Lecithin promotes adipocyte differentiation and hepatic lipid accumulation

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Abstract. Lecithin is an essential biological component and widely used as a nutritional supplement for protecting cells from oxidation, increase fat burning and preventing cardiovascular disease. Lecithin contains fatty acids identified as the peroxisome proliferator-activated receptor (PPAR) agonists. However, the role of lecithin in adipogenesis and lipogenesis remains elusive. 3T3-L1 cells and mouse primary preadipocytes were used to characterize the properties of lecithin related to adipogenesis and lipogenesis. We found that lecithin promoted adipocyte differentiation and differentiation-specific gene expression, and increased triglycerides and free fatty acid levels in the adipocytes. These effects are independent of the clonal expansion of 3T3-L1 cells and the upstream PPARγ regulator, CCAAT-enhancer-binding protein ß. Furthermore, lecithin induced lipid accumulation in human hepatoma HepG2 cells. Our data suggest that lecithin is involved in adipogenesis, lipogenesis and hepatic lipid accumulation and it is implicated in obesity and hepatic steatosis.

Introduction

Lecithin, also known as phosphatidylcholine, is a naturally-occurring mixture of diglycerides of stearic, palmitic, lenoleic, and oleic acids, which links to the choline ester of phosphoric acid (1). Lecithin is used as a nutritional supplement and thought to protect cells from oxidation, prevent cardiovascular disease and steatosis (2-6). It was also used in phospholipid-structured system for topical drug delivery (7). A study showed that the components of lecithin, such as oleic, palmitic and linoleic acid, activates peroxisome proliferator-activated receptor γ (PPARγ) (8). PPARγ is a member of the nuclear receptor superfamily of ligand-dependent transcription factors and plays key role in the regulation of adipocyte differentiation.

Past studies suggest that PPARγ is the master regulator of the formation of adipocytes during the development of obesity (9). Activation of PPARγ causes adipocyte differentiation and obesity in animals, while inhibition of PPARγ results in the loss of adipocyte in mice (10-14). Several PPARγ agonists have been identified, including thiazolidinedione (TZD) and 15 deoxy-$\Delta^{12,14}$ prostaglandin J2 (10,11), which stimulates 3T3-L1 adipocyte differentiation and causes weight gain in patients. However, the role of lecithin in adipogenesis and lipogenesis remains elusive. Herein we provide evidence that lecithin not only stimulates adipocyte differentiation in both 3T3-L1 adipocytes and primary preadipocytes but also increases lipid accumulation in human HepG2 hepatocytes.

Materials and methods

Cell culture. 3T3-L1 and HepG2 cells were purchased from ATCC and maintained in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT). For adipocyte differentiation, 3T3-L1 cells were grown to full confluence for 2 days and cultured for additional days at 37˚C, and 8% CO_2 after the addition of differentiation medium (DM, 10 μg/ml insulin, 0.5 μM dexamethasone, and 0.8 mM isobutylmethyl xanthine). Lecithin, obtained from MSE Pharm (Bad Homburg, Germany), was dissolved in 10% alcohol, 10% glycerol and H_2O, and added to the medium at various concentrations.

The primary preadipocytes were isolated using a previously described method with minor modifications (15). Briefly, epididymal fat pads from 8-week-old male ICR mice were removed and washed with PBS twice under sterilized conditions and vessels were carefully removed. The fat pads were then finely minced and incubated with lysis buffer (PBS containing 0.1 M HEPES, 1 mM $CaCl_2$, 5 mM glucose and 0.2% type IV collagenase (Sigma, St. Louis, MO) in airbath of 37˚C, for 60 min while rotating at 100 rpm. After centrifugation at 1,000 rpm for 2 min, the floating adipose tissue and lysis buffer were removed by pipeting. The remaining cells were washed with DMEM containing 10 mM HEPES, 2% fetal calf serum, 1% bovine serum albumin, penicillin (20 units/ml), and streptomycin (20 mg/ml), and subsequently filtered through a nylon mesh. The cells passing through were collected and cultured in 10-cm dishes to 90% confluence, and then plated into 12-well plates for further processing.

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Oil red O staining. The cells were washed twice with PBS, and then fixed with 10% formalin at room temperature for 10 min before stained with oil red O (Sigma, St. Louis, MO) at 60°C for 10 min. The photographs were taken using an Olympus microscope (Tokyo, Japan).

Cell and supernatant chemistry analysis. Cells were homogenized in PBS-T on ice and extracted with chloroform. After centrifugation for 10 min at 13200 rpm, the upper layer was removed and the chloroform layer was dried and then dissolved in isopropanol. Triglycerides (TG) and free fatty acids (FFA) were determined using a Hitachi 7020 Automatic Analyzer, and an Ultrospec 2100 Pro UV/Visible spectrophotometer (Amersham, England), respectively.

Cell proliferation analysis. The proliferation of 3T3-L1 cells was evaluated by [³H]-TdR incorporation assay. 3T3-L1 cells (1x10⁴) were plated in 96-well flat-bottomed microplates. Lecithin and 0.5 μCi/well [³H]-TdR (Amersham, Little Chalfont Buckinghamshire, England) were added to the wells after cells were attached. After 12 h of incubation time, the cells were washed with cold PBS 3 times and then detached from the plates using trypsin-EDTA (Invitrogen, Carlsbad, CA). The incorporated thymidine in cells was measured using a MicroBeta Trilux liquid scintillation counter (Wallac, Gaithersburg, MD).

Quantitative real-time PCR. Real-time PCR methods and primers were used as described (16). Total RNA was extracted with a spin column (Qiagen, Hilden, Germany) and treated with DNase I to avoid genomic DNA contamination. The gene expression levels were evaluated by quantitative real-time RT-PCR using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). After an initial incubation for 2 min at 50°C, the cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C, 15 sec; 60°C, 60 sec). The mRNA levels of all genes were normalized using ß-actin as an internal control.

2-Deoxy-[³H]-D-glucose uptake assay. Glucose uptake was performed using the 2-deoxy-[³H]-D-glucose method (17). Lecithin-treated 3T3-L1 cells were incubated with media containing media overnight and replaced with serum-free medium the next morning at 37°C for 3 h. The cells were then washed 3 times in warm KRHB, and exposed to insulin (10 nM) or medium (control) for 30 min in KRHB. 2-Deoxy-[³H]-D-glucose (0.5 μCi) (Amersham) was then added to the cells and incubated for 10 min at 37°C. The reaction was terminated by washing twice in ice-cold KRHB. The cells were digested in 0.2 M NaOH for 1 h at 60°C, and then transferred to scintillation vials and counted using a MicroBeta Trilux liquid scintillation counter.

Western blot analysis. 3T3-L1 cells treated with lecithin for 24 h were homogenized in 2X SDS sample buffer and boiled for 5 min. Protein (10 μg) was separated by 10% SDS-PAGE and transferred to PVDF membrane, blocked for 1 h at room temperature in 5% non-fat dry milk/PBS-T. The membrane was incubated with antibodies to PPARγ (Upstate, Charlottesville, VA) and ß-actin (Sigma, St. Louis, MO) in 2% BSA containing PBS-T for 1 h at room temperature, followed by washing with PBS-T 3 times, 10 min for each, and incubated with HRP conjugated secondary antibodies (Kangchen, Shanghai, China) for 30 min at room temperature. The signals were detected by ECL (Amersham) according to the manufacturer's specifications.

Statistical analysis. Data analyses were performed using SPSS12.0 for Windows. All data were presented as means ±SE. Statistical analysis was done by one-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

Results

Lecithin promotes adipocyte differentiation and elevates TG and FFA levels. To clarify whether lecithin promotes adipocyte differentiation, we adopted the 3T3-L1 cell culture system, a widely used *in vitro* adipocyte differentiation model, with or without lecithin stimulation. Under 4 days of DM induction, confluent 3T3-L1 cells proceeded 2 cycles of proliferation, and then started adipocyte differentiation. Most of the cells completed their differentiation cycles at day 5 (Fig. 1B). However, when lecithin was added to the DM induction medium, we found that adipocyte differentiation of 3T3-L1 was completed on day 1 (Fig. 1C), while the cells induced with DM but without lecithin did not show any signs of differentiation at this time point (Fig. 1A).

To investigate whether lecithin alone without DM induction medium induces 3T3-L1 adipocyte differentiation, lecithin was added to the cells at various concentrations. The results showed that lecithin alone also induces 3T3-L1 differentiation in 24 h (Fig. 1D and E). To further verify this adipocyte differentiation promoting effect on primary adipocytes, we separated the preadipocytes from mouse epididymal fat pads and treated them with lecithin. The results demonstrated that the primary adipocyte differentiation and lipid accumulation were induced with lecithin alone within 24 h in a dose-dependent manner (Fig. 1G, H, I and J), while there were no obvious signs of adipocyte differentiation with DM induction at the same time point (Fig. 1F).

To determine whether lecithin has any influence on other characters of adipocytes in addition to differentiation, TG and FFA levels were measured in lecithin-treated 3T3-L1 cells. We found that the TG level increased significantly after 24-h induction in the cells treated with 3 and 6‰ of lecithin (Fig. 1K), and the FFA contents in cells and supernatant were moderately elevated (Fig. 1L and M).

Lecithin increases the mRNA expression and protein levels of adipocyte differentiation specific markers. The lecithin components were reported to activate the key transcription factor of adipocyte differentiation, PPARγ (8). To examine whether lecithin regulates PPARγ expression, total RNA purified from 3T3-L1 cells treated with 6‰ lecithin were transcribed into first strand cDNA followed by real-time PCR analysis. The results demonstrated that 24 h treatment of lecithin of the cells increased PPARγ expression at both mRNA (Fig. 2A) and protein levels (Fig. 2B and C), indicating that lecithin acts on PPARγ directly. The expression levels of
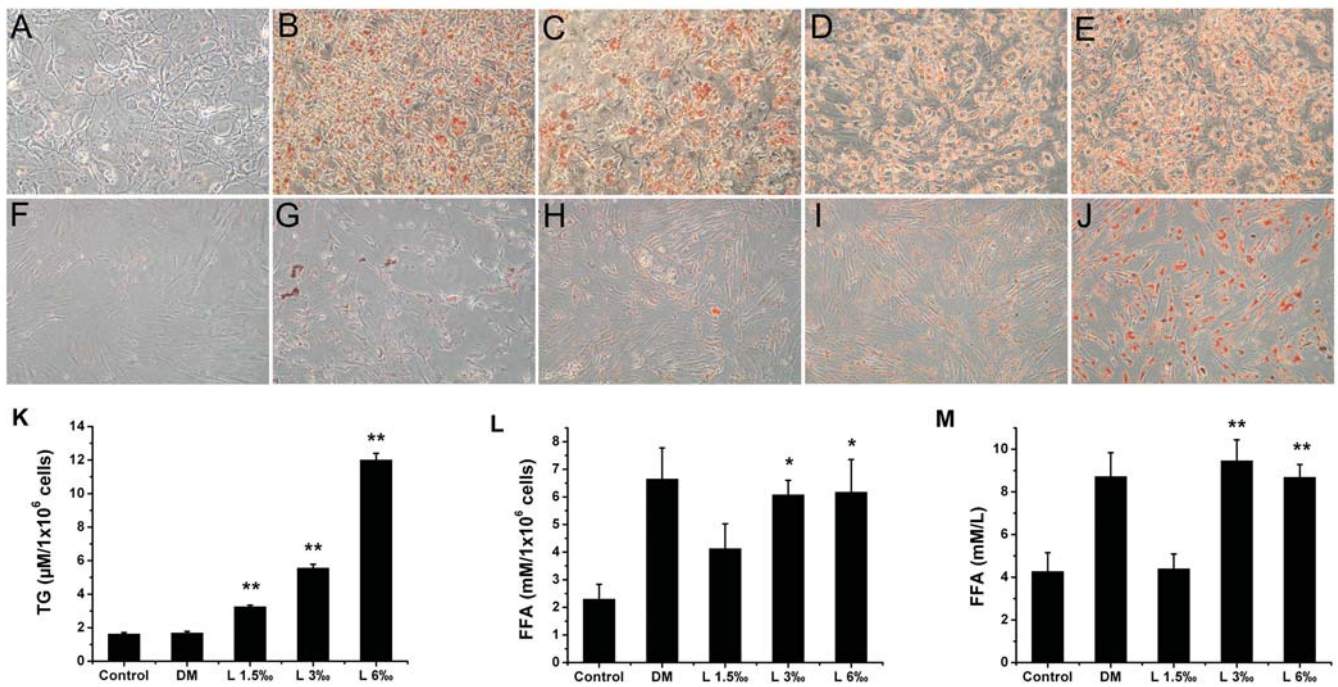
Figure 1. Lecithin promotes adipocyte differentiation. (A-J) Oil red O staining of 3T3-L1 adipocyte cell line. (A) Differentiation induction with DM alone at day 1; (B) Differentiation induction with DM alone at day 5; (C) Differentiation induction with DM plus 3‰ of lecithin at day1; (D and E) Differentiation induction with lecithin alone at 3 and 6‰, respectively without DM at day 1. (F-J) Oil red O staining of mouse primary preadipocytes on day 1. (F) Differentiation induction with DM alone; (G-J) Differentiation induction with 0, 1.25, 2.5, and 5‰ of lecithin, respectively. (K) TG level in 3T3-L1 cells; (L and M) FFA levels in 3T3-L1 cells and supernatant.
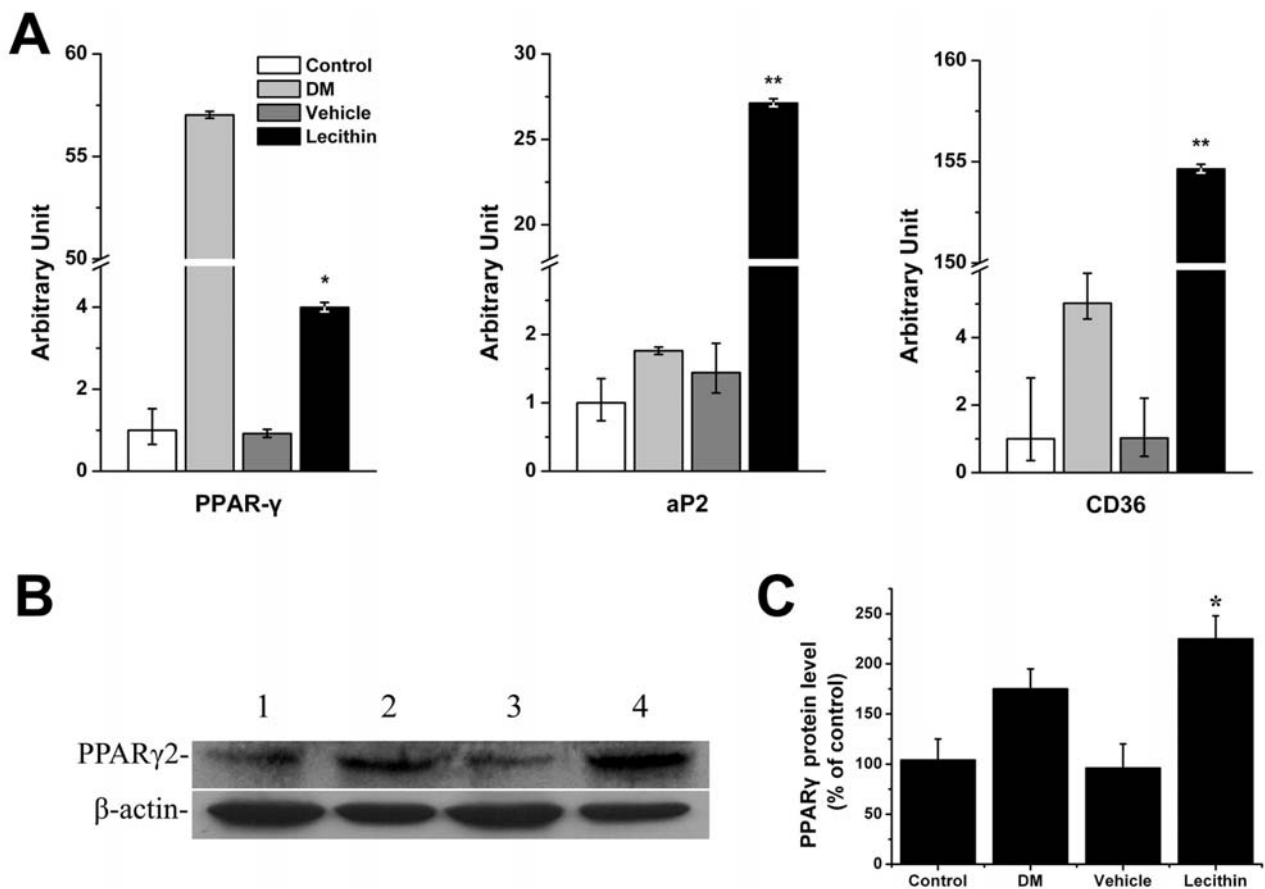

Figure 2. Lecithin increases the expression of PPARγ and its target genes. (A) Real-time PCR analysis of lecithin-treated 3T3-L1 cells at day 1, ß-actin was used as an internal control. The results represent at least three independent experiments. Data are presented as means ±SE. *P<0.05; **P<0.01. (B) PPARγ2 protein level analyzed by Western blotting in lecithin-treated 3T3-L1 cells. 1, Cells without induction as control; 2, cells induced with DM alone for 24 h; 3, cells treated with vehicle but without lecithin; 4, cells treated with 6‰ of lecithin for 24 h. (C) Quantification of PPARγ2 intensity in Western blotting.

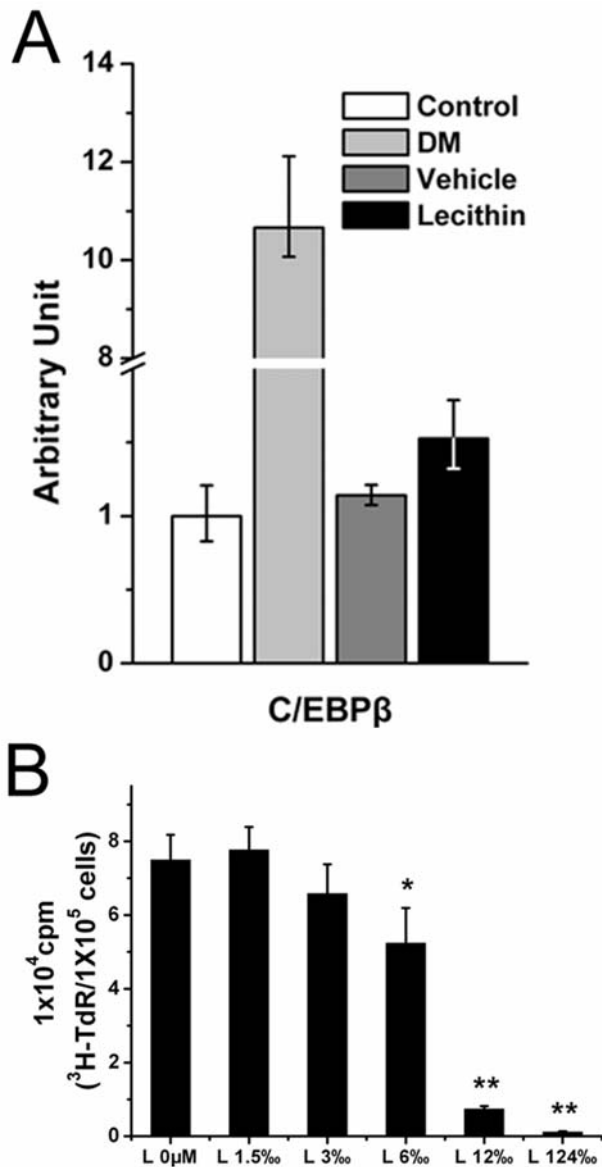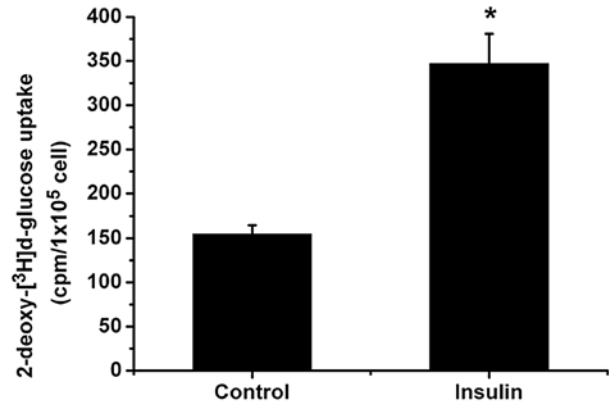Figure 4. Lecithin induced adipocytes possess glucose uptake ability. Cells treated with lecithin for 24 h were exposed to insulin (10 nM) or medium (control) for 30 min, 2-deoxy-[³H]-D-glucose was then added into cells and incubated for 10 min. The remaining radioactivity in cells was measured as the indicator of glucose uptake. The data are presented as mean ±SE. n=8 for each group. *P<0.001 compared to vehicle control.

Figure 3. Lecithin showed no effect on mRNA expression level of C/EBPß and preadipocyte proliferation. (A) Real-time PCR analysis of lecithin-treated 3T3-L1 cells at day 1, ß-actin was used as an internal control. (B) Adipocyte proliferation assay. Cells (1x10⁴) were plated into 96-well plates and lecithin was added at indicated concentrations. [³H]-TdR (0.5 µci) was added 12 h before the cells were harvested and counted with a MicroBeta Trilux liquid scintillation counter. Data are presented as means ±SE. *P<0.05; **P<0.01, n = 6 for each group.

cellular toxicity (Fig. 3B). These data indicate that the promoting effect of lecithin on adipocyte differentiation is independent of the early adipocyte differentiation signal, C/EBPß, and subsequent clonal expansion.

Lecithin enhances insulin sensitivity of 3T3-L1 adipocytes. As matured adipocytes are usually insulin sensitive, we next evaluated the ability of glucose uptake by lecithin-induced adipocytes in the presence of insulin. Fig. 4 demonstrated that lecithin-treated cells exhibited a significant increase in insulin-induced glucose uptake (P<0.001), suggesting that lecithin-induced adipocytes are insulin sensitive, similar to 3T3-L1 adipocytes induced by DM.

Lecithin promotes lipid accumulation in hepatic cells. It was reported that excess dietary intake of lecithin exacerbates alcohol-induced onset of hepatic steatosis (18). Therefore, we examined the TG and FFA levels in the human hepatoma cell line, HepG2, after 24 h of lecithin treatment. As shown in Fig. 5B and C, the cells treated with lecithin contained more lipid droplets compared to the untreated ones (Fig. 5A). TG concentrations in lecithin-treated cells were also markedly elevated (Fig. 5D), and FFA levels in the cells and supernatant were also increased by lecithin induction (Fig. 5E and F). These results suggest that lecithin induces rapid lipid accumulation in hepatocytes.

Discussion

We described, for the first time, that lecithin has unique properties of stimulating adipocyte differentiation in both 3T3-L1 and primary preadipocytes, and inducing hepatic lipid accumulation. The conclusion is supported by several lines of experimental evidence. Firstly, lecithin promoted adipocyte differentiation in a rapid and dose-dependent manner and escalated the glucose uptake ability of the cells. Secondly, lecithin stimulation induced lipid accumulation and increased levels of TG and FFA in hepatic cells. Thirdly, lecithin activated PPARγ and its target gene expression, such as aP2

PPARγ downstream genes, such as adipose fatty acid-binding protein (aP2) and CD36, were also increased up to 154- and 27.1-fold, respectively (Fig. 2A).

Since early events like CCAAT-enhancer-binding protein ß (C/EBPß) expression and the consequent clonal expansion, are the key steps for 3T3-L1 adipocyte differentiation induced by DM (18), we examined whether lecithin induces C/EBPß expression and cell proliferation during differentiation. Unlike DM induction, lecithin induction did not increase the mRNA expression level of C/EBPß (Fig. 3A), and had no effect on cell proliferation at the concentration of 3 and 6‰ (Fig. 3B). However, an inhibitory effect occurred with extreme high concentrations of lecithin treatment which may have caused
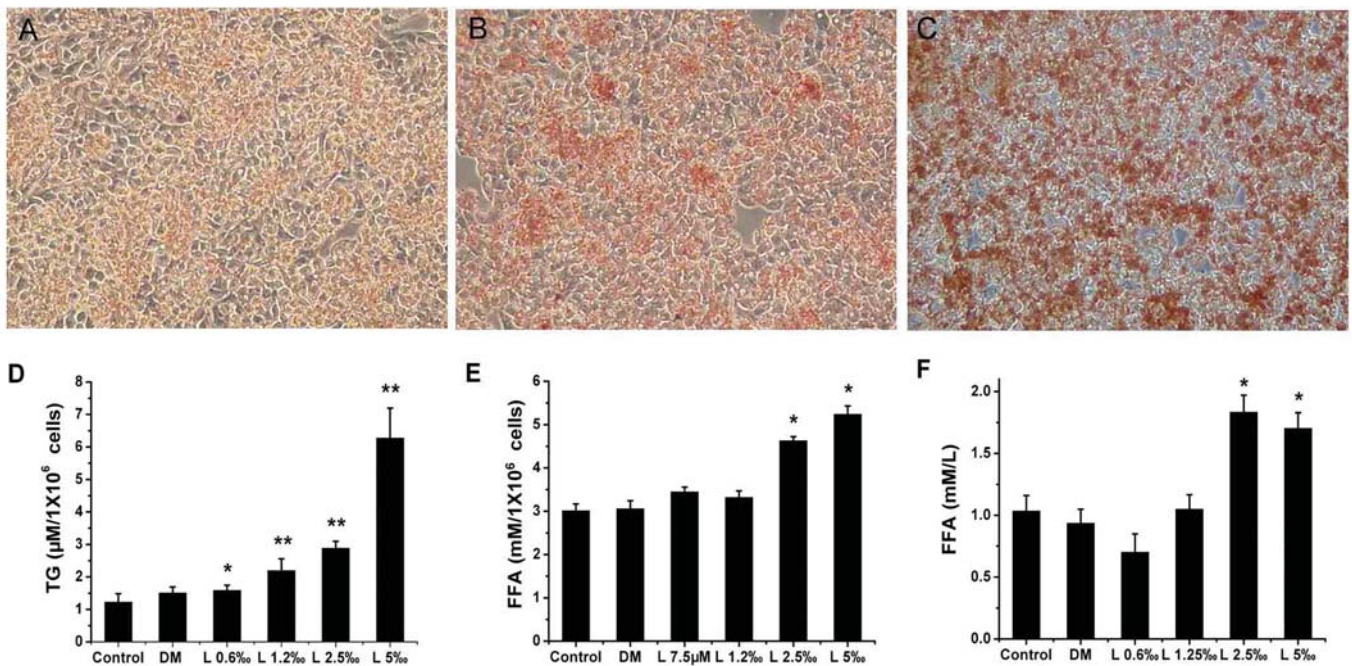
Figure 5. Lecithin induces lipid accumulation in hepatic cells. Human hepatic cells, HepG2, were treated with lecithin for 24 h. (A-C) Oil red O staining of cells. (A) Absence of lecithin as control; (B and C) cells treated with 2.5 and 5‰ of lecithin, respectively. (D) TG levels in HepG2 cells; (E) FFA level in HepG2 cells; (F) FFA level in supernatant. The data are presented as mean ±SE. n=6 for each group. *$P<0.05$ compared to vehicle control.

and CD36. The adipocyte differentiation induced by lecithin was completed within 24 h, whereas the classical differentiation induction by insulin requires at least 3 to 4 days and rosiglita-zone and 15 deoxy-$\Delta^{12,14}$ prostaglandin J2 promoting 3T3-L1 adipocyte differentiation require 7 days (10,11). Thus, the differentiation induced by lecithin is relatively faster compared to standard inducers. It is important to note that adipocyte differentiation induced by lecithin is independent of the early differentiation marker, CEBP/ß, which is usually seen with DM-induced adipocyte maturation. The exact underlying mechanism requires further clarification.

During the process of adipocyte differentiation by insulin induction, 3T3-L1 cells often undergo two cycles of mitotic clonal expansion which is essential for adipocyte differentiation (19). At the early stage, at least one PPARγ agonist is synthesized to induce 3T3-L1 adipocyte differentiation (20). In our study, we found that clonal expansion is not necessary for adipocyte differentiation when lecithin is used as the inducer, since the proliferation of 3T3-L1 preadipocytes was inhibited by lecithin, indicating the adipocyte differentiation induced by lecithin is independent of the clonal expansion.

Several reports have shown that a high intake of lipids increases membrane lipid contents, influences the activity of signaling pathways, regulates appetite, satiety, body weight and metabolism through binding to nuclear receptor PPARs (21). Since the components of lecithin, such as oleic and leoleic acids, are reported as PPARγ agonists (8), we postulate that lecithin is a PPARγ activator and induces adipocyte differentiation through activation of the PPARγ pathway.

Since over-expression of PPARγ (22,23) or activation of PPARγ by the anti-diabetic drug rosiglitazone (24) causes obesity as well as fatty liver in mice, we investigated whether lecithin causes lipid accumulation in HepG2 hepatocytes. Our results strongly suggest that lecithin causes a rapid increase of TG and FFA levels in hepatocytes. These findings are different from the report that lecithin has protective effects on fatty liver induced by high fat diets (4), but agree that lecithin may exacerbate alcohol-induced onset of hepatic steatosis. Taken together with the PPARγ activating effect, our data suggest that excess intake of lecithin induces adipocyte differentiation and lipid accumulation. However, the correlation between over-consumption of lecithin and obesity and hepatic steatosis awaits further investigation.

Acknowledgments

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