*). Briefly, 7.0×10^7 macrophages were collected and suspended on ice in 10 ml of phosphate-buffered saline (PBS) (pH 7.4) containing 10 mM 2-mercaptoethanol. After sonication six times for 10 s at 100 W (model XL 2020, Misonix), the membrane fraction was pelleted by centrifugation at $100,000\times g$ for 1 h at 4°C and then resuspended in 500 μl of the buffer described above.

Assay for Acyl-CoA Synthetase Activity—The isotope method for long chain acyl-CoA synthetase activity (*29*) was used with slight modifications. Briefly, an assay mixture containing 0.1 M Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 150 mM KCl, 15 mM $MgCl_2$, 10 mM ATP, 1 mM CoA, 0.1 mM [^{14}C]oleic acid (0.5 μCi), together with triacsins A or C (added in 2.5 μl methanol solution to make a final concentration of 0-5.1 μM), and the macrophage membrane fraction (30 μg of protein) in a total volume of 100 μl was incubated at 37°C for 20 min. The reaction was stopped by adding 1.25 ml of an isopropanol: heptane: 1 M sulfuric acid mixture (40:10:1, v/v), H_2O (0.5 ml), and the radioactive free oleic acid was extracted twice with 0.75 ml heptane. The aqueous layer (0.5 ml) containing synthesized [^{14}C]oleoyl-CoA was counted with a liquid scintillation spectrometer (Aloka).

Preparation of Liposomes—Multilamellar liposomes were prepared as described previously (*30*). In brief, a lipid mixture of phosphatidylcholine (1 μmol), phosphatidylserine (1 μmol), dicetylphosphate (0.2 μmol), and cholesterol (1.5 μmol) in chloroform was dried and then suspended in 1 ml of 0.3 M glucose. To prepare [^{14}C]phosphatidylcholine-containing liposomes, 1,2-di[1-^{14}C]palmitoyl-glycerophosphocholine (0.01 μmol, 1.1 μCi) was added to the lipid mixture.

Analysis of Neutral Lipids in Liposome-Loaded Macrophages—Mouse peritoneal macrophages (1.2×10^7 cells) attached to 100 mm cell culture dishes (Costar) after 2 h of primary culture were washed with HBSS and then placed in 6.0 ml of medium A. After a 2-h preincubation, 240 μl of liposomes were added to each culture. Following a 14-h incubation, the medium was removed, and the cells in each dish were washed three times with HBSS. The cellular lipids were extracted three times with hexane/2-propanol (3:2, by volume). After concentrating the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck). The first chromatogram was developed 2 cm from the origin with a solvent of chloroform/methanol/acetic acid (65:25:10, by volume). After drying, the plate was re-developed 8 cm from the origin with hexane/diethyl ether/acetic acid (70:30:1). Under these conditions, the R_f values of CE, TG, fatty acids (oleic acid or palmitic acid), and phospholipids (PL) were 0.66, 0.55, 0.38, and 0.05, respectively. The spots were visualized by staining with 0.1% primulin vapor, and the spots for CE and TG were scraped off, and CE and TG were extracted from the gel by the method of Bligh and Dyer (*31*). By this method of extraction, the lipids were collected quantitatively. The amounts of CE and TG were measured with cholesterol E-test WAKO and triglyceride G-test WAKO (WAKO), respectively. The range of detection was 10-150 nmol/assay, which was sufficient for our experiments.

Assay for [^{14}C]Neutral Lipid Synthesis by Macrophages—Macrophages were cultured in a 48-well plastic microplate with 2.5 μl of inhibitors (MeOH solution) and 10 μl of liposomes together with 5 μl (0.05 μCi) of [^{14}C]-oleic acid (10% EtOH/PBS solution) or 10 μl of [^{14}C]phosphatidylcholine-containing liposomes. Following a 14-h incubation, the medium was removed and the cells in each well were washed three times with HBSS. The cells were lysed by adding 0.25 ml of PBS containing 0.1% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by the method of Bligh and Dyer. After concentrating the organic solvent, the total lipids were separated on a TLC plate as described above. The distribution of radioactivity on the TLC plate was analyzed with a radioscanner (AMBIS Systems). The radioactivity measurable by the radioscanner was about one thirtieth that measurable by usual liquid scintillation spectrometers. In control experiments, the incorporation of [^{14}C]oleic acid into CE, TG, and PL was about 23, 20, and 10% of the total radioactivity added, respectively. In experiments using [^{14}C]phosphatidylcholine-containing liposomes, the lipids in the medium after a 14-h incubation were extracted and analyzed by the same method as described above.

Cellular Neutral Lipid Staining—Macrophages were cultured in a tissue culture chamber with liposomes and inhibitors as described above. After 14-h incubation, the cells were washed three times with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin and oil red O, respectively, and the stained cells were examined under a light microscope (Vanox-S model, Olympus).

Other Analytical Methods—Protein concentrations were determined by Bradford's method (*32*) using a Protein Assay Kit (Bio-Rad).

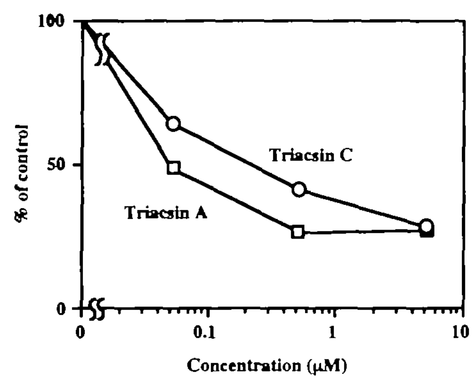

Fig. 1. **Inhibition of acyl-CoA synthetase activity in the mouse macrophage membrane fraction by triacsins.** Mouse peritoneal macrophages (7×10^7 cells) in 10 ml of cold PBS containing 10 mM 2-mercaptoethanol were sonicated (for 10 s six times at 100 W) on ice. The membrane fraction precipitated by centrifugation (at $100{,}000\times g$ for 1 h) was used as the enzyme source. Acyl-CoA synthetase activity was assayed in a 100 μl mixture containing 0.1 M Tris-HCl (pH 8.0), 5 mM DTT, 150 mM KCl, 15 mM $MgCl_2$, 10 mM ATP, 1 mM CoA, 0.1 mM [^{14}C]oleic acid (0.5 μCi), together with triacsins A (□) or C (○) (0-5.1 μM), and the membrane fraction (30 μg of protein). After a 20-min incubation at 37°C, the [^{14}C]oleoyl CoA produced and unreacted [^{14}C]oleic acid were separated and the radioactivity of [^{14}C]oleoyl CoA was counted by a liquid scintillation spectrometer.

RESULTS

Inhibition of Acyl-CoA Synthetase Activity by Triacsins—The characteristics of long chain acyl-CoA synthetase activity were examined using a macrophage membrane fraction. ATP, CoA, and Mg^{2+} are required to elicit full activity (data not shown), similar to the characteristics of the enzyme activity present in rat liver and other animal and insect cells (*15, 17, 20*). Triacsins A and C inhibit the activation of oleic acid in a dose-dependent manner with calculated IC_{50} values of 0.05 and 0.22 μM, respectively (Fig. 1).

Effect of Inhibitors on Macrophage Viability—The viability of macrophages cultured in the presence of inhibitors under foam-cell inducing conditions was tested by the alamar Blue assay. No significant difference was observed relative to untreated controls with up to 10 μM of triacsins A or C, compactin, or 1233A, up to 30 μM pregnenolone, up to 1 μM cerulenin, or up to 100 nM CL-283,546 (data not shown). Accordingly, lower doses of inhibitors were used for further experiments described below.

Lipid Composition of Liposome-Loaded Macrophages—Cellular CE and TG levels were measured in mouse macrophages cultured in the presence (liposome-loaded) or absence (unloaded) of liposomes (Table I). In unloaded

TABLE I. **Comparison of CE and TG contents in liposome-loaded and unloaded macrophages.**

Condition	g (mol)^a/cell			
	CE		TG	
	(g)	(mol)	(g)	(mol)
(−) Liposome	7.7×10^{-13}	1.2×10^{-15}	8.6×10^{-13}	0.96×10^{-15}
(+) Liposome	35×10^{-13}	5.4×10^{-15}	25×10^{-13}	2.8×10^{-15}
(+) − (−)	27×10^{-13}	4.2×10^{-15}	16×10^{-13}	1.8×10^{-15}

^{a}Estimated as cholesteryl oleate (MW 651) for CE and triolein (MW 885) for TG. Mouse macrophages (1.2×10^7 cells) were cultured for 14 h in the presence (+) or absence (−) of liposomes (240 μl) comprising phosphatidylcholine, phosphatidylserine, dicetylphosphate, and cholesterol at a molar ratio of 10:10:2:15 as described in "EXPERIMENTAL PROCEDURES" for 14 h. The cellular lipids were extracted and separated by TLC. The CE and TG spots were scraped off, and their amounts were determined according to the protocols of the kits as described in "EXPERIMENTAL PROCEDURES." In this experiment, the protein content was 360 μg/1.2×10^7 cells. Each value represents the average of duplicate determinations which varied by less than 10% under the conditions used.

macrophages, no lipid droplets were observed and similar levels of CE and TG (about 1×10^{-15} mol/cell) were found. However, in liposome-loaded macrophages, which contained a number of lipid droplets in the cytosolic area, CE and TG levels were increased by 4.5- and 2.9-fold, respectively. In liposome-loaded macrophages, the relative ratio of CE to TG was 2:1.

Effect of Inhibitors on the Synthesis of [^{14}C]Neutral Lipids from [^{14}C]Oleic Acid in Macrophages—When macrophages were incubated with liposomes and [^{14}C]oleic acid, the [^{14}C]fatty acid was activated and incorporated mainly into [^{14}C]CE (23% of [^{14}C]oleic acid added) and [^{14}C]TG (20% of [^{14}C]oleic acid). The effects of triacsins were tested on neutral lipid synthesis by macrophages. As shown in Fig. 3, triacsins inhibit both [^{14}C]CE and [^{14}C]TG syntheses in a dose-dependent fashion. The IC_{50} values of [^{14}C]CE and [^{14}C]TG syntheses were similar for each triacsin, 0.19 and 0.17 μM, respectively, for triacsin A, and 0.18 and 0.10 μM for triacsin C. Under the same conditions, pregnenolone inhibits [^{14}C]CE synthesis with an IC_{50} value of 5.0 μM, but [^{14}C]TG synthesis is enhanced. The results are consistent with those reported by Aikawa *et al.* (*8*). An ACAT inhibitor, CL-283,546, was found to inhibit [^{14}C]CE synthesis (IC_{50}; 0.035 μM) without significantly affecting [^{14}C]TG synthesis. However, compactin (10 μM), 1233A (10 μM), and cerulenin (1 μM) showed no effects on [^{14}C]CE or [^{14}C]TG synthesis (data not shown).

Effect of Triacsin C on the Synthesis of [^{14}C]Neutral Lipids from [^{14}C]Phosphatidylcholine-Containing Liposomes in Macrophages—When macrophages were incubated with liposomes containing 1,2-di[1-^{14}C]palmitoyl-glycerophosphocholine, the radiolabeled fatty acyl chains were metabolized in the cells to form [^{14}C]CE (cholesteryl [^{14}C]-palmitate) and [^{14}C]TG (*21*). The effect of triacsin C on the metabolic pathway of [^{14}C]phosphatidylcholine-containing vesicles was examined. The amounts of radioactivity recovered from the cells ([^{14}C]CE, [^{14}C]TG, and [^{14}C]PL) and medium ([^{14}C]fatty acid) in each experiment are shown in Fig. 4, A and B. The formation of [^{14}C]CE and [^{14}C]TG was suppressed in a dose-dependent manner, with calculated IC_{50} values of 0.10 and 0.045 μM, respectively (Fig. 4C). In contrast, the release of free [^{14}C]palmitic acid into the medium increased significantly in response to the amount of triacsin C. These data indicate that triacsin C does not affect the hydrolysis of [^{14}C]phosphatidylcholine and that the [^{14}C]palmitic acid produced is activated by acyl-CoA synthetase to be utilized in neutral lipid formation.

Effect of Inhibitors on Lipid Droplet Formation by Macrophages—When macrophages were incubated with liposomes and stained with oil red O, large numbers of cytosolic neutral lipid droplets were observed microscopically (*21*). The effect of inhibitors on lipid droplet formation was examined under the same conditions described above except that no [^{14}C]lipid was added. In the presence of triacsin C (0.059–0.59 μM, Fig. 4, B–D), macrophages show a dose-dependent reduction in the size and number of cytosolic lipid droplets relative to untreated control cells (Fig. 4A). Surprisingly, lipid droplets disappear almost completely in the macrophages at a concentration of 0.59 μM triacsin C, a level that causes about 90% inhibition of CE and TG syntheses as indicated in Figs. 2 and 3, and the morphology is ostensibly the same as that of the macrophages cultured without liposomes (Fig. 4E). On the other

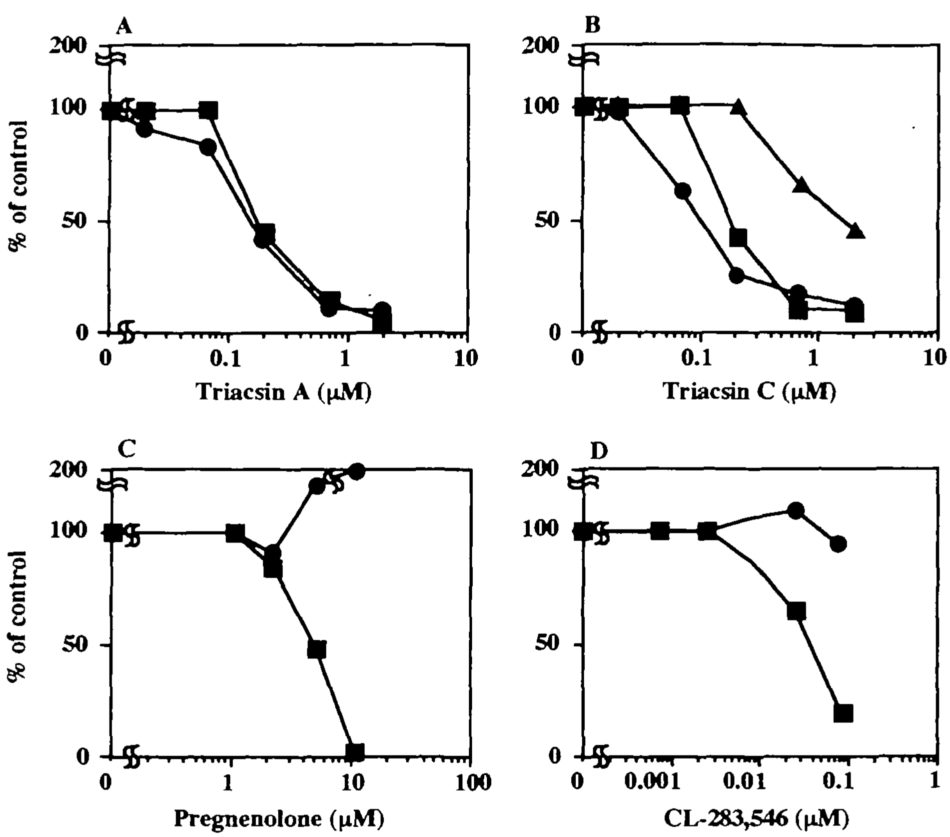

Fig. 2. **Effect of inhibitors on the synthesis [^{14}C]neutral lipids from [^{14}C]oleic acid in macrophages.** Macrophage monolayers obtained from 5×10^{5} cells/well in a microplate were incubated in 0.25 ml of medium A with liposomes composed of phosphatidylcholine, phosphatidylserine, dicetylphosphate, and cholesterol at a molar ratio 10:10:2:15 and [^{14}C]oleic acid in the absence or presence of the indicated amounts of either triacsin A (A), C (B), pregnenolone (C), or CL-283,546 (D). After a 14-h incubation, cholesteryl [^{14}C]oleate (■), [^{14}C]triacylglycerol (●), and [^{14}C]phospholipids (▲) were separated by TLC, and measured with a radioscanner as described in "EXPERIMENTAL PROCEDURES." The results are plotted as % of control (without drug).

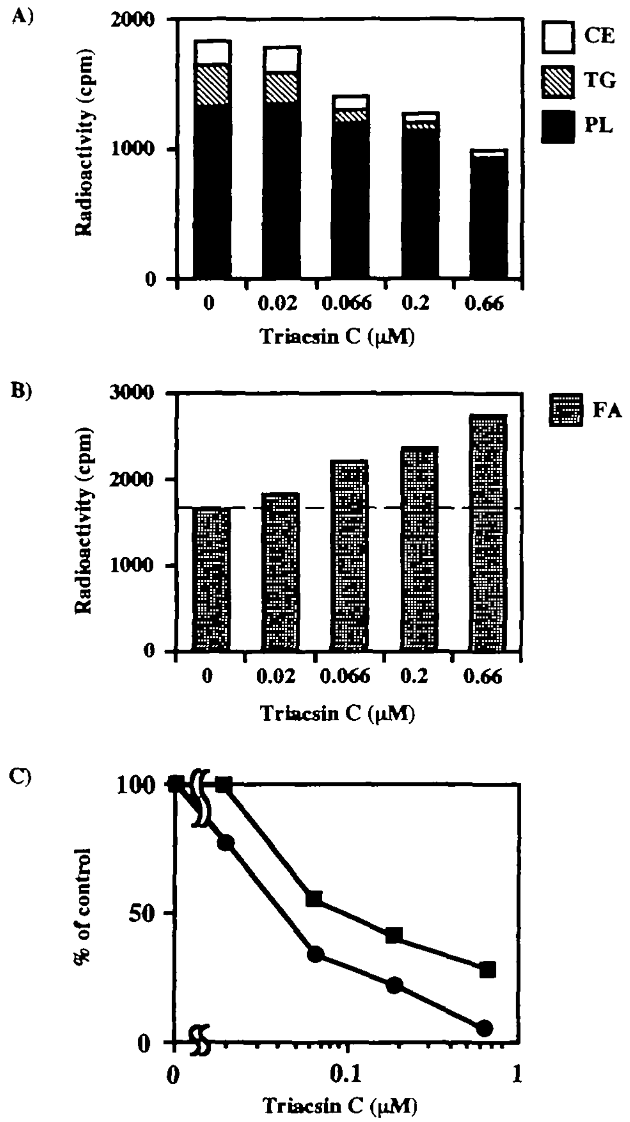

Fig. 3. **Effect of triacsin C on the synthesis of [^{14}C]neutral lipids from [^{14}C]phosphatidylcholine-containing liposomes in macrophages.** Macrophage monolayers obtained from 5×10^5 cells/well in a microplate were incubated in 0.25 ml of medium A with 10 μl of liposomes containing 0.1 μCi of 1,2-di[1-^{14}C]palmitoyl glycerophosphocholine in the absence or presence of triacsin C (0-0.58 μM). After a 14-h incubation, cholesteryl [^{14}C]palmitate (CE), [^{14}C]triacylglycerol (TG), and [^{14}C]phospholipids (PL) recovered from the cells (A), and [^{14}C]palmitic acid (FA) released into the medium (B) were determined with a radioscanner as described in "EXPERIMENTAL PROCEDURES." Cholesteryl [^{14}C]palmitate (■) and [^{14}C]-triacylglycerol (●) produced in the cells are indicated as % of control (C).

hand, partial inhibition of cytosolic lipid droplet formation was observed at the highest dose of 10 μM pregnenolone (Fig. 5C), a level that inhibits CE synthesis completely while it enhances TG synthesis (Fig. 2), and 0.085 μM CL-283,546 (Fig. 5D), which inhibits CE synthesis without any effect on TG synthesis (Fig. 2). Compactin (10 μM), 1233A (10 μM), and cerulenin (1 μM) showed no effects on cytosolic lipid droplet formation (data not shown).

DISCUSSION

We have established a model of mouse peritoneal macrophage-derived foam cell formation using lipid liposomes containing acidic phospholipids (*21, 33*), in which massive amounts of neutral lipid droplets can be observed microscopically in the cytosol of the macrophages. Similar models have been reported using J774 cells (*34*) and other cell lines (*35, 36*). These models are useful not only for investigating the mechanisms of cholesterol metabolism and foam cell formation, but also for evaluating compounds as pharmaceutical agents for the treatment or prevention of atherosclerosis.

The amounts of CE and TG were quantitated in mouse macrophages (Table I). When macrophages were cultured for 14 h in the absence of liposomes (phosphatidylcholine, phosphatidylserine, dicetylphosphate, and cholesterol at a molar ratio 10:10:2:15), no cytosolic lipid droplets were observed (Fig. 4E), and the amounts of CE and TG were calculated to be 7.7×10^{-13} and 8.6×10^{-13} g/cell, respectively. Under these conditions, [^{14}C]oleic acid was incorporated into CE (<1.0% of [^{14}C]oleic acid added) and TG (6.0%). In contrast, when macrophages were cultured in the presence of liposomes, lipid droplets were formed in the cytosolic area (Figs. 4A and 5A), and the amounts of CE and TG were increased to 35×10^{-13} (4.5-fold) and 25×10^{-13} g/cell (3-fold), respectively. Furthermore, the rates of incorporation of [^{14}C]oleic acid into CE and TG were also increased to 23 and 20% of the added [^{14}C]oleic acid, respectively. On the other hand, the rate of incorporation of [^{14}C]oleic acid into phosphatidylcholine remained almost constant regardless of the absence or presence of liposomes (14 *vs.* 10%, respectively). Ishii *et al.* reported that the CE and TG content of lipid droplets from β-VLDL-loaded mouse macrophages is 58 and 27% (w/w), respectively (*5*). Based on their data, CE and TG contents were calculated to be 23×10^{-13} and 11×10^{-13} g/cell, respectively, in good agreement with the net increases in the amounts of CE and TG in the present assay (Table I). These data strongly suggest that liposome-induced lipid droplets in mouse macrophages are comprised mainly of CE and TG. Furthermore, the molar ratio of [^{14}C]oleic acid incorporated into CE and TG in lipid droplets was estimated as 1 to 2. In the present study, inhibitors with different modes of action were used to show clearly a good correlation between CE and TG syntheses and cytosolic lipid droplet formation in mouse peritoneal macrophages. It was found that pregnenolone, a well-known inhibitor of intracellular cholesterol transport (*8, 10*), and CL-283,546, an ACAT inhibitor (*22, 37*), never inhibit the lipid droplet formation completely. In the presence of 10 μM pregnenolone, which almost completely inhibits CE synthesis but enhances TG synthesis by about 2-fold (Fig. 2), the macrophages contained fewer oil red O-positive lipid droplets (Fig. 5C) relative to untreated macrophages, which exhibited extensive lipid droplets in the cytosol (Fig. 5A). In the presence of 0.085 μM CL-283,546, which inhibits CE synthesis by 80% with no effect on TG synthesis (Fig. 2), the macrophages produced fewer lipid droplets, and the size of most of the droplets stained was considerably smaller (Fig. 5D). The reason why CL-283,546 is more effective than pregnenolone in inhibiting the lipid droplet formation may be that

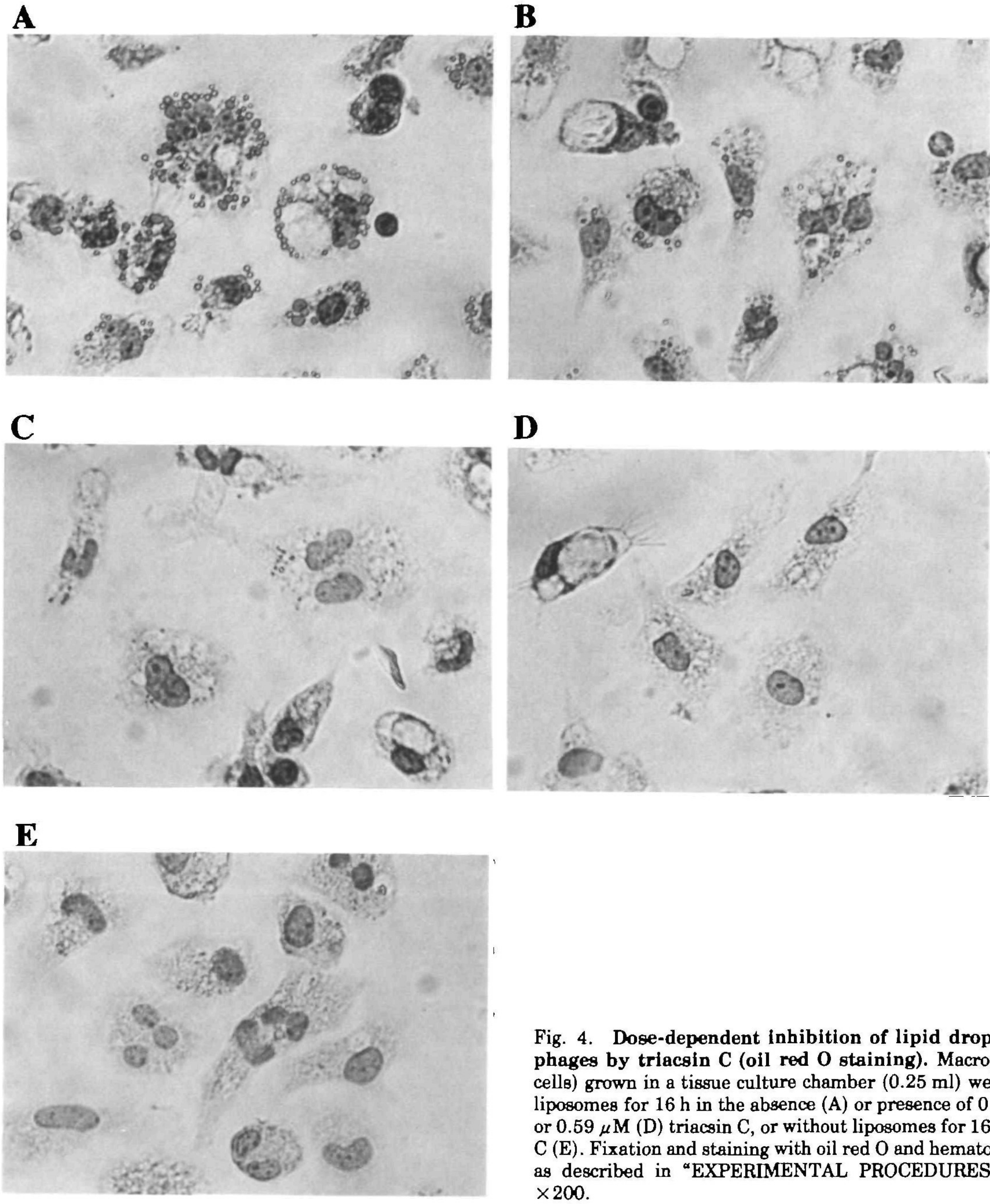

Fig. 4. **Dose-dependent inhibition of lipid droplet formation in macrophages by triacsin C (oil red O staining).** Macrophage monolayers (5×10^5 cells) grown in a tissue culture chamber (0.25 ml) were incubated with 10 μl of liposomes for 16 h in the absence (A) or presence of 0.059 μM (B), 0.20 μM (C), or 0.59 μM (D) triacsin C, or without liposomes for 16 h in the absence of triacsin C (E). Fixation and staining with oil red O and hematoxylin were then performed as described in "EXPERIMENTAL PROCEDURES." Original magnification, $\times200$.

pregnenolone has a stimulatory effect on TG synthesis, whereas CL-283,546 has no effect. Similar observations have been reported using a number of compounds, including inhibitors of oxidized LDL uptake such as ikarugamycin (*38*), inhibitors of intracellular cholesterol transport such as U18666A (*6*) and progesterone (*8, 10*), inhibitors of vacuolar-type H^+-ATPase such as bafilomycins (*39, 40*) and destruxin (*40*), and ACAT inhibitors such as 58-035 (*41*), beauvericin (*37*), and terpendoles (*42*). These inhibitors of cholesterol metabolism were found to inhibit CE synthesis in cell-based assays, but morphological observation suggests their partial inhibition of the cytosolic lipid droplet formation even at the highest doses which almost completely inhibited CE synthesis (*38-40*). Thus, it was clear that complete inhibition of the lipid droplet formation is not achieved by any inhibitors of CE synthesis, strongly suggesting that TG synthesis contributes to the lipid droplet formation as well as CE synthesis. Furthermore, neither compactin (*23*), 1233 A (*24*), nor cerulenin (*25*) affects CE or TG synthesis, indicating that the *de novo* biosynthesis of cholesterol and fatty acids is not involved in lipid droplet formation by mouse macrophages.

TG synthesis in mouse macrophages has rarely been reported. However, acyltransferases involved in TG synthesis, such as glycerolphosphate acyltransferase, monoacylglycerolphosphate acyltransferase, and diacylglycerol acyltransferase, are reported to be localized in microsomal fractions (*43, 44*), suggesting that TG synthesis occurs

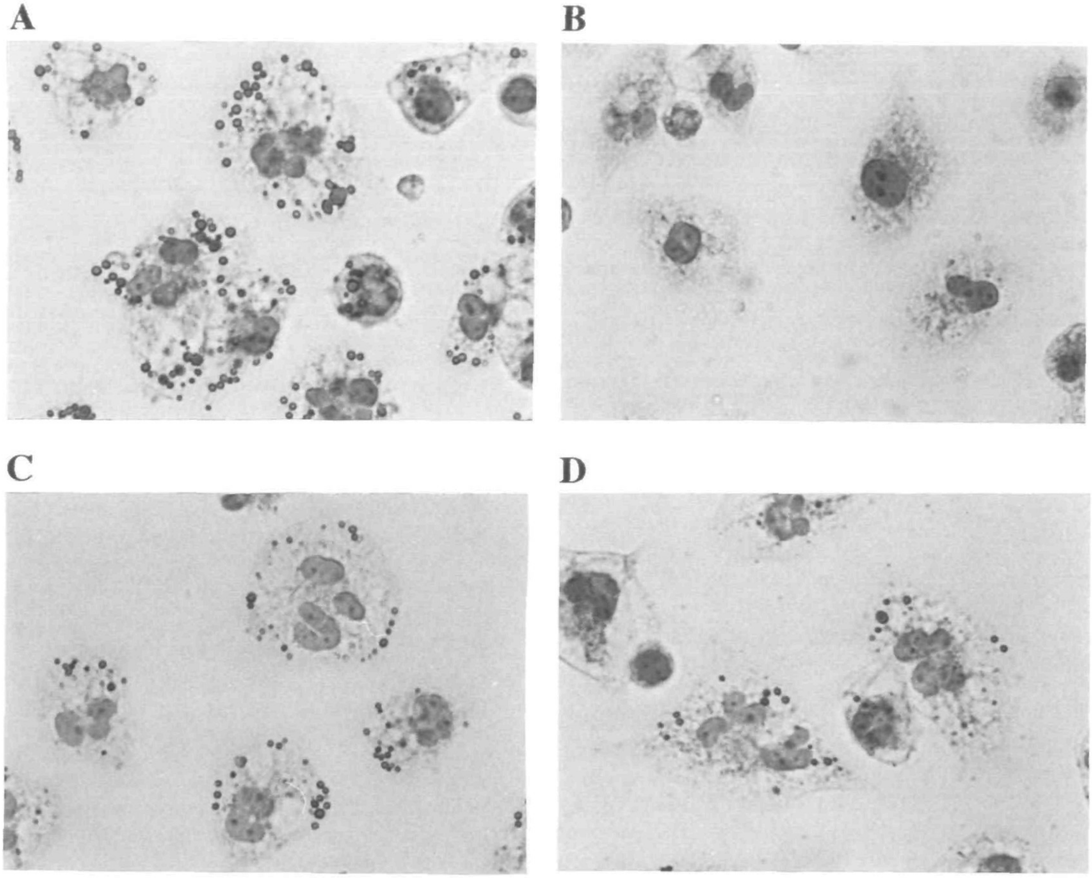

Fig. 5. **Effect of inhibitors on lipid droplet formation in macrophages (oil red O staining).** Macrophage monolayers (5×10^5 cells) grown in a tissue culture chamber (0.25 ml) were incubated with 10 μl of liposomes for 16 h in the absence (A) or presence of 0.59 μM triacsin C (B), 10 μM pregnenolone (C), or 85 nM CL-283,546 (D). Fixation with 10% formaldehyde and double staining with oil red O and hematoxylin were then performed as described in "EXPERIMENTAL PROCEDURES." Original magnification, $\times$200.

actively in ER. In addition, long chain fatty acids as donors for acyltransferase are supplied mostly *via* the degradation of phospholipids (*33*), and are activated by acyl-CoA synthetase to yield acyl-CoA.

On the other hand, triacsin C causes a dose-dependent inhibition of lipid droplet formation in mouse macrophages (Fig. 4), and at 0.59 μM the inhibition is complete with almost no cytosolic lipid droplets observed. The mechanism of action has been demonstrated. Triacsins inhibit long chain acyl-CoA synthetase activity in macrophage membrane fractions (Fig. 1). Accordingly, both CE and TG syntheses in macrophages are blocked by triacsins (Fig. 2) due to the depletion in the cellular levels of acyl-CoA, the common substrate for CE and TG syntheses, leading to the eventual disappearance of cytosolic lipid droplets. Additionally, it was shown that the ACAT and acyl-CoA synthetase reactions are closely linked to the production of CE in macrophages. All these findings show that cytosolic lipid droplet formation is closely correlated with the total amount of CE and TG synthesized in mouse peritoneal macrophages.

Macrophages under the culture conditions used are not proliferative and differentiated to foam cells. Accordingly, it is plausible that the PL synthesis essential for cell growth is not so active, whereas CE and TG syntheses are very fast, an assumption supported by the high rate of incorporation of [^{14}C]oleic acid into CE (23% of the total radioactivity added) and TG (20%) and the low rate of incorporation into PL (10%). The depletion in cellular acyl-CoA caused by triacsins might affect other cell processes such as PL synthesis (*17*) and β-oxidation where acyl-CoA is degraded to CO_2 and H_2O for energy production. In fact, the incorporation of [^{14}C]oleic acid into the PL fraction is inhibited by triacsin C (Fig. 2B), and the total radioactivity recovered from the cells and the medium seems to increase in the presence of triacsin C (Fig. 3), suggesting the inhibition of CO_2 production *via* β-oxidation. Further study is necessary to determine the effect of triacsin on β-oxidation. However, these essential metabolic reactions are not so sensitive to triacsins as CE and TG syntheses. At 0.59 μM triacsin C, both CE and TG syntheses are inhibited by 90%, whereas PL synthesis is inhibited by only 35%. The IC_{50} value (1.5 μM) for PL synthesis is about 10 times higher than those for CE and TG syntheses in [^{14}C]oleic acid incorporation experiments (Fig. 2B). Therefore, triacsin is not so lethal to macrophages because PL is still produced even though neutral lipid synthesis is completely inhibited by the drug. As a result, triacsins show no toxic effect on macrophages, at least up to 10 μM. Furthermore, the triacsin-treated macrophages without cytosolic lipid droplets (Fig. 4D) are morphologically indistinguishable from control cells cultured without liposomes (Fig. 4E).

As shown in Fig. 3, triacsin C has no effect on the scavenger receptor-mediated endocytosis and hydrolysis of [^{14}C]phosphatidylcholine in lysosomes, because the amount of [^{14}C]fatty acid (palmitic acid) released increases in response to increasing amounts of triacsin C. The drug does not inhibit ACAT activity in mouse liver microsomes or diacylglycerol acyltransferase activity in rat liver microsomes even at 30 μM (data not shown). Thus, triacsin action is very specific to long chain acyl-CoA synthetase. Recently, multiple types of acyl-CoA synthetase genes have been cloned from eukaryotic cells, and their distinct functions in cells are suggested. Gordon and coworkers (*45, 46*) reported at least five acyl-CoA synthetases designated Faa in *Saccharomyces cerevisiae* exhibiting distinct functions and different sensitivities to triacsin. Triacsin C shows potent inhibitory activity against Faa2p (K_i; 15 nM), less potent activity against Faa4p (K_i; 2 μM), but has no inhibitory effect on Faa1p and Faa3p (*46*). Faa2p is shown to activate endogenous fatty acids. Yamamoto and coworkers (*47-49*) reported three acyl-CoA synthetases (ACS) in mammals. ACS1 (*47*) is most abundant in liver, heart, and adipose tissue, while ACS2 (*48, 49*) and ACS3 (*48*) are present in brain. ACS1 and ACS2 are structurally related and exhibit similar characteristics, and this type of ACS is predominantly expressed in many tissues. Therefore, this type of ACS, possibly observed in mouse macrophages, might be inhibited by triacsins. It remains to be investigated whether or not triacsins inhibit all mammalian ACSs and which ACS(s) is eventually involved in macrophage-derived foam cell formation.

Taken together, TG synthesis, as well as CE synthesis, by macrophages is responsible for cytosolic lipid droplet formation, and inhibition of the syntheses of these neutral lipids is effective in blocking lipid droplet formation, possibly leading to the prevention of macrophage-derived foam cell formation. In this sense, the TG synthetic pathway is a potential target for new antiatherosclerotic agents. Triacsins, due to their unique mode of action, are the only compounds that simultaneously inhibit the syntheses of both these neutral lipids.

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