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Long-Chain Fatty Acids Induce Lipid Droplet Formation in a Cultured Human Hepatocyte in a Manner Dependent of Acyl-CoA Synthetase

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Lipid droplets (LDs) are intracellular storage sites of neutral lipids, which accumulate in fatty liver disease. Here, we investigated the effects of fatty acids and glucose on LD formation in a cultured human hepatocyte, HuH7, by adding them to culture media. Fatty acids with carbohydrate chains C12—C18 efficiently induced LDs, but those of C8 and C10 were ineffective. Glucose did not induce LD formation even in the presence of insulin. Oleic acid induced significant increases in cellular neutral lipids, and cell fractionation revealed that most of the newly synthesized neutral lipids were concentrated in LDs together with LD proteins. The LD formation was not abrogated by removal of medium glucose but was significantly inhibited by an ACSL inhibitor, triacsin C. These results demonstrate that long-chain fatty acids contribute to LD formation to a greater extent than glucose, possibly by being taken up into the cells, activated by ACSL, reconstituted into neutral lipids and then stored in LDs. Pregnenolone and lithium did not suppress oleic acid-dependent LD formation, despite previous reports of their ability to inhibit LD formation in macrophages and adipocytes suggesting differences among LD formations in these cells.

Key words lipid droplet; neutral lipid; fatty acid; hepatocyte; triacsin C

Various organisms store energy in a form of neutral lipids such as triacylglycerol (TG) and cholesteryl ester (CE). In mammals, these neutral lipids are intracellularly stored as typically seen in adipocytes, hepatocytes, steroidogenic cells and macrophage-derived foam cells. Storage of lipids has merits allows for energy preservation and synthesis of lipoproteins and steroid hormones. However, excess accumulation often leads to disorders such as obesity, fatty liver and atherosclerosis.[1] It is therefore important to understand how neutral lipids are stored and mobilized intracellularly, as these mechanisms largely remain unclear. In animal cells, neutral lipids are deposited in intracellular compartments called lipid droplets (LDs, also called lipid storage droplets, lipid bodies or lipid particles). TG and CE are packed in LDs coated with phospholipid monolayer.[2,3]

The liver is the center of lipid metabolism in the body. It recovers and synthesizes lipid molecules and reconstitutes them as lipoproteins such as very low-density lipoprotein (VLDL), which are secreted into the circulation and distributed throughout the body. Thus, hepatic cells are important stores of neutral lipids and act as a physiological buffer. Increased accumulation of neutral lipids often causes fatty liver disease accompanied by LD formation in the hepatocytes. Recently, attention has been focused on hepatic steatosis as a part of the metabolic syndrome since this is intimately related to other signs and symptoms of the syndrome including obesity, hypertension, insulin resistance, hyperglycemia and hyperlipidemia.[4] Hepatic steatosis is also induced by factors other than nutrients, including drugs and toxic reagents.[5]

In this study, we tested LD-induction activity of fatty acids and glucose, which can cause hepatic steatosis, using a human hepatocyte cell line; this suggested that long-chain free fatty acids (FFA) most effectively induce LDs. We also tested various compounds as to whether they could suppress LD formation induced by oleic acid (OA), a long chain FFA. The LD formation was effectively blocked by an inhibitor of long chain acyl-CoA synthetase (ACSL). These results suggest that long chain FFAs and ACSLs are significant factors in LD formation in hepatocytes.

MATERIALS AND METHODS

Materials HuH7, a human hepatocyte cell line, was obtained from Health Science Research Resources Bank (cell no. JCRB0403; Osaka Japan). Fatty acids (as sodium salt) and 5-pregnen-3β-ol-20-one (pregnenolone) were purchased from Sigma (U.S.A.). Caffeine, capsaicin, carbon tetrachloride and *dl*-isoproterenol hydrochloride were purchased from Wako (Japan). Triacsin C was purchased from Kyowa Medex (Japan). 4,4,10β-Trimethyl-*trans*-decal-3β-ol (AMO 1618) was purchased from Calbiochem (U.S.A.). Anti-ADRP monoclonal antibody was purchased from PROGEN (Germany). Anti-17βHSD11 antiserum was prepared as described previously.[6] Peroxidase-conjugated goat anti-IgG antibodies were purchased from Biosource International (Camarillo, California). ECL Western Blotting detection system was purchased from Amersham Pharmacia Biotech (U.K.).

Culture and Oil Red O Staining of HuH7 Cells HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). In every case, the medium was supplemented with 10% fetal bovine serum (FBS). In some cultures, fatty acids (as sodium salt, conjugated with bovine serum albumin (BSA) at a molar ratio of FFA/BSA=5/1) and/or other reagents were added to the medium at the indicated concentrations. For Oil red O staining experiments, HuH7 cells were inoculated at 5×10^4 cells/well in four-well culture slides

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(Falcon, U.S.A.) and cultured at 37 °C under the above conditions. Following incubation, cells were fixed and stained using the method described by Mori *et al.*[7]

Determination of Quantities of Cellular TG Cells were cultured in 10 cm plastic dishes with various compounds as indicated and collected by centrifugation following treatment with trypsin (0.2% trypsin, 0.02% EDTA and 0.2% glucose in PBS) and washed three times with PBS. Collected cells were disrupted and homogenized by sonication in a buffer solution containing 1 mM EDTA, 10 mM tris (pH 8.0), 20 μg/ml phenylmethylsulfonylfluoride and 10 μg/ml each of antipine, pepstatin, leupeptin and chymostatin. The amounts of TG in the cell lysates were measured using a Nescote TG kit GN (AZWELL, Japan).

Fractionation of HuH7 Cells Cells were cultured and collected as mentioned above. The collected cells were disrupted by homogenization using a Dounce type glass-Teflon homogenizer in buffer A (3 mM EDTA in 10 mM tricine (pH 7.4), containing 20 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml antipine, pepstatin, leupeptin and chymostatin) containing 250 mM sucrose. The lysate was centrifuged for 10 min at 1000×$\boldsymbol{g}$ at 4 °C and the supernatant was recovered as post nuclear supernatant (PNS). The sucrose concentration of the PNS was adjusted to 26% by adding buffer A containing 70% sucrose (26% sucrose-PNS). In a 16 ml-ultracentrifuge tube (16PA, Hitachi, Japan), 1.1 ml of buffer A containing 51% sucrose and 2.4 ml of buffer A containing 43% and 35% sucrose were layered. Subsequently, 2.0 ml of 26% sucrose-PNS were layered on top of this. Following this, 2.4 ml of buffer A containing 18% and 10% sucrose were layered sequentially onto the PNS fraction. Finally, 3.0 ml of 1 in 5 diluted buffer A containing 2% sucrose was loaded on top. The step-wise gradient was centrifuged at 24000 rpm, at 4 °C for 3 h using a SRP28-SA1 rotor and 65P-7 ultracentrifuge (Hitachi, Japan). Following centrifugation, the samples were fractionated from the top (0.8 ml each).

Lipid Analysis by Thin Layer Chromatography Samples were concentrated using a rotary evaporator and lipids were extracted sequentially with chloroform/methanol (2/1 by volume), chloroform and diethyl ether. The extracted lipids were spotted and developed on a TLC plate (Silica gel 60, Merck) with hexane/diethyl ether/acetic acid (80/20/1 by volume). The TLC plate was soaked in 8% phosphoric acid containing 3% cupric acetate and the lipids were visualized by heating at 130 °C.[8]

Other Procedures Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (9.5% acrylamide gel) and immunoblot analysis was performed by the method described previously.[6] Protein amounts were measured using a BCA protein assay reagent (PIERCE, U.S.A.). Statistic analysis was performed using Williams multiple comparison test.

RESULTS

Effects of Fatty Acids and Glucose on LD Formation in HuH7 Cells HuH7 cells were cultured in the presence of OA, glucose and both glucose and insulin. Twenty hours of culturing the cells with 0.6 mM oleic acid markedly induced LD formation and was detectable with Oil red O staining (Fig. 1). Glucose appeared ineffective in inducing LD formation even in the presence of insulin (Fig. 1), and no obvious toxicity was detected for these compounds. Next, we tested FFAs of various carbohydrate chain lengths. FFAs longer than C10 induced LD formation, while octanoic acid (C8:0) and decanoic acid (C10:0) were ineffective (Fig. 2A). There was a tendency for longer FFAs to induce larger amounts of TG accumulation (Fig. 2B). These results indicate that long chain FFAs are highly potent inducers of LD formation and their potencies are much greater than those of ethanol, glucose and carbon tetrachloride using the same culture conditions. In addition, two variants of C18 FFA, stearic acid (C18:0) and linoleic acid (C18:2), induced LDs as effectively as OA (C18:1) indicating that both saturated and unsaturated FFAs with long carbohydrate chains are highly potent inducers. In cells cultured with stearic acid (C18:0) and palmitic acid (C16:0), the color of LDs turned from red to brown soon after the staining. Also, LDs gradually turned brown in cells treated with myristic acid (C14:0) and lauric acid (C12:0) within a few days. Conversely, most of the LDs kept their vivid red color for longer periods in cells loaded with unsaturated FFAs (C18:1, C18:2). Hence there may be differences of the quality of LDs among cells loaded with saturated and unsaturated FFAs. OA was used in the subsequent experiments, since OA is a major component of plasma and tissue lipids, and since OA gives rise to color-stable LDs. Generally, LD formation depends on dose of OA (Figs. 3A, B) and culturing period (not shown).

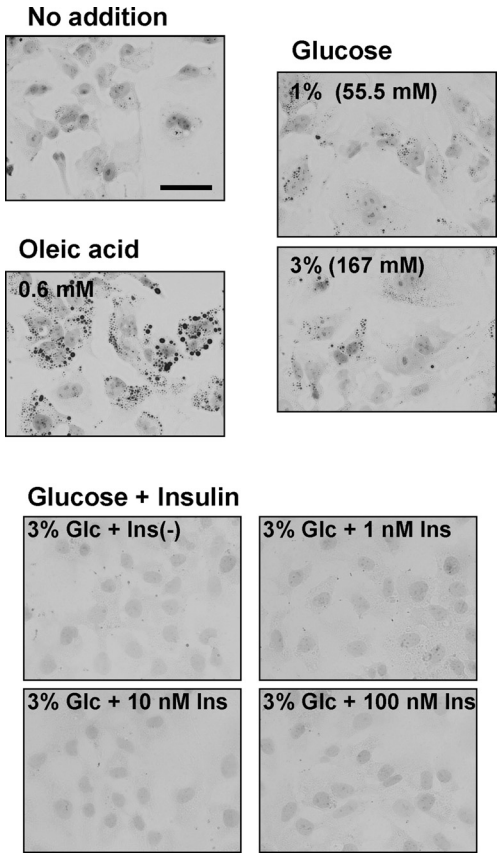

Fig. 1. Effects of Oleic Acid, Glucose and Insulin on LD Formation in HuH7 Cells

The human hepatocyte cell line HuH7 was cultured for 20 h in DMEM containing 10% FBS in the presence or absence of oleic acid (0.6 mM at final concentration), glucose (1, 3%) and both 3% glucose and insulin (at indicated concentrations). After culturing, cells were fixed with formalin and stained with Oil red O/Mayer's hematoxylin. The bar indicates 50 μm.

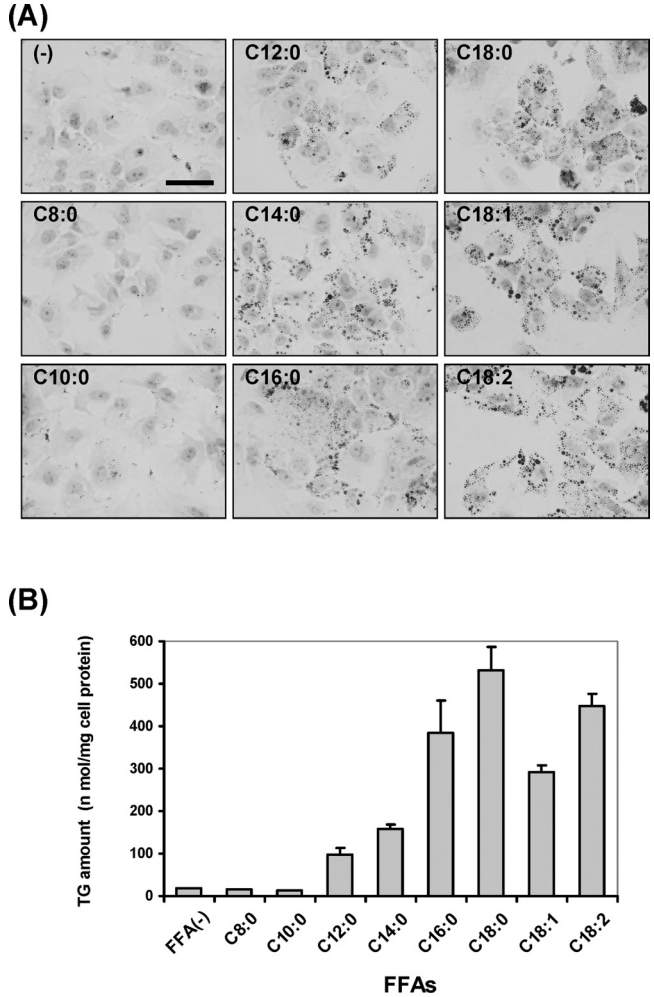

Fig. 2. LD Formation is Dependent on Chain Lengths of Fatty Acids

HuH7 cells were cultured for 20 h in DMEM in the presence of saturated fatty acids of various chain lengths or unsaturated ones with a chain length of C18 at the final concentration of 0.6 mM. The fatty acids tested were octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). (−) indicates control cells without fatty acid. (A) After culturing, cells were fixed and stained with Oil red O/Mayer's hematoxylin. The bar indicates 50 μm. (B) TG amount in each cell sample was shown as normalized with amount of cellular protein. The results are shown as mean±S.D. from two experiments.

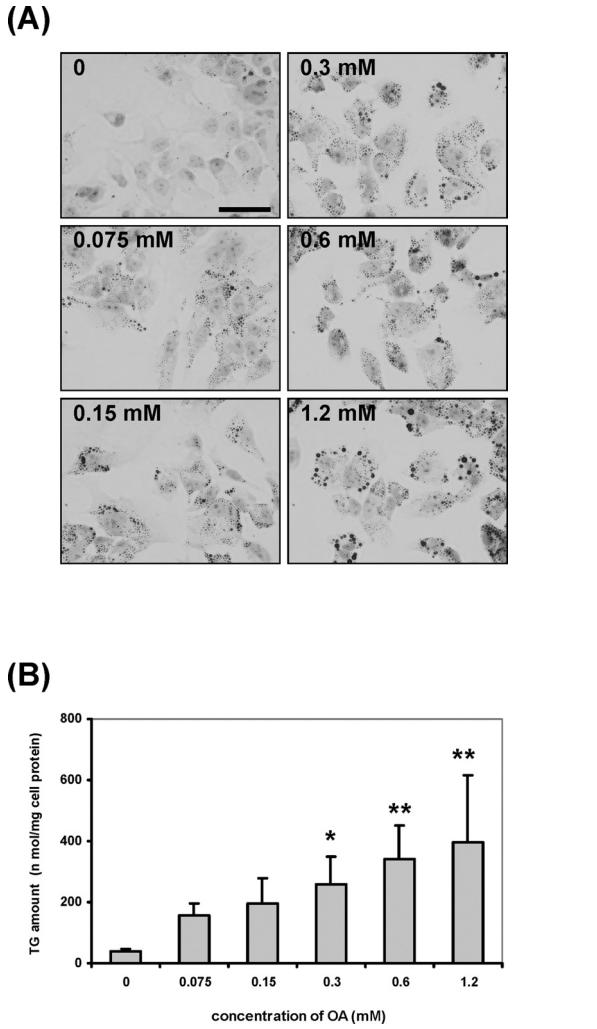

Fig. 3. LD Formation is Dependent on Concentrations of Oleic Acid

HuH7 cells were cultured for 20 h in DMEM containing various concentrations of oleic acid (0—1.2 mM). (A) After culturing, cells were fixed and stained with Oil red O/Mayer's hematoxylin. The bar indicates 50 μm. (B) TG amount in each cell sample was determined as normalized with amount of cellular protein. The results are shown as mean±S.D. from three experiments. * $p<0.05$; ** $p<0.01$.

Subcellular Fractionation of HuH7 Cells Next, we surveyed whether the newly synthesized neutral lipids were concentrated in LDs or dispersed throughout the cells. To do this, distributions of neutral lipids were analyzed by fractionating HuH7 cells using sucrose density gradient centrifugation. In cells cultured without OA, most of the cellular TG and CE were detected in fraction 1 together with adipose differentiation-related protein (ADRP),[9,10] a specific marker protein for LDs (Fig. 4). This indicates that cellular neutral lipids are located mainly in LDs in the basal state. On the other hand, most of the cellular proteins were distributed in the denser fractions corresponding to fractions 9—19 in Fig. 4 (data not shown). When cells were cultured with OA, amounts of cellular TG, CE and ADRP increased significantly, retaining their distribution in fraction 1 (Fig. 4). Therefore, neutral lipids newly synthesized by OA-loading mostly accumulate in LDs. To confirm that the fraction 1 does consist of LDs, we compared proteins in the tube top fraction prepared from OA loaded cells with those from control cells by means of SDS-PAGE. OA loaded cells contained larger amount of proteins in the top fraction than the control cells (Fig. 5). The major proteins in the top fractions in the OA-treated cells were comparable to those in the cells without OA (Fig. 5, lanes 4, 2). These proteins corresponded to LD proteins we previously identified by proteome (Fig. 5, ref. 6). We also confirmed the cellular distribution of 17β-hydroxysteroid dehydrogenase 11 (17βHSD11),[11,12] one of the proteins recently identified in the LDs.[6] Immunoblot analysis revealed that its amount increased in OA-loaded cells while keeping the tube top specific location (Fig. 4). These results of protein analysis indicate that the top fractions correspond to LDs and that neutral lipids produced from OA are mostly deposited in LDs.

Inhibition of OA-Induced LD Formation In order to identify factors involved in the OA-induced LD formation, we investigated the conditions necessary to inhibit LD formation. First, restriction of medium glucose was tested since TG synthesis requires not only FFAs but also glycerol 3-phosphate (G 3-P), and one of the major routes of cellular G 3-P supply is the glycolytic pathway.[13] However, glucose

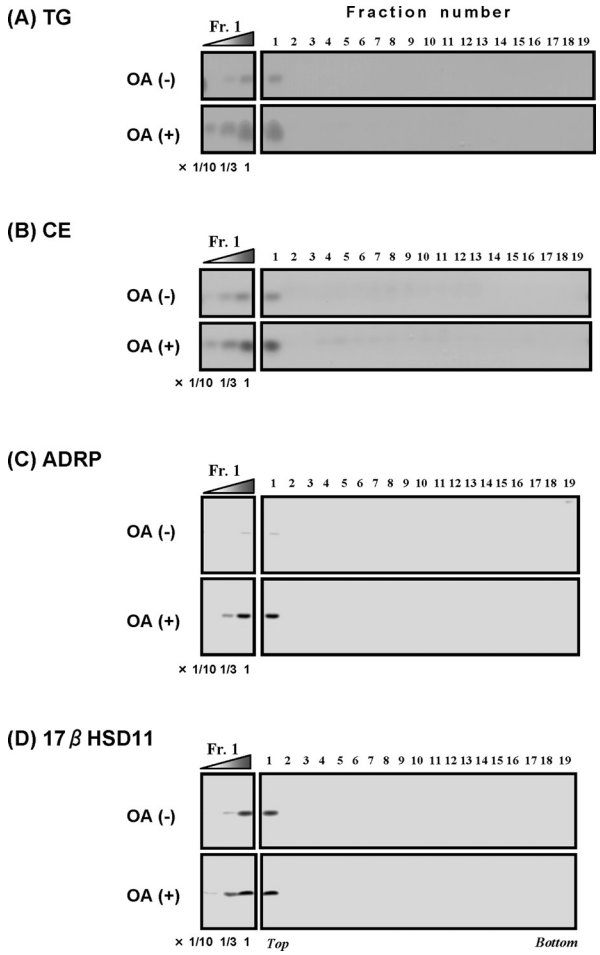

Fig. 4. Subcellular Distributions of Neutral Lipids and LD Proteins

HuH7 cells were cultured in DMEM for 20 h in the presence or absence of 0.6 mM oleic acid and then harvested. Post-nuclear supernatants prepared from the cells underwent sucrose density gradient centrifugation and were fractionated into 19 fractions (0.8 ml each) (see procedure). Triacylglycerol (A) and cholesteryl ester (B) in each fraction were detected using TLC. ADRP (C) and 17βHSD11 (D) were detected in immunoblots. The small panels in the left side indicate dose dependency of the signals found in the fraction 1 at dilutions of ×1, ×1/3 and ×1/10.

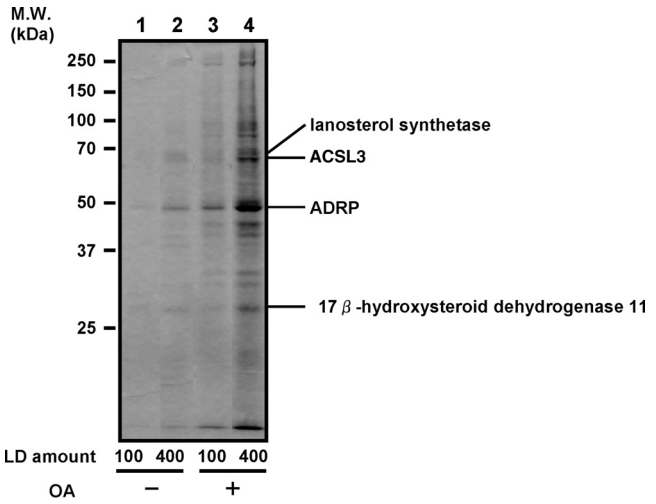

Fig. 5. Analysis of LD Proteins in Cells Cultured with or without Oleic Acid

HuH7 cells were cultured in the presence or absence of 0.6 mM oleic acid for 20 h and fractionated as shown in Fig. 4. The tube top LD fraction isolated from each cell sample was applied to SDS-PAGE and proteins in each LD sample were compared. Lanes 1 and 2; LDs isolated from cells cultured without OA. Lanes 3 and 4; LDs from OA loaded cells. For the quantitative comparison, LDs from equal amount of the cell extracts (post nuclear supernatant (PNS)) were applied to the electrophoresis. Lanes 1 and 3 correspond to LDs isolated from 100 μg PNS samples (as protein amounts). Lanes 2 and 4 correspond to LDs from 400 μg PNS samples. Proteins were stained with Coomassie blue. Molecular weight standards are indicated on the left. Identification of each protein is shown in the right.[6]

deprivation did not affect the LD formation induced by OA (Fig. 6).

Next, we screened for compounds which might inhibit OA-dependent LD formation. Previously, we performed proteomic analysis of LDs isolated from HuH7 cells and found that lanosterol synthetase and ACSL3,[14] a long chain acyl-CoA synthetase, are the major protein components of LDs (Fig. 5).[6] Thus, an inhibitor of lanosterol synthetase, AMO 1618 (4,4,10 beta-trimethyl-*trans*-decal-3 beta-ol),[15] and an acyl-CoA synthetase inhibitor, triacsin C,[16,17] were tested. The former compound failed to inhibit the formation of LDs, but the latter efficiently inhibited LD formation (Fig. 7), suggesting participation of acyl-CoA synthetase in LD formation. Interestingly, analysis of LD proteins demonstrated that protein band corresponding to ACSL3 significantly increased in OA loaded cells (Fig. 5). In addition, we tested pregnenolone and lithium chloride, which inhibit LD formation in macrophages and adipocytes, respectively,[18,19] but found both to be ineffective (Fig. 7). In addition, we tested isoproterenol (an agonist for the adrenaline beta receptor) and 1-butanol (an inhibitor of phospholipase D[20]), as well as two compounds of food origin, caffeine (an adenosine receptor

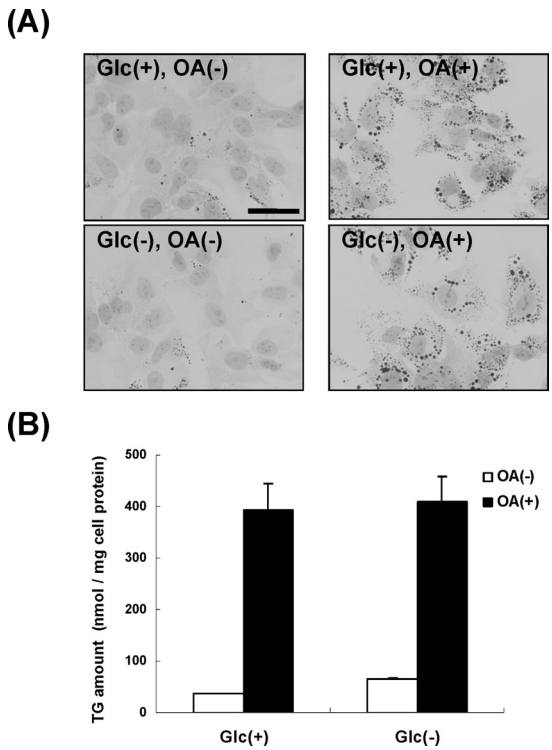

Fig. 6. Oleic Acid-Induced LD Formation in the Presence or Absence of Glucose

HuH7 cells were cultured for 20 h in DMEM containing or lacking 0.6 mM oleic acid and/or 0.1% glucose. (A) After the culture, cells were fixed and stained with Oil red O/Mayer's hematoxylin. The bar indicates 50 μm. (B) TG amount in each cell sample was shown normalized with amount of cellular protein. The graphs represent mean±S.D. from two experiments. Glc(+), normal DMEM containing 0.1% glucose. Glc(−), DMEM lacking glucose.

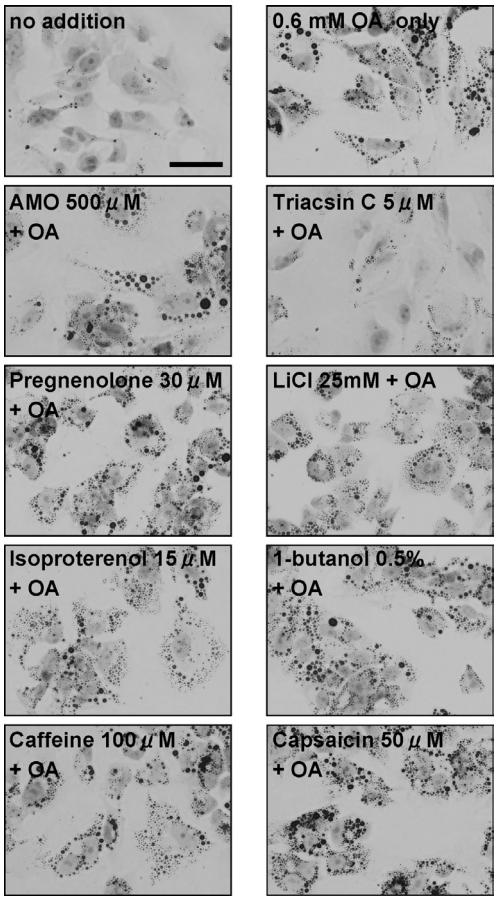

Fig. 7. Effects of Various Compounds on OA-Dependent LD Formation

HuH7 cells were cultured for 20 h in DMEM containing 0.6 mM oleic acid (except for 'No addition') and various compounds at indicated concentrations. After culturing, cells were fixed and stained with Oil red O/Mayer's hematoxylin. The bar indicates 50 μm.

antagonist and a phosphodiesterase inhibitor) and capsaicin (an agonist of vanilloid receptor). Phosphatidic acid, a product of phospholipase D, has been reported to promote LD formation.[21] However, none of these compounds were found to inhibit LD formation (Fig. 7).

DISCUSSION

In the current report, we have demonstrated that long-chain fatty acids with chain lengths of C12—C18 are highly potent inducers of lipid droplet (LD) formation in a cultured hepatocyte cell line of human origin. A concentration of oleic acid (OA, C18:1) of 75 μM and a period of culture of 20 h was sufficient to cause LD formation clearly detectable via light microscopy. Using these experimental conditions, glucose was not found to be essential for LD formation even though it is a source of glycerol 3-phosphate, another substrate for TG synthesis. Cell fractionation revealed that most of the newly synthesized neutral lipids accumulated in LDs. These results strongly suggest that hepatocytes vigorously take up long chain FFAs and store them after they are esterified to neutral lipid molecules. Hence, increased levels of extracellular long-chain FFAs are associated with hepatic steatosis. Elevation of blood FFAs most probably occurs in both nutrient-poor and -rich conditions. In the presence of excess lipid nutrients, TG-rich lipoproteins are taken up into the hepatocytes, hydrolyzed and re-esterified intracellularly. This is the primary route of hepatic TG generation. Lipoprotein lipase, an extra-cellular enzyme, can produce FFAs by hydrolyzing the neutral lipids in the lipoproteins and these FFAs secondary induce hepatic LD formation. In starvation states, FFAs are supplied from adipocytes by hydrolyzing stored neutral lipids and then transported to the liver, where they induce LD formation.[1] Indeed, hepatic LD formation has been seen in mice fasted for 2 d.[22] Decrease of insulin sensitivity in insulin resistance or diabetes mellitus causes increase of serum FFA levels since insulin inhibits release of FFAs from adipose tissues. This elevation appears to induce steatosis.[4] In diabetes, defects in glucose utilization leads to a shift from glucose-based to lipid-based energy metabolism. Peripheral TGs stored in the adipose tissues are hydrolyzed and FFAs are released into the circulation. In diabetes, decreased insulin sensitivity also leads to increased blood glucose levels. However, as shown in Fig. 1, glucose did not induce LD formation even at very high concentrations and even in the presence of insulin. In contrast to this, physiological concentration of glucose is sufficient for LD formation in differentiating 3T3-L1 cultured adipocytes and higher concentration of glucose induces significantly larger amount of LDs.[23] This glucose efficacy, as well as lithium sensitivity mentioned below, is a difference between glucose-based LD formation in the adipocytes and FFA-based one in the hepatocytes.

Of the compounds tested in this study, only triacsin C inhibited the formation of LDs. Triacsin C is a specific inhibitor of fatty acyl-CoA synthetase,[17] a family of enzymes which catalyze the production of fatty acyl-CoAs from FFAs and coenzyme A. Fatty acyl-CoAs are activated forms of fatty acids and can be utilized as substrates for the synthesis of various lipid molecules including TG and CE. Therefore, it can be assumed that FFAs are taken up into cells, modified to fatty acyl-CoAs, and then incorporated into neutral lipids. Mammalian fatty acyl-CoA synthetases are mainly classified into four groups based on their sequence and substrate specificities.[24] These groups include acetyl-CoA synthetase (C2), medium-(C4—C14), long-(C10—C20) and very long-chain acyl-CoA synthetase ($\geq$C22). Interestingly, a recent study revealed that triacsin C preferentially inhibits long-chain acyl-CoA synthetase (ACSL) but only weakly inhibits medium-chain acyl-CoA synthetase and is an ineffective inhibitor of short-chain acyl-CoA synthetase.[25] These findings, together with our current experimental results using triacsin C, suggest that ACSL and its substrates, namely long-chain fatty acids, are the important factors in LD formation. Previously, we identified ACSL3 as a major protein component of LDs in HuH7 hepatocytes.[6] Recent proteomic studies have revealed that ACSL3 is commonly found in LDs isolated from various kinds of cells including CHO fibroblasts, A431 epithelial cells and 3T3-L1 adipocytes.[26—28] Since ACSL3 is a long-chain acyl-CoA synthetase,[14] this enzyme may be responsible for a part of the neutral lipid synthesis in the course of LD formation. It is noteworthy that the quantities of ACSL3 found in LDs increased as the LDs developed (Fig. 5). However, it has not been clearly elucidated yet what is the factor determining the preference of FFAs in LD formation. Possible candidates of the factor are (i) substrate specificities of FFA transporters in plasma membrane deter-

mining rates of uptake of FFAs, (ii) binding specificities of fatty acid bindin proteins and acyl-CoA binding proteins forming cytoplasmic pools of FFAs and acyl-CoAs, (iii) substrate specificities (acyl-CoA preference) of DGAT enzymes, as well as (v) substrate specificities of ACSLs. Also, FFA utilizations in other cellular compartments such as mitochondrial beta oxidation may indirectly contribute to the FFA utilization in LDs.

In HuH7 cells, pregnenolone and LiCl were ineffective inhibitors of LD formation induced by OA. In mouse peritoneal macrophages, pregnenolone at a concentration of 10 μM efficiently inhibited LD formation induced by liposomes containing phospholipids and cholesterol.[17] Pregnenolone is thought to inhibit intracellular transport of cholesterol,[29] inhibiting the cellular accumulation of CE but not TG.[17] Since LDs of macrophages are often rich in CE for example, as seen in foam cells in lesions of atherosclerosis,[30] inhibition of cellular CE accumulation leads to suppression of LD formation. Conversely, in this study, the rate of TG increase was greater than that of CE when cells were cultured with OA (Fig. 4) forming TG-rich LDs. Therefore, inhibition of cholesterol transport by the pregnenolone was insufficient to block LD formation. LiCl at the concentration used in this study can significantly inhibit LD formation in the murine adipocyte cell line 3T3-L1.[18,19] In adipocytes, vigorous LD formation occurs during cell differentiation from precursor state (pre-adipocytes) to the mature state (differentiated adipocytes). This differentiation requires phosphorylation of CCAAT enhancer binding proteins by glycogen synthase kinase 3 (GSK3).[31,32] Lithium is thought to inhibit LD formation by blocking GSK3 and mimicking inhibition of GSK3 by Wnt signaling.[19] The inability of lithium to suppress LD formation in the hepatocyte suggests that LD formation by long-chain FFAs is not mediated by GSK3 signaling. Isoproterenol, an agonist at the adrenaline β receptor, increases cellular energy metabolism through activation of adenylate cyclase. Caffeine, an inhibitor of phosphodiesterase and an antagonist of adenosine receptor, can stimulate lipolysis and release of cellular energy. Treatment of hyperlipidemic rats with capsaicin reduces levels of serum- and hepatic lipids, accompanied by high- and low activity of hepatic TG lipase and fatty acid synthetase, respectively.[33] In our study, these compounds did not block LD formation. A possible interpretation of this inconsistency is that the potency of OA to induce LDs is so high that the inhibitory effects of the compounds are overwhelmed. Or, they could simply be ineffective using the LD formation system tested in this study. In addition, it is likely that signaling system involved in cellular TG metabolism are different among the cell types. For example, LDs in adipocytes are surrounded with a protein, perilipin A, which is crucially involved in regulation of lipid metabolism in a manner dependent on adrenergic stimulation. In hepatocytes, however, LDs are wrapped with ADRP but not with perilipin A.

In conclusion, in the current study we have demonstrated that long chain FFAs are the key factors required for LD formation in the hepatocytes. While glucose is significant for LD formation in adipocytes and is closely related to metabolic disorders such as insulin resistance and diabetes mellitus, it appears that long chain FFAs contribute much more to the cellular accumulation of neutral lipids *via* ACSL-dependent manner.

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