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Effects of a fatty acid synthase inhibitor on adipocyte differentiation of mouse 3T3-L1 cells

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KEY WORDS adipocytes; 3T3-L1 preadipocyte; cell differentiation; fatty acid synthetase complex; C75 cpd; peroxisome proliferators-activated receptor γ

ABSTRACT

AIM: To investigate the influence of C75, a fatty acid synthase inhibitor, on adipocyte differentiation. **METHODS:** Mouse 3T3-L1 preadipocytes were induced to differentiation by insulin, isobutylmethylxanthine, and dexamethasone. Oil red O staining was performed and activity of glycerol-3-phosphate dehydrogenase (GPDH) was measured. The level of peroxisome proliferators-activated receptor γ (PPAR γ) mRNA was assayed by semi-quantitative reverse transcription PCR. **RESULTS:** C75 blocked the adipogenic conversion in a dose-dependent manner and the inhibitory effects occurred both in the early phases (48 h) and in the latter phases (8 d) of the process. Treatment with C75 for 8 d induced more decrease in lipid content than 48 h ($P < 0.01$). Treatment with C75 50 mg/L for 48 h or 8 d decreased GPDH activity by 52.8 % and 31.2 % of Vehicle, respectively. Treatment with C75 10-50 mg/L for 48 h or 8 d down-regulated PPAR γ mRNA expression compared with control ($P < 0.01$). **CONCLUSION:** C75 blocked the adipocyte differentiation, which was related with down-regulation of PPAR γ mRNA.

INTRODUCTION

Preadipocyte cell lines are useful models for investigating the adipogenesis process. 3T3-L1 preadipocyte, which can be induced to differentiate into adipocyte in cell culture, is one of the most studied preadipocyte cell line^[1,2]. When mouse 3T3-L1 preadipocytes are treated with an inducing mixture that includes insulin (Ins), dexamethasone (Dex), and isobutylmethylxanthine (IBMX) in the presence of NBS^[3,4], they change from an extended fibroblast-like morphology to a round one with cytoplasmic lipid vesicles made of newly biosynthesized triglyceride. The sequence of events that lead to expression of adipocyte-specific genes

included peroxisome proliferators-activated receptor γ (PPAR γ), C/EBPs, and ADD1/SREBP1^[5-7]. Free fatty acid (FFA) can promote the preadipocyte differentiation, and PPAR γ was overexpressed in islets cultured with high FFA levels^[8].

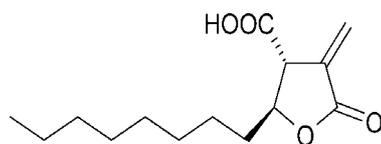
C75, an α -methylene- γ -butyrolactone, is a known inhibitor of fatty acid synthase (FAS)^[9]. Treatment of mice with C75 alters the expression of hypothalamic neuropeptide, leading to reversible inanition and weight loss^[10-12]. In addition to its central action, C75 treatment causes changes in peripheral tissues, including inhibition of hepatic fatty acid synthesis, reduction of fatty liver, and diminution of adipose tissue mass. In addition to the increase of fatty acid oxidation and stimulation of *O*-carnitine palmitoyltransferase-1 (CPT-1) activity^[13], we hypothesized that C75 might have other effects on adipogenesis process by reducing FFA levels of adipose.

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Chemical structure of C75

Adipose tissue mass is a reflection of the number of adipocytes and their amount of fat stored^[14]. The development of obesity is associated with coordinated cellular process including adipocyte precursors and new fat cell differentiation^[15,16]. The study aims to determine whether the FAS inhibitor, C75, affects the adipocyte differentiation of 3T3-L1 cells.

MATERIALS AND METHODS

Reagents Preadipocytes (3T3-L1) were obtained from Institute of Geriatrics, Beijing Hospital. C75 was synthesized in our laboratory and was resolved in Me₂SO. Me₂SO concentration in the final cell culture medium never exceeded 0.1 %. NADH, Oil Red O, dihydroxyacetone phosphate, Ins, IBMX, and DEX were purchased from Sigma. Reagent Trizol was a product of GIBCO. The PCR primers for human β -actin and PPAR γ were synthesized by SBS. AMV and Oligo (dT) were purchased from Promega.

Synthesis of C75 Trans-C75 was synthesized and separated as described^[9].

The physical parameters were as follows. mp: 75.5-77 °C; purity: 99 % (HPLC); FAB-MS [M+H]⁺: 255.1 m/e, ¹HNMR (600 Hz, CDCl₃) δ 0.88 (t, 3H, J=6.8 Hz, 1.2-1.8 (m, 14H), 3.63 (dt, 1H, J=2.8,5.6, 12.8 Hz), 4.81 (dt, 1H, J=6, 12.8 Hz), 6.01 (d, 1H, J=2.8 Hz), 6.47 (d, 1H, J=3.2 Hz).

Purification of FAS and FAS enzyme assay FAS was purified from rat liver without enzyme or protease inhibitors by using stepwise polyethylene glycol and ammonium sulfate precipitations, anion exchange chromatography and gel filtration as described^[17]. FAS was 90 % pure as estimated from SDS/PAGE with Coomassie blue staining. FAS activity (for the overall reaction) was determined with a S500P spectrophotometer at 37 °C^[18]. Briefly, 23.2 μ g FAS, 50 μ L of 1 mol/L K₂PO₄ (pH7.6) at 37 °C, 15 μ mol/L of acetyl-CoA, and 72.5 μ mol/L of NADPH were reacted in 0.5 mL reaction volume and the absorbance was monitored at 340 nm in a heated chamber spectrophotometer at

37 °C for 3 min to measure background NADPH. After the addition of malonyl-CoA 52 μ mol/L, the reaction was assayed for an additional 3 min to determine FAS-dependent oxidation of NADPH. One unit of fatty acid synthase was defined as that amount of enzyme which catalyzes the oxidation of NADPH 0.8 μ mol/L in 1 min at 37 °C. To detect slow-binding inhibition, FAS, C75, water, and K₂PO₄ buffer were incubated at 37 °C for 0 to 30 min before standard FAS assay. Control consisted of Me₂SO vehicle without drug.

3T3-L1 cell culture and differentiation 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) NBS. On d 0 (2 d after 3T3-L1 preadipocytes reached confluence), cells were induced to differentiation by Ins 1.7 μ mol/L, IBMX 0.5 mmol/L, and DEX 1 μ mol/L. On d 2 and every other day afterwards, fresh medium was substituted until d 8^[19]. In some cases, post-confluent 3T3-L1 cells were incubated with C75 0 to 50 mg/L for 48 h for adipogenic stimulation, and then in C75-free medium until d 8 (short-term treatment). In other cases, adipocytes were exposed to C75 0 to 50 mg/L for 8 d (long-term treatment). And in all experiments cells were exposed to an identical concentration of vehicle. Differentiation was assessed by microscopy and Oil red O staining^[20]. Cells were washed twice with PBS, fixed in 3.7 % formaldehyde for 1 h, and stained for 30 min with 0.2 % (w/v) oil red O solution in 60 % (v/v) isopropanol. They were then washed several times with water. Excess water was evaporated by placing the stained cultures at a temperature of about 32 °C. In order to determine the extend of adipose conversion, 0.2 mL of isopropanol was added to the stained culture dish. The extracted dye was immediately removed by gentle pipetting and its optical density was monitored spectrophotometrically at 490 nm.

Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity^[21] For 3T3-L1 adipocytes 5 \times 10⁵ cells per plask were incubated with C75 0 to 50 mg/L for 48 h or 8 d. On d 8 cells were washed with cold PBS (pH 7.4), scraped from dishes, and collected in Trishydrochloride buffer 25 mmol/L (pH 7.4) with edetic acid 1 mmol/L, and mercaptoethanol 1 mmol/L. After homogenization with a ultraschallprozessor for 10 s at 30 W, the suspension was centrifuged at 10 000 \times g at 4 °C for 1 h. The final supernatants were stored at -70 °C for several weeks without any change in enzyme activity. GPDH activity was determined spectrophotometrically. The assay mixture contained

triethanolamine HCl buffer 100 mmol/L (pH7.5), edetic acid 2.5 mmol/L, mercaptoethanol 0.1 mmol/L, NADH 0.12 mmol/L, and dihydroxyacetone phosphate 0.2 mmol/L was added to start the reaction. After mixture, samples were incubated at 25 °C for 10 min and optical density was measured at 340 nm. Results are expressed as kIU·g⁻¹ protein. One international unit of GPDH activity was equal to the oxidation of 1 μmol/L NADH per min.

Reverse transcriptase-PCR (RT-PCR) Total RNA was extracted from 3T3-L1 cells according to manufacture's introduction(GIBCO). The RNA content was quantified by UV spectrophotometer at 260 nm. Two microgram aliquot of total RNA was reverse-transcribed into first-strand complementary DNA(fs cDNA) by using oligo-dT primer. The fs cDNA was amplified by PCR. The PCR was carried out in a total volume of 100 μL containing Tris-HCl 20 mmol/L, KCl 50 mmol/L, MgCl₂ 1.5 mmol/L, dNTP 0.2 mmol/L, 0.6 mmol/L of each primer, and 2.5 units of *Taq* DNA polymerase. The expression of house keeping gene, β-actin mRNA, was considered as an internal standard. The following are primer sequences for PPARγ (sense 5'-GCT CTA GAC GTG ACA ATC TGT CTG AGG TCT GTC AT-3', antisense 5'-CGG GAT CCG TTG TCG GTT TCA GAA ATG CCT TGC AGT G -3' 870 bp); β-actin (sense 5'-GTG GGC CGC TCT AGG CAC CAA-3', antisense 5'-CTC TTT GAT GTC ACG ACG ATT TC-3' 540 bp). PCR were run 30 cycles for PPARγ and β-actin in Eppendorf Mastercycler. Denaturing, annealing, and extension reactions were performed at 94 °C for 1 min, at 50 °C for 1 min, and at 72 °C for 1 min. The PCR products were electrophoresed on 1 % agarose gels, stained with ethidium bromide, and revealed by UV irradiation. The images were taken by Kodak digital camera and analyzed with 1D image analysis software. The levels of PPARγ mRNA were expressed as the ratio of PPARγ to β-actin.

Statistical analysis Data were represented as mean±SD. Differences between individual groups were analyzed by *t*-test.

RESULTS

Inhibitory effects of C75 FAS activity Trans-C75 inhibited FAS activity in a dose-dependent manner and exhibited characteristics of a slow-binding inhibitor of FAS. Slowing-binding inhibitors have been defined as compounds in which equilibrium among

enzyme, inhibitor, and enzyme-inhibitor complex occur on a scale of seconds to minutes and include a number of commonly prescribed drugs^[22]. Under conditions of the standard assay for FAS activity, enzyme and inhibitor were pre-incubated at 37 °C for 3 min, before initiation of the enzyme reaction by the addition of malonyl-CoA^[23], C75 inhibited FAS activity by about 27 % in this standard assay. C75 100 mg/L inhibited FAS activity in a time-dependent manner at 37 °C, which was consistent with slowing-binding inhibition, up to 90 % after 30 min (Fig 1).

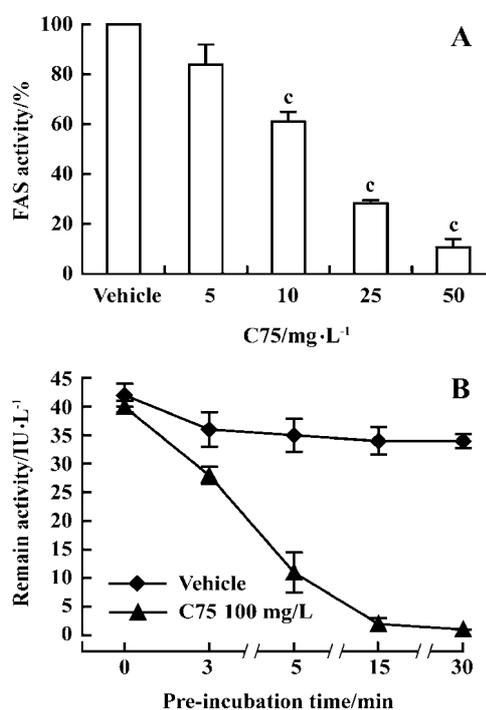


Fig 1. Inhibitory effects of C75 on FAS activity in a dose-(A) and time (B)-dependent manner. *n*=8. Mean±SD. ^c*P*<0.01 vs vehicle.

Effects of C75 on adipose differentiation

Initially, the adipocyte precursor cells had a fibroblast-like appearance and no intracellular lipids were visible under microscopy (Fig 2A). During the following days, cell rapidly acquired a round shape and accumulated lipid droplets. On d 8, most of the cells were completely filled with multiple lipid droplets (Fig 2B). C75 50 mg/L inhibited adipocyte differentiation greatly (Fig 2C).

C75 10-50 mg/L reduced both triglyceride content (Fig 3A) and GPDH activity (Fig 3B) in a dose-dependent manner. The inhibitory effects of C75 50 mg/L for 8 d were stronger than that for 48 h (*P*<0.01).

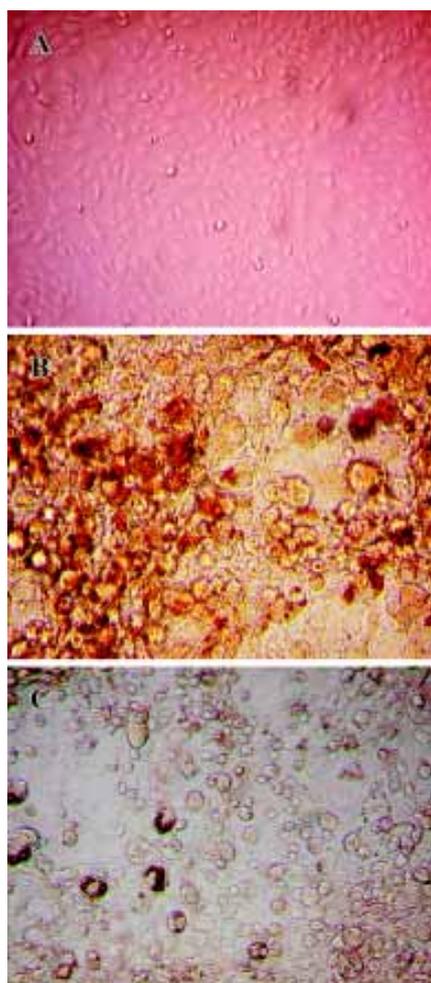


Fig 2. Morphological examination of adipocyte differentiation influenced by C75. (A) Undifferentiated adipocytes on d 2 ($\times 40$); (B) Fully differentiated 3T3-L1 adipocytes treated with vehicle ($\times 100$). (C) Fully differentiated 3T3-L1 adipocytes treated with C75 50 mg/L for 48 h ($\times 100$). Oil Red O staining.

Effect of C75 on PPAR γ gene expression following induction of adipocyte differentiation The level of PPAR γ mRNA transcript was markedly increased in vehicle cells after adipogenic induction. In contrast, in cells incubated with C75 for 48 h (short-term treatment), a slight decrease was induced, whereas long-term treatment (8 d) of differentiated 3T3-L1 adipocytes with C75 induced an obvious decrease of PPAR γ mRNA (Fig 4).

DISCUSSION

Fatty acid synthase is the key enzyme in *de novo* lipogenesis. Both human liver and adipose tissue exhibit substantially lower FAS activity than that found in rats^[24]. Nonetheless, significant *de novo* lipogenesis is

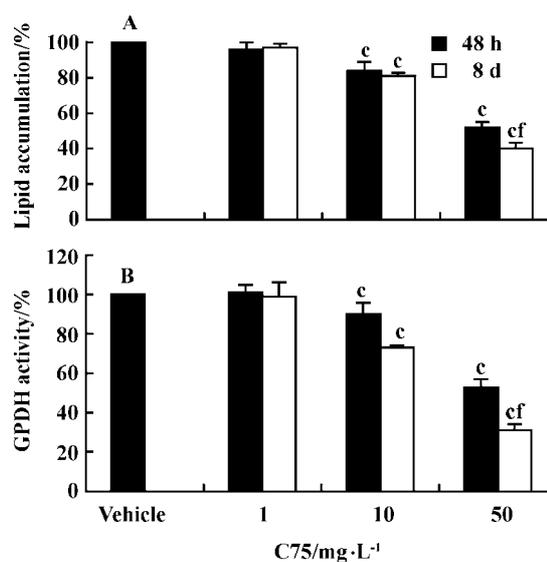


Fig 3. Inhibitory effects of C75 on lipid accumulation (A) and GPDH activity (B) in adipocytes for 48 h or 8 d. (A) $n=8$. (B) $n=6$. Mean \pm SD. ^c $P<0.01$ vs vehicle. ^f $P<0.01$ vs C75 50 mg/L group for 48 h.

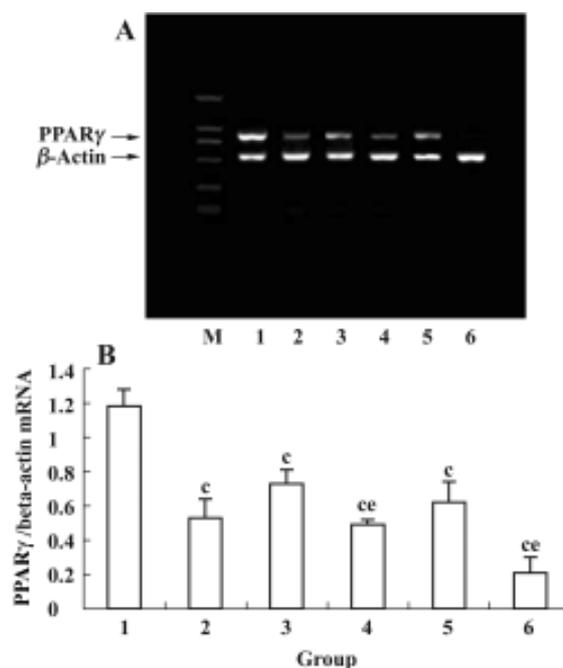


Fig 4. Effect of C75 on PPAR γ gene expression following induction of adipocyte differentiation. (A) RT-PCR results. β -actin was used as internal standard. (B) Densitometric scanning for DNA bands of each group. Lane 1: Vehicle; lane 2: C75 10 mg/L for 8 d; lane 3: C75 25 mg/L for 48 h; lane 4: C75 25 mg/L for 8 d; lane 5: C75 50 mg/L for 48 h; lane 6: C75 50 mg/L for 8 d. $n=6$. Mean \pm SD. ^c $P<0.01$ vs vehicle; ^e $P<0.05$ vs 48-h treatment at the same dose.

well documented in human adipocytes. Human adipocytes contain substantial levels of FAS activity which is

sensitive to both nutritional and hormonal modulation^[25]. Studies in humans fed with a high carbohydrate diet demonstrated that total body fat synthesis significantly exceeded hepatic *de novo* lipogenesis, suggesting that adipose tissue may be the major site for fat synthesis^[26]. Furthermore, another study showed that adipose tissue accounted for up to 40 % of whole-body lipogenesis under this condition^[27]. The effect of carbohydrate intake on adipose tissue lipogenesis was similar between humans and rats. But rats have higher rates of lipogenesis, which may be explained in part by the higher metabolic rate in rats than in humans^[28]. Moreover, FAS levels are elevated in obese animals^[29]. Thus, induction of adipocyte lipogenesis may contribute to obesity.

As reported in recent studies that in central nervous system, C75 inhibited FAS, leading to changes in neuropeptide expression which results in an anorexigenic signal; in the periphery, C75 increased CPT-1 activity, fatty acid oxidation, and energy production. In this report, we propose another action for C75-blocking adipocytic differentiation which lead to the reduction of adipocyte number.

Adipose tissue mass is dependent on both the average volume and the number of its constituent adipocytes. Significant alteration in body mass involves alteration in both adipocyte volume and number. Decrease in adipocyte number occur via preadipocyte and adipocyte apoptosis, and possibly adipocyte de-differentiation^[14].

Our data showed that when cells were triggered to differentiation, C75 significantly diminished the increase in GPDH activity and markedly reduced the proportion of newly formed adipocytes, as well as the lipid contents of the cultures, and the blocking effect of conversion was in a dose-dependent manner. In the meantime treatment of 3T3-L1 adipocytes with C75 for 8 d induced more obvious decrease in triglyceride content and GPDH activity than that for 48 h. These results lead to a conclusion that inhibitory action of C75 on FAS activity resulted in the reduction of 3T3-L1 cells differentiation, and the blocking effect occurred both in the early phases and in the latter phases of the process.

PPAR γ is a nuclear hormone receptor that is expressed at highest levels in adipose tissue. PPAR γ is a major coordinator of adipocyte gene expression and differentiation^[30], and its downregulation has been shown to inhibit the 3T3-L1 cells into adipocytes. Our RT-PCR results showed that the differentiation blockage of 3T3-L1 cells was associated with absence of PPAR γ

induction, and long-term treatment of terminally differentiated 3T3-L1 adipocytes with C75 induced an obvious decrease of PPAR γ mRNA. This result leads to proposition that PPAR γ is the target gene in the reduction of adipogenesis by C75, and there should be other transactivation gene such as C/EBP α or SREBP families.

In conclusion, FAS inhibitor C75 blocked the adipocyte differentiation in a dose-dependent manner which was accompanied with down-regulation of PPAR γ mRNA level. The diet-induced obese mouse model will be used to clarify the inhibitory effects of C75 on adipocyte differentiation *in vivo* in the future.

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