Involvement of ACSL in local synthesis of neutral lipids in cytoplasmic lipid droplets in human hepatocyte HuH7

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Abstract Lipid droplets (LDs) function as intracellular storage depots of neutral lipids. Recently, we identified long-chain acyl-coenzyme A synthetase 3 (ACSL3) as a major LD-associated protein in the human hepatocyte cell line HuH7. In this study, we investigated whether droplet-associated ACSL is involved in lipid metabolism in LDs. Addition of oleic acid (OA) to culture medium was shown to enhance the intracellular accumulation of LDs in the cells, which was accompanied by an increase of droplet ACSL3. When LD-enriched cells induced by OA were further incubated without OA for 3 days, ~80% of LDs were retained in the cells. Conversely, cellular LD content was greatly decreased after the addition of an ACSL inhibitor, triacsin C. This was accompanied by a concomitant decrease of the droplet ACSL3. Incubation of isolated LD fractions with ^{14}C-labeled OA or palmitic acid resulted in [^{14}C]acyl-CoA generation in vitro, indicating the presence of ACSL activity in LDs. The droplet ACSL activity varied according to the quantity of LDs in their emergence and disappearance in cells. Incubation of the LD fraction with [^{14}C]oleoyl-CoA resulted in radioactive triacylglycerol and cholesteryl esters. These results suggest that LD ACSL activity is involved in local synthesis of neutral lipids and LD formation.— Fujimoto, Y., H. Itabe, T. Kinoshita, K. J. Homma, J. Onoduka, M. Mori, S. Yamaguchi, M. Makita, Y. Higashi, A. Yamashita, and T. Takano. **Involvement of long-chain acyl-CoA synthetase in local synthesis of neutral lipids in cytoplasmic lipid droplets in human hepatocyte HuH7. *J. Lipid Res.* 2007.** 48: **1280–1292.**

Supplementary key words triacsin C • triacylglycerol • acyl-coenzyme A

Lipid droplets (LDs; lipid storage droplet, lipid body, or lipid particle) are intracellular compartments storing neutral lipids such as triacylglycerol (TG) and cholesteryl ester (CE) with surfaces surrounded by phospholipid monolayer (1, 2). LDs are detected in cytoplasmic spaces of various types of cells, typically adipocytes, hepatocytes, steroidogenic cells, and foam cells developed in atherosclerotic lesions. LDs have important roles as energy reservoirs and sources of lipid molecules necessary for the production of lipoproteins and steroid hormones. Accumulation of excess amounts of LDs, however, often leads to disorders such as obesity, fatty liver, and atherosclerosis (3). Thus, it is important to understand how LDs are formed and metabolized.

Recently, we performed a proteomic analysis of LDs isolated from the HuH7 human liver cell line for the systematic understanding of LDs (4). This revealed that LDs have a unique protein profile rich in lipid metabolic enzymes and that long-chain acyl-coenzyme A synthetase 3 (ACSL3) exists abundantly in the LDs (4). ACSL is a family of enzymes that catalyze the synthesis of acyl-CoA using long-chain fatty acids, ATP, and CoA as their substrates. Long-chain acyl-CoAs produced by the enzymes are essential substrates for the synthesis of various lipid molecules, including TG and CE. Five isoforms of mammalian ACSLs have been identified (ACSL1 and ACSL3–ACSL6), which are encoded by separate genes (5). Studies by Coleman and colleagues (6) demonstrated that some of these isoforms are located in distinct subcellular compartments. For example, ACSL1 is found in cytoplasm, endoplasmic reticulum (ER), and mitochondria-associated

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Abbreviations: ACSL, long-chain acyl-coenzyme A synthetase; ADRP, adipose differentiation-related protein; CE, cholesteryl ester; DG, diacylglycerol; DGAT, acyl-coenzyme A:diacylglycerol acyltransferase; ER, endoplasmic reticulum; FC, free cholesterol; LD, lipid droplet; LDH, lactate dehydrogenase; MAM, mitochondria-associated membrane; NSDHL, NAD(P)H steroid dehydrogenase-like protein; OA, oleic acid; PNS, postnuclear supernatant; TG, triacylglycerol.

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membrane (MAM), whereas ACSL4 and ACSL5 are much more specific to MAM and mitochondria, respectively. The cellular localization of ACSL6 is not yet known. We found that there are two ACSL isoforms in LD fractions prepared from HuH7 cells, with ACSL3 as one of the most abundant proteins in the LDs and ACSL4 being a minor protein (4). Proteomic studies by other groups have also demonstrated the presence of ACSL3 in LDs isolated from various types of mammalian cells (7–9).

LDs contain various kinds of proteins, such as PAT family proteins [named for the initials of three proteins: perilipin, adipose differentiation-related protein (ADRP; also known as adipophilin), and TIP-47] (10–13), caveolins (14, 15), and Rab family proteins (16, 17). Some of these proteins have been reported to be involved in the cellular storage of neutral lipids using gene expression techniques and disruption studies (18–20). In addition to this, recent studies identified several kinds of lipid-metabolizing enzymes in LDs, including lanosterol synthetase and hydroxy steroid dehydrogenases, as well as ACSLs (7–9). However, the importance of these enzymes in LDs has not been well studied except for a few examples (21). In this study, we investigated whether the droplet ACSL is involved in LD formation and maintenance through its enzymatic activity. We found that ACSL activity is present in LDs and that neutral lipids can be produced by isolated LDs using long-chain fatty acyl-CoA as substrates.

MATERIALS AND METHODS

Materials

HuH7 (human hepatoma cell line) was obtained from the Health Science Research Resources Bank (JCRB0403; Osaka, Japan). Anti-ADRP monoclonal antibody was purchased from PROGEN. Anti-ACSL3 antiserum was prepared as described previously (4). Anti-calnexin and anti-GS28 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Peroxidase-conjugated and rhodamine-labeled goat anti-IgG antibodies were purchased from Biosource International (Camarillo, CA). The ECL Western Blotting Detection System was purchased from Amersham Pharmacia Biotech, and [^{14}C]oleic acid (OA), [^{14}C]oleoyl-CoA, and [^{14}C]palmitoyl-CoA were purchased from Perkin-Elmer. Triacsin C was purchased from Kyowa Medex.

Culture and Oil Red O staining

HuH7 cells were cultured at 37°C in DMEM supplemented with 10% FBS. To induce LD formation in the cells, OA (complexed with BSA at a molar ratio of OA/BSA 5:1) was added to the medium at a final concentration of 0.6 mM. To inhibit ACSL activity, triacsin C was added at a final concentration of 5.4 μM. For Oil Red O staining, HuH7 cells (4×10^4 cells/well) were cultured on four-well culture slides (Falcon), fixed in formalin, and stained using a method described previously (22). For analysis of total cellular lipids, cells were treated with trypsin solution (0.2% trypsin, 0.02% EDTA, and 0.2% glucose in PBS) and recovered by centrifugation. The collected cells were homogenized by sonication in buffer solution containing 1 mM EDTA, 10 mM Tris (pH 7.4), 20 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml each of antipine, pepstatin, leupeptin, and chymostatin. Protein concentrations were measured using the BCA protein assay reagent (Pierce).

Cell fractionation

Generally, cell fractionations were performed as described previously (4) with minor modifications. HuH7 cells were collected by centrifugation and disrupted by homogenization using a Dounce-type glass-Teflon homogenizer in buffer A (10 μg/ml each of antipine, pepstatin, leupeptin, and chymostatin, 20 μg/ml phenylmethylsulfonyl fluoride, and 3 mM EDTA in 10 mM Tricine, pH 7.4) containing 250 mM sucrose. The homogenate was then centrifuged for 10 min at 1,000 *g* at 4°C to remove nuclei and cell debris, and the postnuclear supernatant (PNS) was recovered. The sucrose concentration of PNS was adjusted to 26% by adding buffer A containing 70% sucrose. Into a 16 ml ultracentrifuge tube (16PA; Hitachi), 1.1 ml of buffer A containing 51% sucrose was placed at the bottom. On top of this, 2.4 ml of buffer A containing 43% or 35% sucrose was layered sequentially, and finally, 2.0 ml of the PNS was layered on this. Subsequently, 2.4 ml of buffer A containing 18% or 10% sucrose was layered sequentially on the PNS layer. Finally, 3.0 ml of diluted buffer A containing 2% sucrose was loaded on the top (see Fig. 2A below). The step-wise gradient was then centrifuged at 24,000 rpm at 4°C for 3 h using a SRP28-SA1 rotor and a 65P-7 ultracentrifuge (Hitachi). After centrifugation, the solution was recovered from the top and fractionated (0.8 ml each). Each fraction was assayed for lactate dehydrogenase (LDH) activity using the LDH-UV Test kit (Wako).

Lipid analysis by thin-layer chromatography

Cell homogenates or cell fractions were concentrated using a rotary evaporator, and lipids were sequentially extracted with chloroform-methanol (2:1, v/v), chloroform, and ethyl ether. The extracted lipids were spotted onto TLC plates (Silica gel 60; Merck) and developed first with chloroform-methanol-acetic acid (98:2:1, v/v) and then with hexane-diethyl ether-acetic acid (80:20:1, v/v). The plates were soaked in 8% phosphoric acid containing 3% cupric acetate, and the lipids were visualized by heating at 130°C (23). For the quantitative analysis, the plates were scanned and the densities of the lipid spots were measured by NIH Image software. Standard curves were created by plotting data of various amounts of standard triolein and cholesteryl oleate spotted and developed on the same TLC plates. Then, the amounts of TG and CE in test samples were determined.

Assay for acyl-CoA synthetase

LDs were incubated at 37°C for the indicated time periods in the reaction mixture (100 μl) containing 15 mM $MgCl_2$, 10 mM ATP, 0.5 mM CoA, 5 mM dithiothreitol, 50 μM [^{14}C]OA (0.25 μCi), and 100 mM Tris-HCl (pH 8.0) to assay ACSL activity. Chloroform-methanol (400 μl; 1:2, v/v) was added to terminate the reaction. The samples were mixed with cold oleoyl-CoA (5 nmol) as carrier and spotted onto TLC plates containing fluorescein (Silica gel 60 F_{254}; Merck). TLC plates were developed with 1-buthanol-acetic acid-water (5:2:3, v/v), and [^{14}C]acyl-CoA was detected with a BAS 1500 imaging analyzer (Fuji Film Co., Ltd.). Oleoyl-CoA was detected under 254 nm of ultraviolet light. ACSL activity was calculated from the linear portion of the standard curve. To measure ACSL activity in fractions from sucrose density gradient centrifugation, each 2 μl of the fractions was applied to the assay with incubation periods of 10 min at 37°C. To examine the effect of troglitazone, reactions were performed in the presence of 10 μM troglitazone.

Assay for TG synthesis

TG synthesis from acyl-CoA. LDs were incubated with [^{14}C]acyl-CoA in the reaction mixture (100 μl) containing 15 mM $MgCl_2$, 50 μM [^{14}C]acyl-CoA (50 mCi/mmol), and 150 mM Tris-HCl

(pH 7.4) at 30°C for 30 min. The reaction was terminated by adding 300 μl of chloroform-methanol (2:1, v/v), and nonlabeled triolein and cholesteryl oleate (1 nmol each) were added as a carrier. The mixture was centrifuged at 10,000 rpm for 1 min at room temperature, and the lower chloroform layer was recovered. The upper aqueous layer was reextracted with 300 μl of chloroform and added to the first extract. The combined extracts were concentrated under argon gas and spotted onto TLC plates (Silica gel 60; Merck). They were developed with hexane-diethyl ether-acetic acid (80:20:1, v/v), and radioactive products were detected using the BAS 1500 imaging analyzer. TG and CE were identified by detecting the nonlabeled triolein and cholesteryl oleate with cupric acetate staining. To examine the effect of diacylglycerol (DG), reactions were performed in the presence of 0.4 mM DG.

TG synthesis from OA. LDs were incubated with [^{14}C]OA in reaction mixtures containing 15 mM $MgCl_2$, 1 mM dithiothreithol, 10 mM ATP, 0.5 mM CoA, 50 μM [^{14}C]OA (0.5 μCi), and 100 mM Tris-HCl (pH 8.0). For some reactions, either ATP or CoA was deleted. The reaction was carried out at 37°C for 30 min and terminated by 300 μl of chloroform-methanol (2:1, v/v). Radioactive products were analyzed as described above.

Other procedures

Immunoblot analysis was performed using the method described previously (4). ACSL3 in isolated LDs was quantitated using SDS-PAGE (Coomassie blue detection) with BSA as a standard. Protein amounts in LD samples were measured using the Protein dotMETRIC™ kit (Geno Technology, Inc., St. Louis, MO) and confirmed by SDS-PAGE with BSA as a standard.

RESULTS

LD formation is induced by OA and inhibited by triacsin C

We previously demonstrated that ACSL3 exists as one of the major proteins in LDs from HuH7 cells, with a proportion of the enzyme also found in cellular membrane fractions (4). In this study, we examined whether inhibition of cellular ACSL activity affects LD formation and the intracellular distribution of ACSL3. HuH7 cells were found to markedly accumulate LDs when cultured in the presence of OA for 1 day, and they continued to grow for up to 3 days [**Fig. 1A**, OA(+)]. However, in the presence of the ACSL inhibitor triacsin C (24–26), LD formation was suppressed to near the level of the control cells, despite the existence of the same concentration of OA in the medium [Fig. 1A, OA(+)/Tri C]. Under these culture conditions, OA and triacsin C did not cause any obvious toxicity. By measuring cellular lipids, it was confirmed that the cellular accumulation of TG and CE was induced by OA (Fig. 1Ba, b, closed circles) and that this accumulation was compromised by culturing the cells with triacsin C (Fig. 1Ba, b, open circles). The amounts of free cholesterol (FC) did not alter under the various culture conditions (Fig. 1Bc).

We investigated whether the culturing of cells with OA and triacsin C affects the subcellular distribution of ACSL3. After cells were cultured for 1 day under the conditions described for Fig. 1, subcellular fractionation of HuH7 cells was carried out using sucrose density gradient centrifugation (**Fig. 2A**). An immunoblot showed that ACSL3 was distributed mainly in both the top fraction and the membrane fractions in untreated cells [Fig. 2Bb and **Fig. 3A**, OA(−), fraction 1 and fractions 14–19]. The top fraction corresponds to LDs, where TG, CE, and the LD marker protein ADRP are concentrated (Fig. 2B). The membrane fraction contained marker proteins of microsomes (calnexin), Golgi apparatus (GS28), and plasma membranes (integrin) (Fig. 2Bd, e) (4). LD fractions from OA-treated cells contained 2.8 times more ACSL3 than those from untreated cells when LD fractions from equal amounts of cells were compared (Fig. 3Ba). In OA-treated cells, some ACSL3 was found in medium density fractions [Fig. 3A, OA(+), fractions 7–12]. Conversely, when cells were cultured in the presence of both OA and triacsin C, there was no OA-induced increase in droplet ACSL3, nor was there any change in distribution. In these culture conditions, the ratios of droplet to cellular ACSL3 were 33% OA(−), 40% OA(+), and 21% OA(+)/triacsin C. The amounts of droplet ACSL3 changed in parallel with those of other droplet components, such as ADRP, TG, and CE (Fig. 3Bb–d). These results suggest that LD formation is mediated by ACSL activity and that the intracellular distribution of ACSL3 proteins changes in a manner coupled with LD formation.

Regression of LDs by triacsin C

Triacsin C effectively inhibited LD formation in HuH7 cells. We then tested whether the inhibitor could also induce the regression of LDs. LD formation was significantly induced by incubating cells with OA for 2 days [**Fig. 4A**, OA(+/−), 4Ba, b, closed circles]. When these cells were cultured for another 3 days in the absence of OA, almost 80% of these LDs were still retained in the cells [Fig. 4A, OA(+/−), 4Ba, b, closed circles]. However, when triacsin C was added to the medium after OA removal, most of the LDs disappeared in the subsequent culture periods [Fig. 4A, OA(+/−)/Tri C, 4Ba, b, open circles]. These results suggest that inhibition of cellular ACSL activity leads to decreases in neutral lipid concentration and the disappearance of LDs.

We analyzed the cellular distribution of ACSL3 during the regression of LDs. After OA-treated cells were incubated in the absence of OA for another 3 days, the ACSL3 level in the LD fraction was found to be ~10 times higher than that in the control cells, which were not loaded with OA (**Fig. 5B**a). This shows that droplet ACSL3 remained high even after the removal of OA. However, in cases in which the cells were incubated with triacsin C for 3 days after OA loading, the ACSL3 in LD fractions decreased to 35% of the level of the OA-treated cells and the distribution shift of ACSL3 in fractions 7–11 was canceled (Fig. 5A, Ba). Under these culture conditions, the ratios of droplet and cellular ACSL3 were 15% OA(−/−), 48% OA(+/−), and 26% OA(+/−)/triacsin C. Treatment of the cells with triacsin C also induced significant decreases of ADRP, TG, and CE in the LD fractions (Fig. 5Bb–d). These results suggest that cellular ACSL ac-

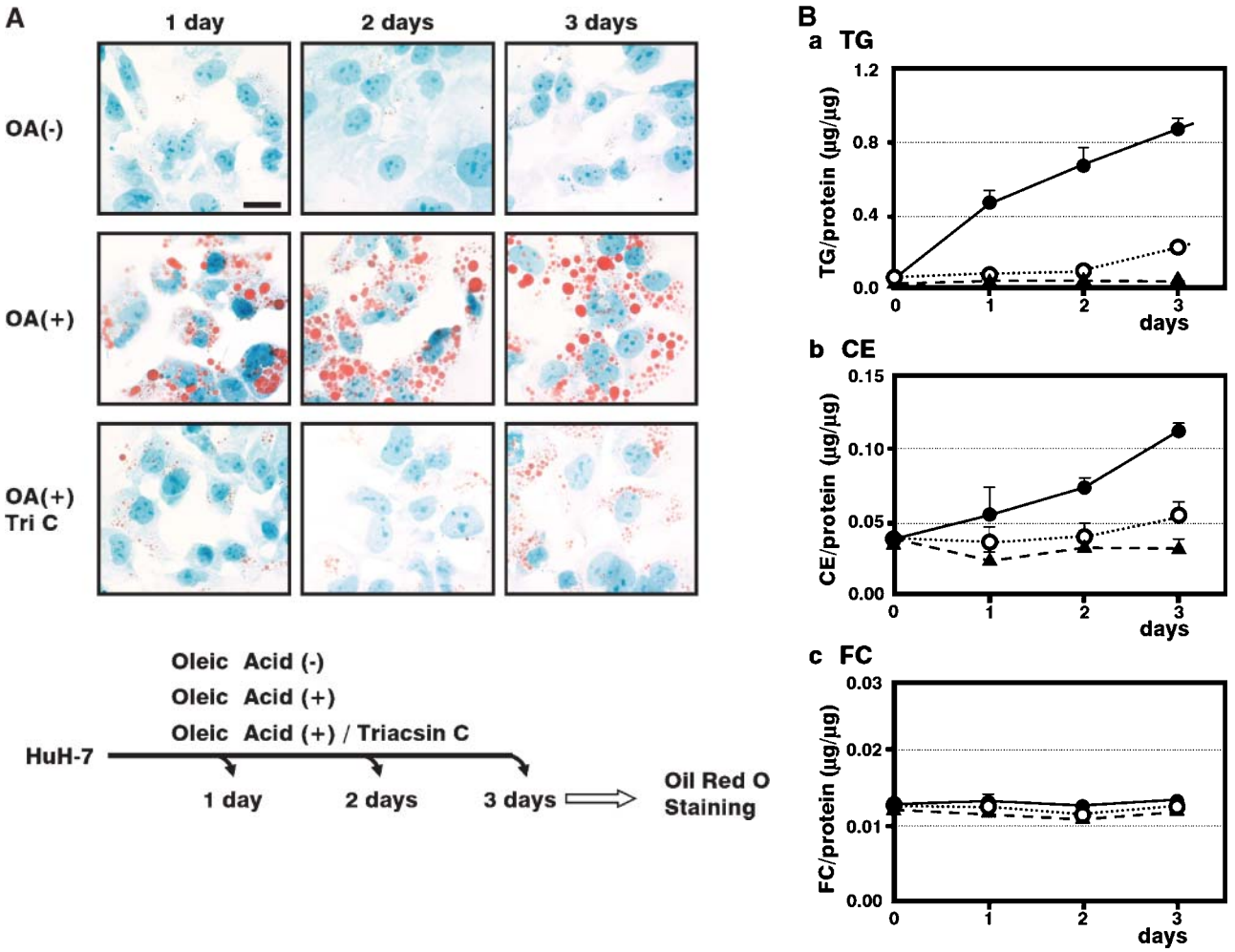

Fig. 1. Lipid droplet (LD) formation is induced by oleic acid (OA) and suppressed by triacsin C. A: HuH7 cells were cultured for 1, 2, or 3 days in basal medium (DMEM containing 10% FBS) supplemented with no OA, 0.6 mM OA, or both 0.6 mM OA and 5.4 μM triacsin C [OA(−), OA(+), and OA(+)/Tri C, respectively]. Cells were fixed with formalin and stained using Oil Red O and Mayer's hematoxylin. Intracellular LDs were stained in red, and nuclei were stained in blue. Bar = 25 μm. Culture conditions and times are shown under the panels. B: Quantitation of cellular triacylglycerol (TG) (a), cholesteryl ester (CE) (b), and free cholesterol (FC) (c). Cells cultured without the addition of OA (closed triangles), with 0.6 mM OA (closed circles), or with both 0.6 mM OA and 5.4 μM triacsin C (open circles) were collected, and lipids were separated using TLC. The amount of each lipid was adjusted for total cell protein, and the values are shown as means ± SD of two independent experiments.

tivity is involved not only in the formation of LDs but also in their maintenance and that the amount and distribution of ACSL3 change in relation to the emergence and disappearance of LDs.

ACSL activity in isolated LDs

Next, we attempted to test whether the LD fraction separated by sucrose density gradient ultracentrifugation showed any ACSL activity. As shown in Fig. 2B, major LD components (TG, CE, and ADRP) were highly concentrated in the top fraction. Plenty of Oil Red O-positive particles were observed in the fraction (4). In contrast, this fraction contained very limited amounts of components of other subcellular compartments, such as the cytosolic marker LDH, the Golgi marker GS28, and the ER marker calnexin (Fig. 2B).

When LDs from untreated cells were incubated with ^{14}C-labeled OA or palmitic acid, production of radioactive acyl-CoA was observed (**Fig. 6**), revealing that LDs show ACSL activity. Based on the concentrations of ACSL3 in LDs, the specific activity of the droplet ACSL was calculated to be 10.8 μmol/min/mg, which is not so different from a value for ACSL3 purified from COS-7 cells (27). When LDs were incubated with [^{14}C]OA in the presence of triacsin C, the activity was inhibited by triacsin C in a dose-dependent manner with maximal inhibition of >80% (Fig. 6B). The IC_{50} value was 4.0 μM, which correlates well with the value of 5.5 μM reported for recombinant ACSL3 (26). These results clearly show that LDs demonstrate ACSL activity.

We surveyed the distribution of ACSL activity in subcellular fractions prepared by sucrose density gradient centrifugation. In untreated cells, 4.5% of the activity was detected in LDs (fraction 1) and residual activity was distributed mainly in fractions 10–19 (Fig. 2Bi). This result demonstrates that ACSL activity is concentrated in LDs,

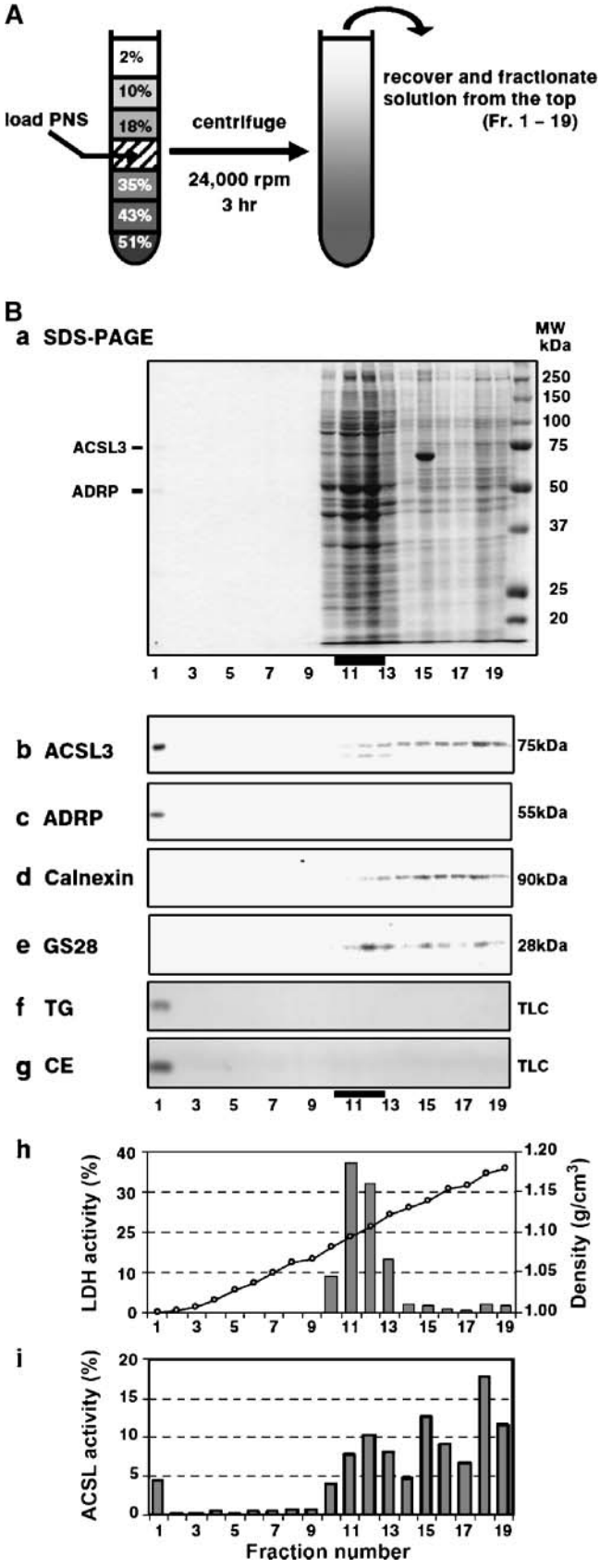

Fig. 2. Subcellular fractionation of HuH7 cells using sucrose density gradient centrifugation. A: Scheme of the cell fractionation procedure. Postnuclear supernatant (PNS) was isolated from HuH7 cells, and its sucrose concentration was adjusted to 26%. The PNS solution was loaded at the middle position of a step-wise sucrose gradient. Percentage values indicate concentrations of sucrose. After ultracentrifugation at 24,000 rpm for 3 h, the solution was recovered from the tube top and separated into 19 fractions (Fr.; 0.8 ml each) (see Materials and Methods). B: Untreated HuH7 cells were fractionated as shown in A, and distributions of various cellular components were analyzed. a: Proteins in each fraction were separated by SDS-PAGE (9.5% acrylamide gel) and stained with Coomassie blue. Two protein bands detected in fraction 1 correspond to two major LD proteins, adipose differentiation-related protein (ADRP) and long-chain acyl-coenzyme A synthetase 3 (ACSL3). b–e: ACSL3, ADRP (LD marker), calnexin (endoplasmic reticulum marker), and GS28 (Golgi marker) in each fraction were detected by immunoblot. f, g: TG (f) and CE (g) were analyzed by TLC. Each analysis of a–g corresponds to the fractionation of 85 μg of PNS (in protein amount). h: Activity of lactate dehydrogenase (LDH; a marker for cytosol) and density in each fraction. i: ACSL activity in each fraction was assayed using [^{14}C]OA as substrate (see Fig. 6 and Materials and Methods). The top and bottom fractions of the gradient are fractions 1 and 19, respectively. The bars above the fraction numbers correspond to the position where the PNS sample was loaded.

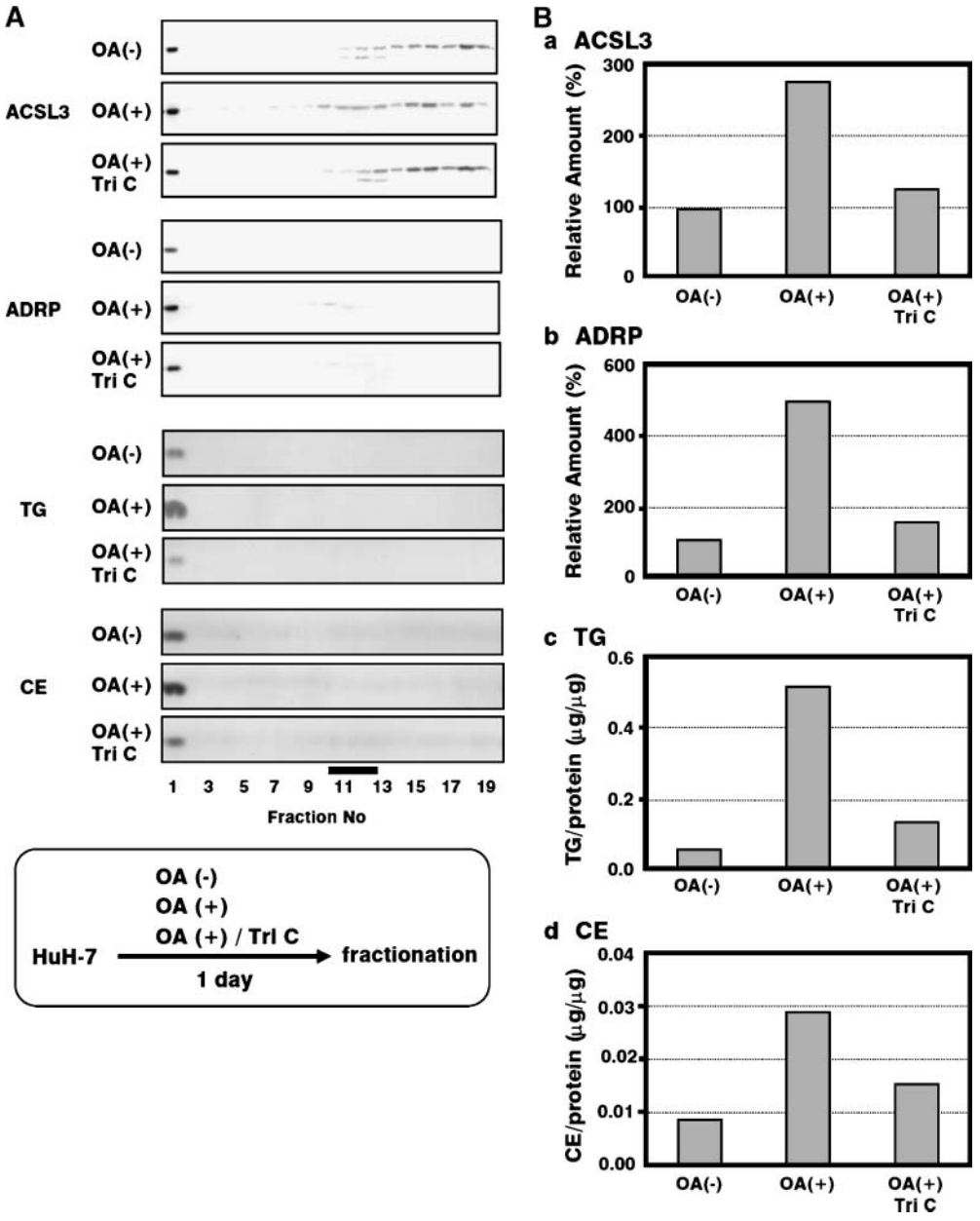

Fig. 3. Distribution of ACSL3 in cells treated with OA and triacsin C. A: HuH7 cells were cultured for 1 day in basal medium (DMEM + 10% FBS) supplemented with no OA, 0.6 mM OA, or both 0.6 mM OA and 5.4 μM triacsin C as indicated at the bottom. PNS prepared from each culture underwent a sucrose density gradient centrifugation as shown in Fig. 2A. ACSL3 and ADRP in each fraction were detected by immunoblot analysis. TG and CE were separated by TLC. Each strip of blot and TLC corresponds to the fractionation of 85 μg of PNS (in protein amount). The top fraction is labeled fraction 1 and the bottom fraction is labeled fraction 19. The bar above the fraction numbers indicates the position where PNS was applied. Strips of blots and TLC indicated by OA(−) are just the same as those in Fig. 2B. B: Amounts of ACSL3 (a), ADRP (b), TG (c), and CE (d) in the LD fraction (fraction 1).

because fraction 1 contains only a small amount of proteins but the subsequent fractions contain much larger amounts (Fig. 2Ba).

We also tested the effects of troglitazone, which inhibits ACSL4 but not other ACSL isoforms (28). Approximately 80% of the droplet ACSL activity was resistant to troglitazone at a concentration of 10 μM (**Fig. 7B**), suggesting that the majority of ACSL activities in LDs were not from ACSL4 but from ACSL3. We tested whether troglitazone could inhibit LD formation in the cells and found that OA-dependent LD formation was not affected by this compound (Fig. 7A). Thus, it is unlikely that ACSL4 is the major enzyme responsible for the LD formation in the cells.

We then compared ACSL activities in LD fractions separated from cells cultured under different conditions. First, the effect of OA loading for 1 day was surveyed as

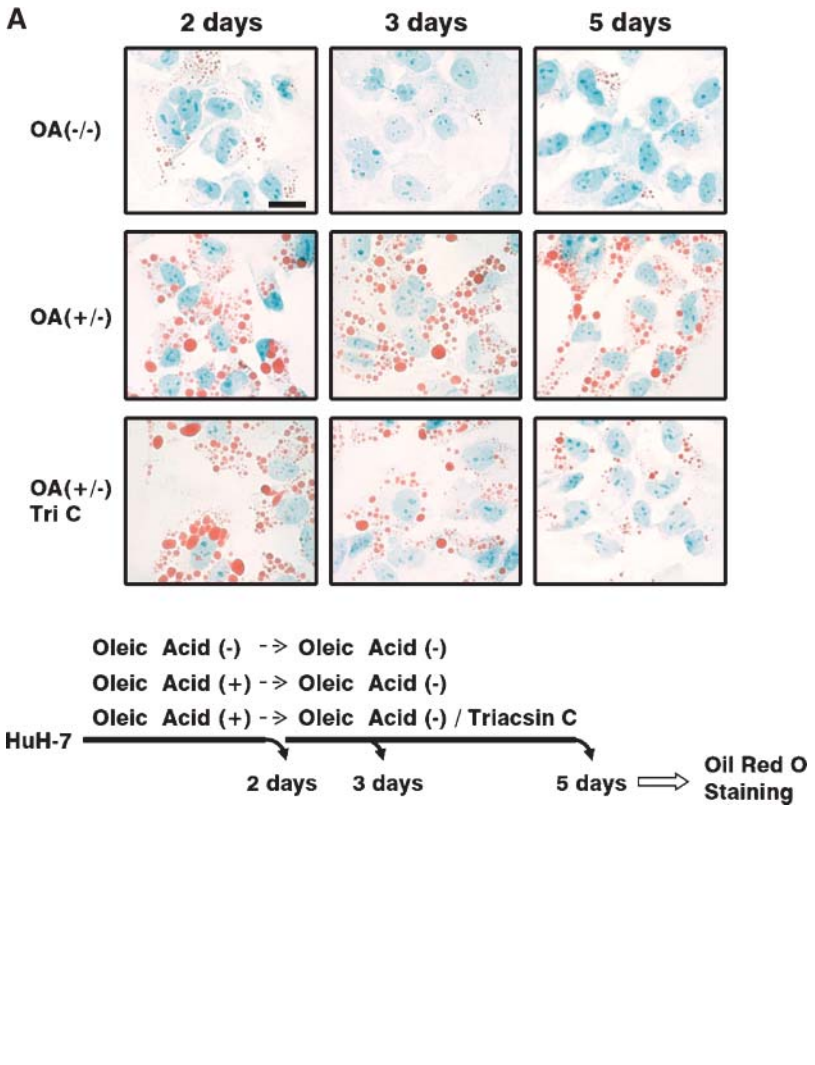

Fig. 4. Triacsin C induces the regression of LDs. HuH7 cells were cultured for up to 5 days using three different conditions as indicated under the panels. In the first condition, the cells were cultured in basal medium without OA for 2 days and then remained in culture for up to 3 days after change to a new medium without OA [OA(−/−); closed triangles]. In the second condition, the cells were cultured in the presence of 0.6 mM OA for 2 days and then in the absence of OA for up to 3 days [OA(+/−); closed circles]. In the third condition, the cells were cultured for 2 days in medium containing 0.6 mM OA and then in new medium containing 5.4 μM triacsin C and no OA [OA(+/−)/Tri C; open circles]. A: Cells were fixed with formalin on each day and stained by Oil Red O/Mayer's hematoxylin. Bar = 25 μm. B: Amounts of TG (a), CE (b), and FC (c) in the cells were measured by TLC. The quantity of each lipid was standardized based on total cell protein, with the indicated values representing means ± SD of two independent experiments.

shown in **Fig. 8A**. The LD fraction in OA-loaded cells had ACSL activities 2.2 times higher than those in untreated control cells (Fig. 8A). This seemed to correlate with the increase in ACSL3 protein detected in the LD fractions (Fig. 3Ba). Conversely, when cells were cultured with both OA and triacsin C, ACSL activity in LDs was only 30% of that found in the control cells (Fig. 8A). Because the amount of ACSL3 in the LD fraction was 1.2 times that in the control cells (Fig. 3Ba), this result suggests that culturing the cells with triacsin C resulted in a strong decrease of ACSL activity in LDs.

Second, we measured droplet ACSL activities in cells cultured in LD-regressing conditions, as depicted in Fig. 4A. Cells were loaded with OA for 2 days to induce LD formation and cultured for another 3 days without OA. Three days after the OA removal, the droplet ACSL activity was four times higher than that of untreated control cells (Fig. 8B), showing that once LD formation was induced by loading with OA, the ACSL activity in the LD fraction was kept at a high level afterward. This is in accordance with the cellular retention of LDs and droplet ACSL3, as observed in Figs. 4, 5. However, when the OA-treated cells were cultured with triacsin C, the activity was reduced to only 24% of that of the untreated cells (Fig. 8B), confirming that treatment of the cells with triacsin C leads to a severe reduction of the droplet ACSL activity.

Activity for neutral lipid synthesis in isolated LDs

As ACSL activity was present in LDs, we then investigated whether acyl-CoAs can be used in LDs. To test this,

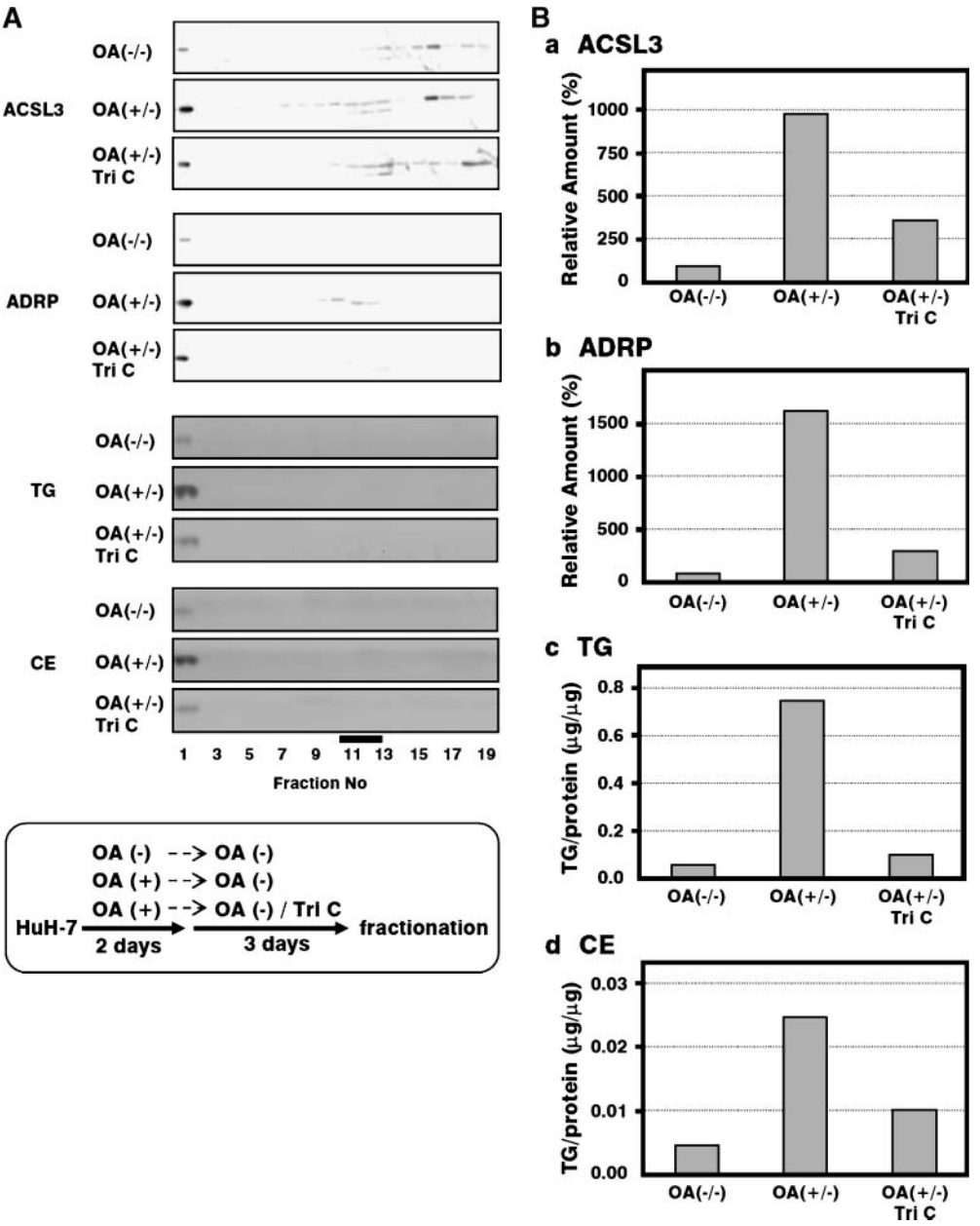

Fig. 5. Effect of triacsin C on the subcellular distribution of ACSL3 after OA removal. A: HuH7 cells were cultured for 5 days under the three conditions as indicated at the bottom. OA(−/−), cells were cultured in basal medium without OA for 2 days and then cultured for an additional 3 days after change to a new medium without OA; OA(+/−), cells were cultured with 0.6 mM OA for 2 days and then for 3 days without OA; OA(+/−)/Tri C, cells were cultured with 0.6 mM OA for 2 days and then for 3 days without OA in the presence of 5.4 μM triacsin C. PNS prepared from each culture underwent sucrose density gradient centrifugation. ACSL3 and ADRP in each fraction were detected by immunoblot analysis. TG and CE were separated by TLC. Each strip of blot and TLC gel corresponds to the fractionation of 85 μg of PNS (in protein amount). The top fraction is labeled fraction 1 and the bottom fraction is labeled fraction 19. The bar above the fraction numbers indicates the position where PNS was applied. B: Relative amounts of ACSL3 (a), ADRP (b), TG (c), and CE (d) in the LD fraction (fraction 1).

we examined whether LDs have the ability to synthesize neutral lipids using acyl-CoA as substrate. When isolated LDs were incubated with ^{14}C-labeled oleoyl-CoA, radioactive TG and CE were produced (**Fig. 9A**). Production of radioactive TG was ~10 times higher than that of CE (Fig. 9A), in accordance with the finding that TG was synthesized more intensely than CE in OA-loaded cells (see TG and CE effects in Figs. 1B, 3B). Interestingly, isolated LDs could produce TG and CE without the addition of other substrate molecules such as DG and FC (Fig. 9A); this suggests that substrates for neutral lipid synthesis can be supplied, at least in part, from LDs themselves. We

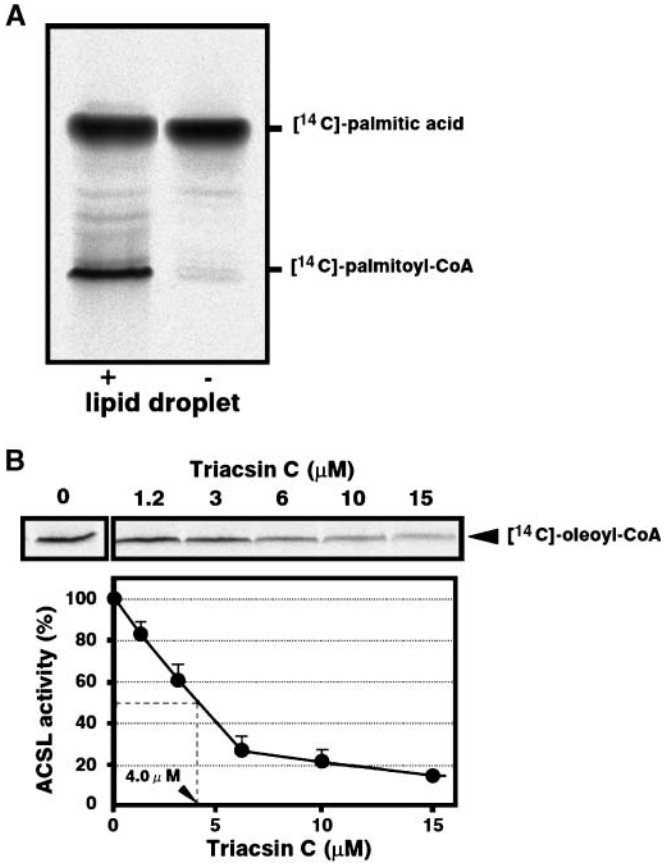

Fig. 6. ACSL activity in isolated LDs. ACSL activity was investigated in the LD fraction isolated from untreated HuH7 cells. A: [^{14}C]palmitic acid was incubated either in the presence or absence of the LD fraction at 37°C for 30 min. After the reaction, [^{14}C]acyl-CoA was separated by TLC and detected using a BAS 1500 imaging analyzer. B: LDs were incubated with [^{14}C]OA in the presence of various concentrations of triacsin C at 37°C for 10 min. [^{14}C]acyl-CoA was separated by TLC and quantified. Relative ACSL activity in the presence of triacsin C is indicated. Values represent means ± SD of two independent experiments.

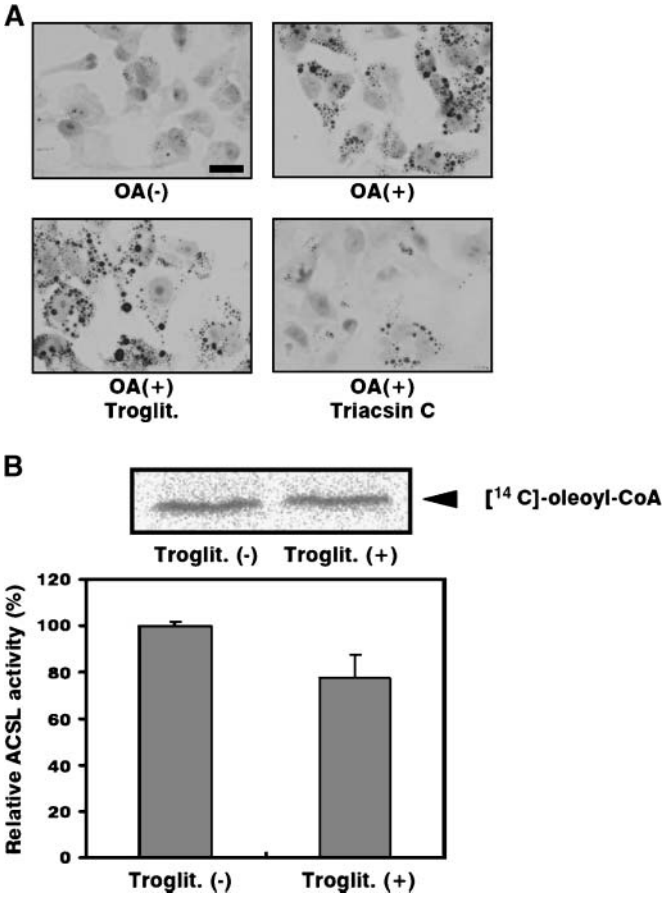

Fig. 7. Effects of troglitazone on LD formation and droplet ACSL activity. A: To investigate the effects of troglitazone on LD formation, cells were cultured for 1 day in basal DMEM containing no OA [OA(−)], 0.6 mM OA [OA(+)], both 0.6 mM OA and 10 μM troglitazone [OA(+)/Troglit.], or both 0.6 mM OA and 5.4 μM triacsin C [OA(+)/Triacsin C]. Cells were fixed with formalin and stained using Oil Red O and Mayer's hematoxylin. Bar = 25 μm. B: The LD fraction from untreated cells was assayed for ACSL activity in the presence or absence of 10 μM troglitazone using [^{14}C]OA as a substrate. Results are expressed as relative activities, with activity in the absence of troglitazone set at 100%. Values represent means ± SD of two independent experiments.

found that isolated LDs contain substantial amounts of FC together with TG and CE (data not shown). Addition of DG in the reaction increased TG production (Fig. 9A), confirming that DG can be a substrate in the reaction. Because the activity for TG synthesis was originally found in cell membrane fractions (29), the activity in LDs was compared with that detected in cell membranes. The level of TG synthesis activity in the LD fraction was 3.6 times that in the cell membrane fraction in the presence of DG when these activities were adjusted for their respective protein concentrations (data not shown).

Finally, we tested whether the OA could be metabolized to neutral lipids in isolated LDs. When isolated LD fractions were incubated with ^{14}C-labeled OA, radioactive TG was produced (Fig. 9B). This reaction required ATP and CoA, supporting the involvement of ACSL in the reaction (Fig. 9B). This suggests that fatty acids in LDs can be modified to acyl-CoAs and then integrated into TG.

DISCUSSION

Involvement of droplet ACSL in lipid metabolism in LDs

Our previous study revealed that ACSL3 is one of the most abundant proteins in LDs of HuH7 cells (4). In this study, we demonstrated that ACSL activity in LDs contributes to local lipid synthesis. In the first half of the study, we found that the degree of change of droplet ACSL3 is closely coupled with the formation and metabolism of LDs. OA induced large amounts of LDs in the cells, and the formation of LDs was reduced significantly when cellular ACSL activity was inhibited by triacsin C. After the accumulation of LDs in the cells, inhibition of ACSL activity resulted in the disappearance of LDs. ACSL3 accumulated in LDs depending on the emergence of LDs, and the droplet ACSL3 disappeared depending on the disappearance of LDs. Therefore, cellular ACSL activity

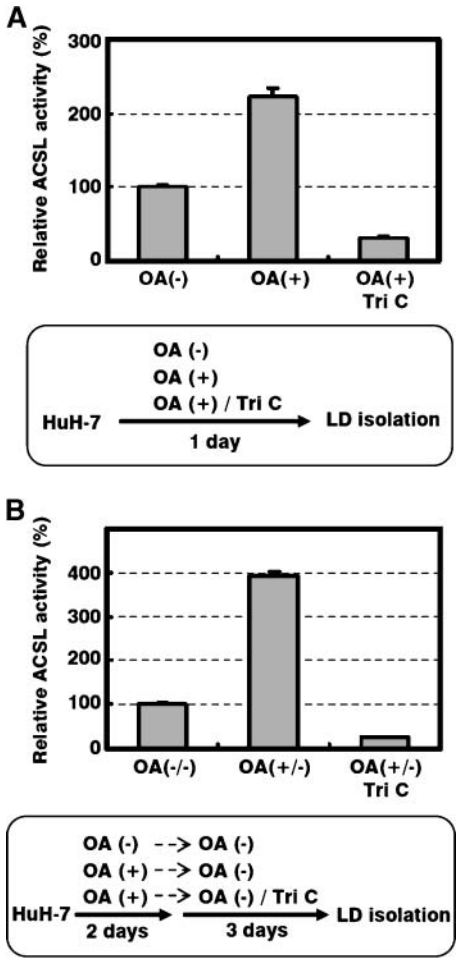

Fig. 8. ACSL activity in LDs isolated from OA- and triacsin C-treated cells. LD fractions were prepared from the cells cultured under the various conditions described for Figs. 3A, 5A. ACSL activities in the LD fractions were measured using [^{14}C]OA as a substrate. Reactions were performed at 37°C for 10 min. A: ACSL activities in LD fractions prepared from cells cultured under the conditions described for Fig. 3A. B: ACSL activities in LD fractions from cells cultured under the conditions described for Fig. 5A. Each graph compares droplet ACSL activities contained in equal amounts of cell extracts, standardized with amounts of PNS. The results are reported as means ± SD of two independent experiments with OA(−) or OA(−/−) as 100%. Culture conditions are also shown under the graphs.

is tightly correlated with both LD formation and the localization of ACSL3 to the droplets. In the second half of the study, we confirmed that LDs isolated from the cells did indeed have ACSL activity. This is the first demonstration, to the best of our knowledge, of ACSL activity in isolated LDs. Furthermore, the LD fraction was shown to be capable of generating not only acyl-CoA but also TG and CE in vitro. These findings suggest that droplet ACSL plays pivotal roles in the formation and maintenance of LDs.

Droplet ACSL activity and contribution of ACSL3

We detected ACSL activity in the isolated LDs using ^{14}C-labeled fatty acids as substrates. This suggests that ACSL proteins abundant in the LDs are active enzymes and that LDs are intracellular spaces for the generation of acyl-CoAs, whereas ACSL activities have been detected in subcellular compartments such as microsomes, mitochondria, and MAM (6).

The majority of the ACSL activity detected in the LD fractions may be attributed to ACSL3 for the following two reasons. First, ACSL3 is one of the most abundant proteins in LDs and is a specific component of LDs, as reported previously. ACSL3 was reproducibly detected in LDs with high and specific content (4). It closely associated with the LDs and did not easily dissociate from them (4). Its amount in LDs is several times greater than that of ACSL4, and the contents of other ACSL isoforms are much lower than these two or at negligible levels (4). No other proteins identified in LDs appear to be ACSLs, because they have no obvious sequence similarities to known ACSLs or ACS-related proteins (4). Second, ~80% of the droplet ACSL activity was unaffected by troglitazone, which preferably inhibits ACSL4 (28). This suggests that ACSL4 contributes to only ~20% of the droplet ACSL activity. This contribution ratio agrees with the protein ratio of ACSL3 and ACSL4 in LDs, taking into account that specific activities of these two enzymes are at similar levels (26). Hence, it is assumed that a large part of droplet ACSL activity is attributed to ACSL3.

We also demonstrated that droplet ACSL activity increases or decreases in a manner closely correlated with changes in the amounts of LDs and droplet ACSL3. The droplet ACSL activity increased in parallel with the increases of cellular LDs and of droplet ACSL3. Treatment of cells with triacsin C blocked LD formation or induced the disappearance of LDs. In these cases, the droplet ACSL activity was reduced very severely.

Triacsin C, at the concentration used in this study, preferentially inhibits ACSL1, ACSL3, and ACSL4 but not ACSL5 and ACSL6 (25). The inhibitor does not inhibit short-chain acyl-CoA synthetase and medium-chain acyl-CoA synthetase (26). This suggests that ACSL1, ACSL3, or ACSL4 is the most probable candidate ACSL isoform responsible for the OA-dependent LD formation. Very recently, we reported that free fatty acids with chain lengths of C12–C18 effectively induced LDs in HuH7 cells, whereas those of C8 and C10 did not (30). This fatty acid preference in LD formation agrees with the reported substrate specificities of ACSL3 and ACSL4 (27, 31). However, it is unlikely that ACSL4 contributes significantly to LD formation, because troglitazone did not inhibit the OA-dependent LD formation (Fig. 7A).

It is noteworthy that ACSL3 is commonly present in LDs of various kinds of mammalian cell types (7–9). Interestingly, even in the yeast *Saccharomyces cerevisiae*, ACSLs are major protein components of LDs (32). The evolutionarily conserved localization of ACSLs in LDs over a wide range of eukaryotic cells appears to support its importance in LDs.

Neutral lipid synthesis in LDs

In isolated LDs, we detected neutral lipid synthesis using ^{14}C-labeled acyl-CoAs as substrates (Fig. 9A). This result suggests that fatty acyl-CoA can be used in LDs. Isolated

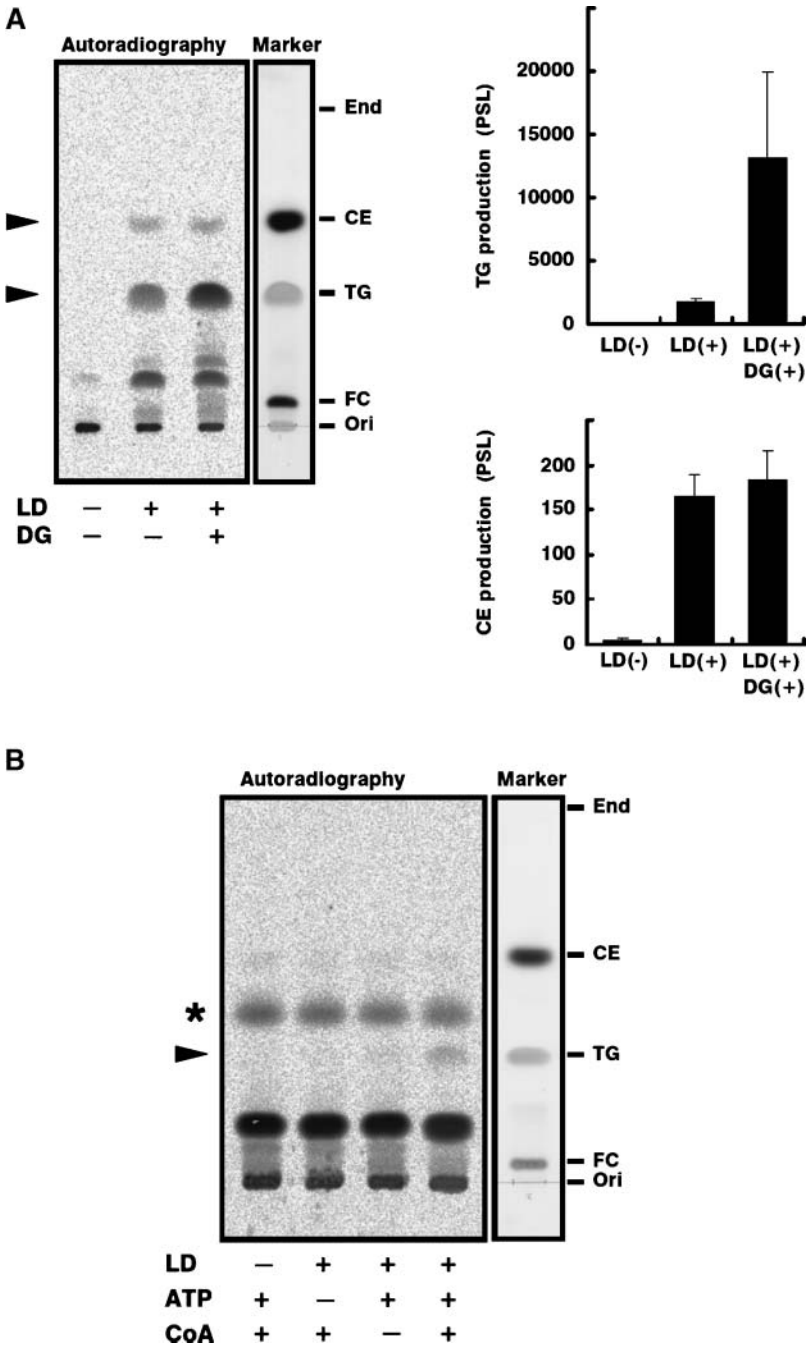

Fig. 9. Neutral lipid synthesis in isolated LDs. The activity of neutral lipid synthesis was investigated in LDs isolated from untreated HuH7 cells. A: Isolated LDs were incubated with [^{14}C]oleoyl-CoA in the presence or absence of 0.4 mM diacylglycerol (DG). Radioactive products were separated by TLC and visualized using a BAS 1500 imaging analyzer. A marker lane indicates nonlabeled standard lipids separated on the same TLC plate and visualized by cupric acetate staining. Radioactive TG and CE are indicated by arrowheads, and the amounts of TG and CE are shown in the graphs. Values represent means ± SD of two independent experiments. PSL (photostimulated luminescence) is a unit of quantity of radioactivity detected by the image analyzer. B: Isolated LDs were incubated with [^{14}C]OA in the presence or absence of 10 mM ATP and 0.5 mM CoA, and radioactive products were analyzed by TLC. The arrowhead indicates labeled TG. The thickest spots correspond to [^{14}C]OA. An asterisk indicates contaminant signals derived from the original solution of [^{14}C]OA.

LDs produced much larger amounts of TG than CE. This finding coincides with our cellular studies, showing that the production of TG was greater than that of CE in the presence of OA (Figs. 1B, 3B). In vitro TG synthesis by LDs was also observed using radiolabeled OA as a substrate. This reaction required ATP and CoA, the other essential substrates of ACSL. Therefore, droplet ACSL appears to metabolize fatty acids to acyl-CoAs in the course of neutral lipid synthesis. In addition to acyl-CoA, the synthesis of TG and CE usually requires DG and FC as substrates. TG synthesis by LDs was enhanced significantly in the presence of DG (Fig. 9A). However, the synthesis of TG and CE was detected even in the absence of DG and FC. These substrates may have been supplied from LDs themselves. TLC analysis of LDs indicated the existence of a substantial amount of FC, whereas a significant amount of DG was not detected (data not shown).

The production of TG and CE from radioactive oleoyl-CoA (Fig. 9A) suggests the presence of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT)- and ACAT-like activities in LDs. In our proteomic analysis, however, neither DGAT nor ACAT protein was found among proteins identified in LDs of HuH7 cells (4). It is thus possible that these enzymes might be present in LDs in very low concentrations. Other proteomic studies also failed to detect DGAT and ACAT in LDs from Chinese

hamster ovary K2 cells (7), OA-treated A431 cells (8), or 3T3-L1 adipocytes (9). DGAT and ACAT mainly locate in ER membranes (33). Ultrastructural and biochemical studies revealed that the surfaces of LDs are surrounded by a monolayer of phospholipids but not by bilayer membranes (2, 34). It is unlikely that isolated LD fractions contain large amounts of cell membranes. In fact, LDs are quite distinct from other membranous organelles in their protein compositions (4). They are also quite distinct in their densities. LDs float and cellular membranes sediment in ultracentrifugation. However, studies demonstrate that some LDs associate intimately with ER-like membrane structures (16, 35, 36). DGAT and ACAT may exist in such membrane structures.

Involvement of ACSL in the cellular maintenance of LDs

As shown in Fig. 4, most LDs were retained in the cells after OA was removed from the medium. However, LDs disappeared by a combination of OA removal and inhibition of ACSL activity by triacsin C. This suggests that the cells tend to retain LDs and that ACSL activity is required for the maintenance of LDs. It is thought that cellular TG and CE turn over in a cycle of hydrolysis and reesterification (37). Presumably, LDs remain in the cells when these two processes are balanced. The droplet ACSL may engage in the reesterification step by providing acyl-CoA. Inhibition of ACSL leads to the inhibition of acyl-CoA production and thus weakens the reesterification process. This leads to the hydrolysis of neutral lipids and the subsequent disappearance of LDs. ACSL activity also appears to be involved in the maintenance of LDs in cell types other than hepatocytes. For example, neutral lipids accumulated in THP-1 macrophages can be removed by treatment with triacsin D, a similar ACSL inhibitor (38).

We have reported that ADRP decreases during the depletion of neutral lipids stored in macrophages and HuH7 cells and that the rapid degradation of ADRP protein is mediated by a proteasome-dependent mechanism (39). Therefore, to understand LD regression more systemically, it is important to consider whether other LD proteins, including ACSL3, are also degraded via similar pathways.

Localization of ACSL3 in LDs and cellular membranes

In our study, ACSL3 protein was detected in both LD fractions and cell membrane fractions. When LD formation was enhanced by OA, the amount of droplet ACSL3 increased, with a proportion of ACSL3 in the membrane fractions shifting to less dense fractions (Fig. 3A). Recently, such a localization shift was observed for a lipid-metabolizing enzyme called NAD(P)H steroid dehydrogenase-like protein (NSDHL) (21). In LD-poor conditions, NSDHL was detected in cell membrane fractions comigrating with an ER marker protein, calnexin. In LD-rich conditions, NSDHL was found in both the LD fraction and the membrane fraction (21). A major hypothesis accounting for LD formation is that LDs originate from the accumulation of neutral lipids in ER membranes. The synthesis of neutral lipids leads to their accumulation in a space between the leaflets of the membranous bilayers of the ER, with subsequent budding off of LDs from it (14, 40–42). A part of proteins identified in LDs may be transferred from ER membranes to LDs in the course of LD formation.

In conclusion, we found ACSL activity in LDs from a cultured human hepatocyte cell line. The quantity of ACSL3 in the LDs, and also droplet ACSL activity, changes in a manner closely related to the formation and metabolism of LDs. These LDs also have the ability to synthesize neutral lipids using fatty acyl-CoA as a substrate. This experimental evidence suggests a local engagement of droplet ACSL in the synthesis of neutral lipids in LDs.

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