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Regulation of adipogenesis by medium-chain fatty acids in the absence of hormonal cocktail☆

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Abstract

We report here that octanoate and decanoate, 8-carbon and 10-carbon medium-chain fatty acids (MCFA), decreased adipogenesis in 3T3-L1 preadipocytes when treated with standard hormonal cocktail, but increased adipogenesis in a dose-dependent manner (with decanoate being more effective) when treated with basal media. Addition of dexamethasone to basal medium with either octanoate or decanoate further increased adipogenesis. In order to understand the adipogenic effects of MCFA in the absence of standard hormonal cocktail, postconfluent 3T3-L1 preadipocytes were treated with octanoate or decanoate, and the change in the expression of several adipogenic transcription factors and enzymes was investigated using real-time RT-PCR. Octanoate and decanoate up-regulated the mRNA expression of peroxisome-proliferator-activated receptor (PPAR) γ, CCAAT/enhancer-binding protein (C/EBP) α, fatty-acid-binding protein, sterol-regulatory element binding protein 1c, lipoprotein lipase and hormone-sensitive lipase, and the protein expression of PPARγ and C/EBPα, with decanoate being more effective. Moreover, the PPARγ antagonist GW9662 inhibited MCFA-induced lipid accumulation by about 50%. Decanoate and octanoate, to a lesser degree, increased lipid accumulation, which was associated with an increase in glycerol-3-phosphate dehydrogenase activity. These results show that octanoate and decanoate may stimulate differentiation of preadipocytes, at least in part, by their influence on the expression of PPARγ and other adipocyte-specific factors.

Keywords: MCFA; Adipocytes; Preadipocytes; Adipocyte-Specific Genes; GPDH activity

1. Introduction

Obesity is characterized by an increase in lipid stores and is generally associated with enhanced lipid consumption, which contributes to its development [1]. Therefore, the study of the metabolic fate of dietary lipid in obese subjects is crucial in the understanding of this disease. Adipocyte differentiation is critical for metabolic homeostasis and nutrient signaling. Growth of adipose tissue mass involves both hypertrophy and hyperplasia of adipocytes [2]. These processes result, respectively, from the increase in lipid accumulation in the adipocytes and the formation of new adipocytes from precursor cells, the preadipocytes. Preadipocytes differentiate into mature adipocytes when treated with a well-characterized inducing cocktail [3]. The sequence of events that leads to the expression of adipocyte-specific genes involves the activation of several transcriptional factors, notably peroxisome-proliferator-activated receptor (PPAR) γ, CCAAT/enhancer-binding protein (C/EBP) α and sterol-regulatory element binding protein 1c (SREBP1c) [4]. PPARγ and C/EBPα control the expression of several adipocyte genes such as fatty-acid-binding protein (aP2) and fatty acid transporter (CD36). SREBP1c increases the expression of many lipogenic genes, including fatty acid synthase [5].

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Lipoprotein lipase (LPL) [6] and hormone-sensitive lipase (HSL) [7] are the two major enzymes regulating the process of lipolysis.

Medium-chain triglycerides (MCT) are triglycerides with fatty acids having a chain length of 6–12 carbons. MCT occur naturally and are especially abundant in coconut and palm oil [8]. Commercial MCT predominantly comprise C8 octanoic and C10 decanoic acids [9]. MCT differ from long-chain triglycerides (LCT), which have fatty acids of more than 12 carbons, in that they are absorbed directly into the portal circulation and transported to the liver for rapid oxidation [10]. Dietary intake of MCT results in the incorporation of medium-chain fatty acids (MCFA) into the triglycerides stored in adipocytes [11]. Exogenous MCFA can also be incorporated into adipocyte triglycerides in culture [12]. It has been reported that octanoate attenuates lipid accumulation in 3T3-L1 preadipocytes [13]. In contrast, octanoate has been used as an adipogenic inducer in bovine preadipocytes [14]. A recent study reported that octanoate also induced lipid accumulation when 3T3-L1 cells were cotreated with dexamethasone [15]. In the present study, we show for the first time that MCFA induce differentiation of 3T3-L1 cells in the absence of standard hormonal cocktail. We report that octanoate (C8), as well as decanoate (C10), promotes differentiation of 3T3-L1 preadipocytes by stimulating the expression of key adipogenic transcription factors.

2. Materials and methods

2.1. Cell culture

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). Cells (preadipocytes) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) containing 10% bovine calf serum until confluent. For the cells in basal medium, postconfluent preadipocytes were treated with 0.1% EtOH or test compounds (octanoate or decanoate) along with DMEM containing 10% fetal bovine serum (FBS). The medium was replaced every 2 days with fresh medium, and the cells were cultured up to Day 6. For cells treated with adipogenic medium, at 2 days after confluency (D0), the cells were stimulated for differentiation with DMEM containing 10% FBS, 167 nmol/L insulin , 0.5 μmol/L isomethylbutylxanthine (IBMX) and 1 μmol/L dexamethasone (standard hormonal cocktail) for 2 days (D2). Cells were then maintained in 10% FBS/DMEM with 167 nmol/L insulin for another 2 days (D4), followed by culturing with 10% FBS/DMEM for an additional 2 days (D6). On D0, the maturing preadipocytes were treated with 0.1% EtOH or test compounds (octanoate or decanoate). Fresh treatment solution was added each time the medium was changed. All media contained 100 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Reagents and antibodies

Phosphate-buffered saline (PBS) and DMEM media were purchased from GIBCO BRL Life Technologies (Grand Island, NY). Octanoate (>99% pure), decanoate (>98% pure) and PPARγ antagonist (GW9662) were purchased from Sigma (St. Louis, MO). AdipoRed assay reagent was purchased from Cambrex, Inc. (Walkersville, MD). Antibodies specific for polyclonal PPARγ, C/EBPα and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RNeasy Mini kit was from Qiagen, Inc. (Valencia, CA).

2.3. Quantification of lipid content and oil red O staining

Lipid content was quantified using AdipoRed assay reagent. In brief, postconfluent preadipocytes and maturing preadipocytes were treated with 0.1% EtOH or test compounds along with 10% FBS/DMEM from Days 0 to 6. On Day 6, cells were washed with PBS (pH 7.4), and 200 μl of PBS and 5 μl of AdipoRed reagent were added to the wells. After incubation for 10 min, fluorescent signal was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm. In order to obtain an estimate of lipid content/viable cells, the relative fluorescence units from the AdipoRed data were divided by absorbance values from the viability data, and the resulting data were expressed as percent control. Oil red O staining was performed as described by Suryawan and Hu [16]. Three images for each dish were captured using ImagePro software (MediaCybernetics, Silver Spring, MD).

2.4. Western blot analysis

Whole-cell extracts were prepared as described elsewhere [17]. Western blot analysis was performed using the commercial NUPAGE system (Novex/Invitrogen, Carlsbad, CA). Samples were heated to 70°C for 10 min, separated by 12% acrylamide gels and analyzed by immunoblotting. Immunoblots were developed using ECL kits (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.5. Quantitative analysis of Western blot analysis data

Measurement of signal intensity on PVDF membranes after Western blot analysis with various antibodies was performed using a FluorChem densitomer with the AlphaEaseFC image processing and analysis software (Alpha Innotech Corporation). For statistical analysis, all data were expressed as integrated density values, which were calculated as the density values of the specific protein bands/β-actin density values and expressed as percentage of control. All figures showing quantitative analysis include data from at least three independent experiments.

2.6. Glycerol-3-phosphate dehydrogenase activity

Postconfluent preadipocytes were treated with 0.1% EtOH or test compounds along with 10% FBS/DMEM from Days 0 to 6. On Day 6, cells were rinsed and scraped into 0.5 ml of

ice-cold sucrose buffer containing 0.28 mol/L sucrose, 5 mmol/L Tris, 1 mmol/L EDTA and 0.002% β-mercaptoethanol, and stored at −70°C. The homogenate was sonicated with three blasts for 15 s and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were used for the assay of glycerol-3-phosphate dehydrogenase (GPDH) activity according to Wise and Green [18]. Protein was measured as described above. GPDH activity is expressed in milliunits per milligram of protein (1 mU is equal to the oxidation of 1 nmol/L NADH per minute).

2.7. Total RNA extraction

Postconfluent preadipocytes were treated with 0.1% EtOH or test compounds along with 10% FBS/DMEM from Days 0 to 6. On Day 6, RNA samples were extracted using the RNeasy Mini kit following the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using RNA 6000 Nano Assay and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.8. Real-time RT-PCR

One hundred nanograms of total RNA in a 20-μl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA) in accordance with the manufacturer's protocols. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative RT-PCR (Taqman) assays were performed using 384-well MicroFluidic Cards on the ABI PRISM 7900 Sequence Detection System. All of the oligonucleotide primer and fluorogenic probe sets for Taqman real-time RT-PCR were made by ABI (Table 1). The cycle conditions were 94.5°C for 15 min, followed by 40 cycles of 97°C for 30 s and 59.7°C for 1 min. mRNA expressions were normalized by using 18S as endogenous control to correct for differences in the amount of total RNA added to each reaction. The RQ values from each gene were used to compare the gene expressions of all groups (control; 0.1% EtOH, octanoate and decanoate).

2.9. Statistical analysis

Analysis of variance (GLM procedure, Statistica, version 6.1; StatSoft, Inc.) was used to determine the significance of treatment effects. Fisher's post hoc least significant difference test was used to determine the significance of differences among means. Statistically significant differences are defined at the 95% confidence interval. Data shown are presented as mean±S.E.

3. Results

3.1. MCFA induce lipid accumulation without adipogenic agents

To test whether MCFA increase preadipocyte differentiation, we used MCFA with 10% FBS/DMEM to induce 3T3-L1 preadipocyte differentiation. First, postconfluent preadipocytes were treated with octanoate or decanoate without adipogenic agents such as insulin, dexamethasone and IBMX for 6 days, and intracellular lipid content was measured and quantified (Fig. 1). Results from the AdipoRed assay showed that both octanoate and decanoate increased lipid content in a dose-dependent manner (Fig. 1A). Decanoate (298.6±31.7%, $P<.001$) was more effective than octanoate (136.0±8.8%, $P<.001$) at 1 mmol/L in stimulating lipid accumulation. Oil red O staining demonstrated increased intracellular lipid accumulation with both octanoate and decanoate.

3.2. MCFA decrease lipid accumulation with adipogenic agents

Since treatment of cells with dexamethasone or insulin promotes lipid accumulation, we treated cells with octanoate or decanoate in the presence of dexamethasone or insulin and measured lipid accumulation. When the postconfluent preadipocytes were treated with octanoate or decanoate in the presence of dexamethasone for 6 days, significant lipid accumulation was observed in a dose-dependent manner (Fig. 1B). Lipid accumulation also increased with decanoate in the presence of insulin, but octanoate in the presence of insulin had no significant effect on stimulating lipid accumulation. Interestingly, when the postconfluent preadipocytes were treated for 6 days with octanoate or decanoate along with dexamethasone, insulin and IBMX (a standard hormonal cocktail), lipid accumulation was significantly decreased

Table 1
List of probes for different adipocyte-specific transcription factors, enzymes and other genes used in real-time RT-PCR

Gene symbol	Gene name	Probe sequence
18S	Eukaryotic 18S rRNA	CCATTGGAGGGCAAGTCTGGTGCCA
PPARγ	Peroxisome-proliferator-activated receptor γ	CTCAGTGGAGACCGCCCAGGCTTGC
CEBPα	CCAAT/enhancer-binding protein (C/EBP) α	GCCACCGCCGCCACCGCCACCGCCG
SREBF1 (*SREBP1*)	Sterol-regulatory element binding factor 1	CGACATCGAAGACATGCTCCAGCTC
FABP4 (*aP2*)	Fatty-acid-binding protein 4, adipocyte	CAAGCTGGTGGTGGAATGTGTTATG
LIPE (*HSL*)	Lipase, hormone sensitive	CACATCGCCCACAGCAGAGTCTGTG
LPL	Lipoprotein lipase	TGGATGGACGGTAACGGGAATGTAT
GPD1 (*GPDH*)	Glycerol-3-phosphate dehydrogenase 1 (soluble)	CCCCAATGTGGTGGCCATCCCAGAC

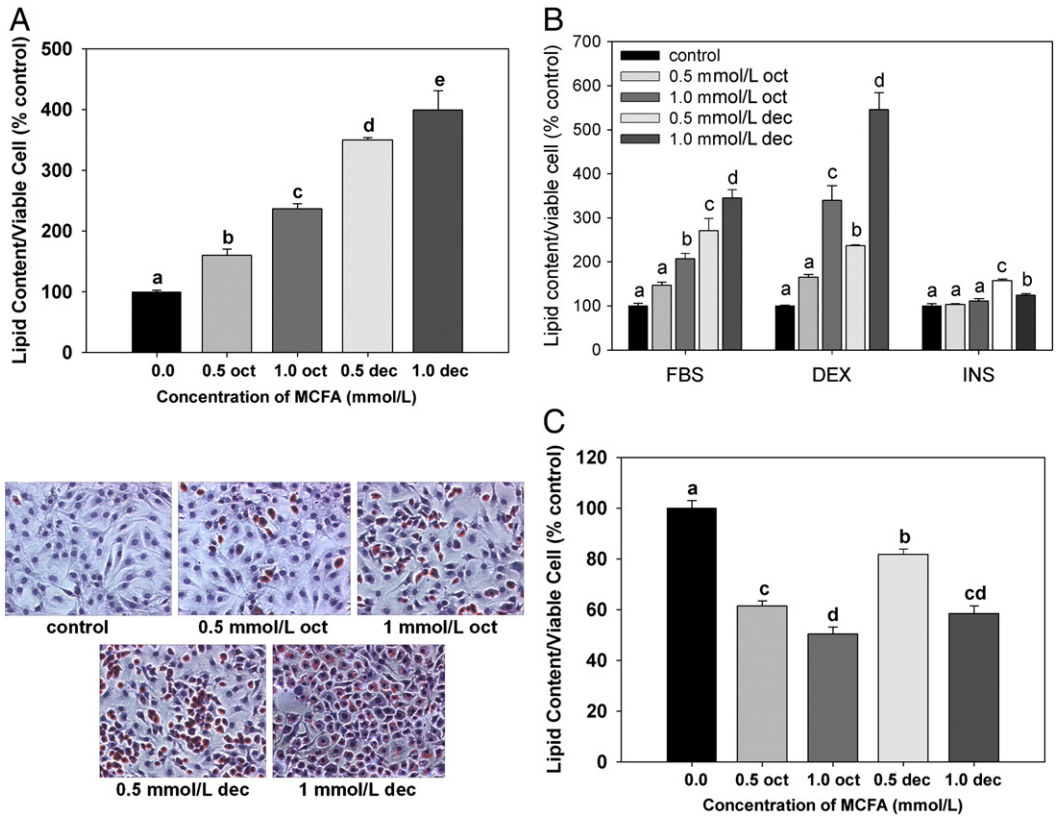

Fig. 1. Effect of octanoate (oct) and decanoate (dec) without adipogenic agents (A) and with adipogenic reagents (B and C) on lipid accumulation. Lipid content was measured by AdipoRed assay. [abcde]Means not designated by a common superscript are different ($P<.05$; $n=8$). Cellular triglyceride was stained with oil red O dye, and cells were photographed at a magnification of ×200. Each treatment was run in triplicate. Representative images are shown.

(Fig. 1C). At the same concentration, octanoate was more effective than decanoate in inhibiting lipid accumulation. All subsequent experiments were carried out in media without dexamethasone, IBMX or insulin present.

3.3. MCFA up-regulate the expression of adipogenic transcription factors

To determine whether the stimulation of cytosolic lipid accumulation by MCFA involves alteration in the expression of genes encoding key adipogenic transcription factors, we used a real-time RT-PCR approach to examine the expression of *PPARγ*, *C/EBPα*, *aP2*, *SREBP1c*, *LPL* and *HSL*. Results from real-time RT-PCR show that decanoate was more effective than octanoate (Fig. 2) in stimulating adipogenesis-related gene expression. At 1 mmol/L concentration, octanoate increased the expression of *PPARγ* (287.9±62.3%, $P<.001$), *C/EBPα* (219.8±39.3%, $P<.001$), *aP2* (327.0±57.1%, $P<.001$), *SREBP1c* (163.6±44.7%, $P<.001$), *LPL* (170.3±39.8%, $P<.005$) and *HSL* (485.0 ±85.1%, $P<.001$). At the same concentration, decanoate induced a greater increase in the expression of *PPARγ* (539.8±30.2%, $P<.001$), *C/EBPα* (286.5±21.2%, $P<.001$), *aP2* (460.4±25.8%, $P<.001$), *SREBP1c* (254.4±20.9%, $P<.001$), *LPL* (232.5±16.7%, $P<.001$) and *HSL* (820.1±42.2%, $P<.001$).

3.4. MCFA increase gene expression and activity of GPDH

Both MCFA increased GPDH activity. At 1 mmol/L concentration, decanoate (108.2±10.9%, $P<.001$) was more effective than octanoate (49.2±16.3%, $P<.01$) in inducing GPDH activity (Fig. 3A). Similar results were observed using real-time RT-PCR to examine the expression of *GPDH*. Fig. 3B showed that *GPDH* mRNA was increased significantly by MCFA. In the group treated with 0.5 and 1 mmol/L octanoate, the expression of *GPDH* was increased by 117.7±22.2% ($P=0.113$) and 265.1±63.9% ($P<.005$), respectively. At the same concentration, decanoate further increased the expression of *GPDH* to 433.4±81.9% ($P<.001$) and 308.7±35.6% ($P<.001$), respectively.

3.5. MCFA increase PPARγ and C/EBPα protein levels

Consistent with the mRNA expression, PPARγ and C/EBPα protein expressions showed similar patterns of increase. PPARγ protein levels were increased dramatically by octanoate and decanoate at 1 mmol/L by 221.6±21.1% ($P<.005$) and 471.8±45.5% ($P<.001$), respectively (Fig. 4A). C/EBPα (p42) was also increased by octanoate and decanoate at 1 mmol/L by 315.9±24.3% ($P<.05$) and 713.7±101.4% ($P<.001$), respectively (Fig. 4B).

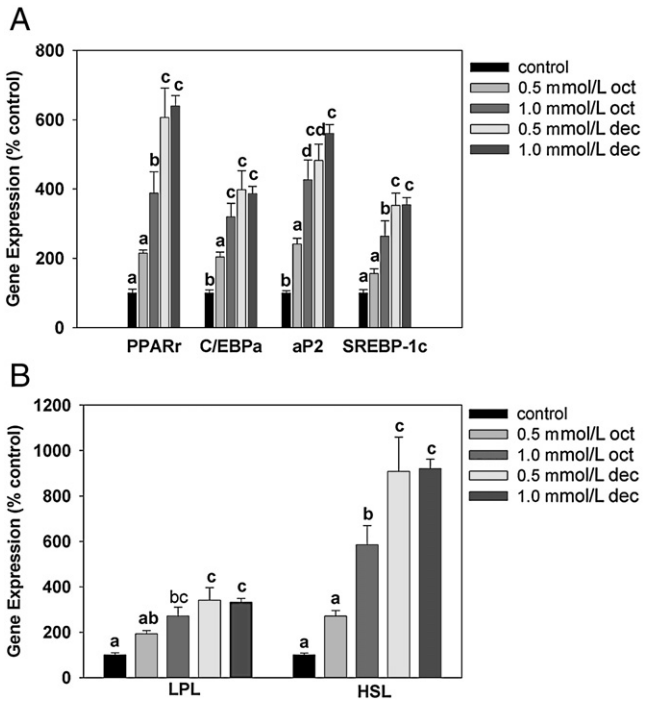

Fig. 2. (A) Effect of octanoate (oct) and decanoate (dec) on adipocyte-specific transcription factors (PPARγ, C/EBPα and SREBP1c) and aP2. (B) Effect of octanoate (oct) and decanoate (dec) on lipolytic enzymes LPL and HSL. Data are expressed as mean±S.E.M. for each group and within each gene. Means without a common letter are different ($P<.05$; $n=6$).

3.6. PPARγ antagonist GW9662 inhibits MCFA-induced lipid accumulation

The postconfluent preadipocytes were treated with octanoate or decanoate for 6 days in the presence of 15 μM GW9662 (a PPARγ antagonist), and intracellular lipid content was quantified (Fig. 5). Adipogenic changes were observed with both MCFA. Results from the AdipoRed assay showed that both octanoate and decanoate increased lipid content at 0.5 mmol/L ($P<.05$). However, GW9662 decreased MCFA-stimulated lipid accumulation by about 50% ($P<.05$).

4. Discussion

The goal of this study was to demonstrate the effects of MCFA on 3T3-L1 adipocyte–lipid accumulation. Although MCFA have been shown to attenuate adipogenesis in the presence of standard hormonal cocktail [19], in our current study, we have shown that treatment of postconfluent 3T3-L1 preadipocytes with octanoate or decanoate without the standard hormonal cocktail increased lipid accumulation in a dose-dependent manner, with decanoate being more effective. Fatty acids of different chain lengths have different effects on cellular processes. We speculate that the chain length differences may be one reason for the difference in the effects of octanoate and decanoate on lipid accumulation. Our study also showed that cotreatment of adipogenic agents such as dexamethasone or insulin separately, with octanoate or decanoate, further increased lipid accumulation. This is in agreement with previous reports [15]. These findings suggest that dexamethasone or insulin may be important factors in regulating actions of MCFA. Furthermore, treatment of postconfluent preadipocytes with octanoate or decanoate with the standard hormonal cocktail (IBMX, dexamethasone and insulin) decreased lipid accumulation. Han et al. [13] also found that octanoate attenuated adipogenesis in 3T3-L1 preadipocytes in the presence of the standard hormonal cocktail. Our data are consistent with these previous findings.

The dramatic changes in gene expression profiles during adipocyte differentiation have been previously described [20]. In the present study, MCFA treatment of 3T3-L1 cells led to potentiation of the increase in expression of adipocyte-specific transcription factors, including *PPARγ*, *C/EBPα* and *SREBP1c*. Moreover, expression of the major adipocyte marker genes such as *aP2* and *GPDH* increased after treatment with octanoate or decanoate, with decanoate being more effective. Fatty acids and certain prostaglandins and prostaglandin metabolites are known to function as ligands of PPARγ [21]. In addition, supply of exogenous fatty acids is required for adipocyte differentiation, unless synthetic

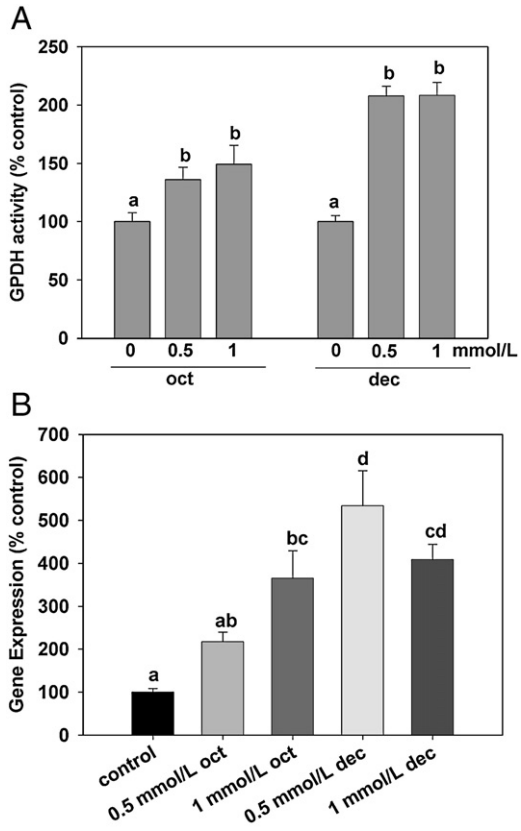

Fig. 3. Effect of octanoate (oct) and decanoate (dec) on GPDH activity (A) and gene expression (B). All assays were performed in hexaplicate for each treatment. Data are expressed as mean±S.E.M. for each group and within each gene. Means without a common letter are different ($P<.05$; $n=6$).

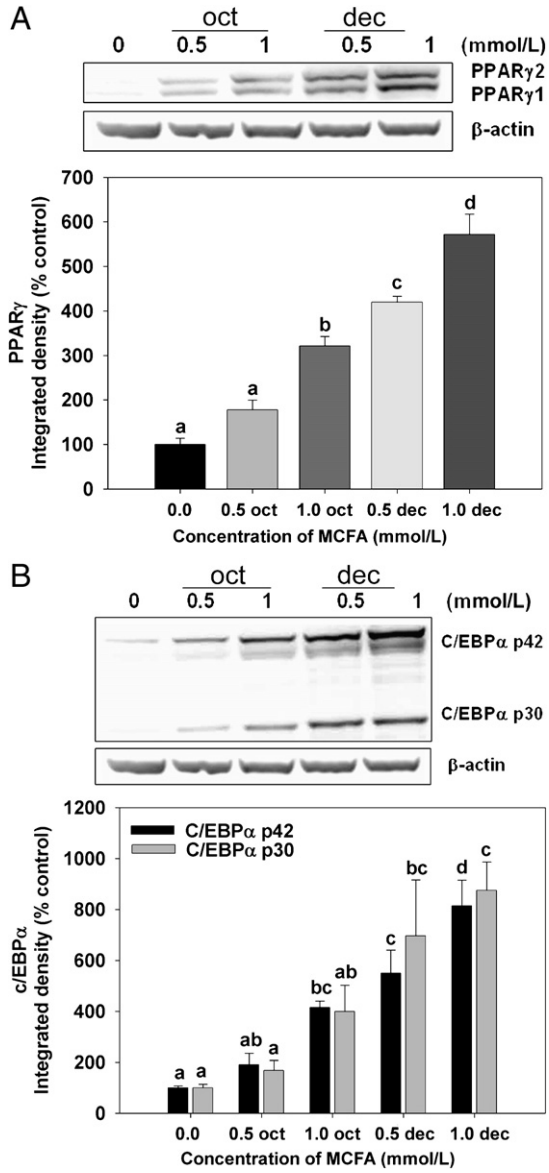

Fig. 4. Effect of octanoate (oct) and decanoate (dec) on PPARγ (A) and C/EBPα (B) protein levels. All assays were performed in triplicate for each treatment. [abcd]Means that are not denoted with a common letter are different ($P<05$).

PPAR agonists are included in the medium [22]. A recent report showed that octanoate may act as a partial ligand for PPARγ because octanoate alone promoted adipogenesis in 3T3-L1 cells stably overexpressing *PPARγ* [13]. It is also possible that suppression of PPARγ might be the secondary event in the inhibition of adipocyte differentiation. Our data show that the PPARγ antagonist (GW9662) inhibited MCFA-induced 3T3-L1 lipid accumulation, confirming that this is a PPAR-mediated process. Therefore, we can deduce that MCFA bind PPARγ and induce cellular PPARγ signaling.

MCFA also caused an enhanced expression of *HSL* and *LPL*, which are the two major enzymes regulating the process of lipolysis, in postconfluent 3T3-L1 preadipocytes. Octanoate was reported to increase basal lipolysis and to facilitate triglyceride hydrolysis in adipocytes [23]. Moreover, triglycerides containing MCFA were shown to be hydrolyzed more rapidly by HSL or LPL than triglycerides containing long-chain fatty acids [24,25], indicating that MCFA treatment of adipocytes might affect the expression of *HSL* and *LPL*.

Under normal dietary conditions, MCFA are virtually absent in adipose tissue and only detectable after supplementation [26]. The incorporation of $C_{8:0}$ is 5–20 times less efficient than that of $C_{10:0}$ [26]. Effects of MCT on body weight and body composition have been inconsistent in vivo. Tsuji et al. [27] observed that 12-week ingestion of low amounts of MCT reduced body weight and fat in human subjects. In contrast, Yost and Eckel [28] found that, compared to a diet containing LCT, administration of MCT for either 4 or 12 weeks failed to improve rates of weight loss in obese women. Other studies also reported no effects of MCT on body weight [29]. It is possible that the variable in vivo effects may be a result of differences in circulating levels of insulin and glucocorticoids. The concentration range tested in the present study was 0.5–1 mmol/L MCFA, and these concentrations were lower than those used in previous in vitro studies [13,30] but comparable to those achievable in vivo [31].

Takenouchi et al. [15] reported that adipogenesis induced by hormonal cocktail is accompanied by up-regulation of *GPDH*, *PPARγ* and *caveolin-1*. It is therefore possible that the hormonal cocktail and MCFA might be stimulating adipogenesis by activating similar signaling pathways. Furthermore, we have noticed that the amount of lipid accumulation with standard hormonal cocktail is significantly more (480% more than noninduced cells) when compared to MCFA-stimulated lipid accumulation (150% and 300% more lipid accumulation than noninduced cells, with octanoate and decanoate at 1 mM, respectively).

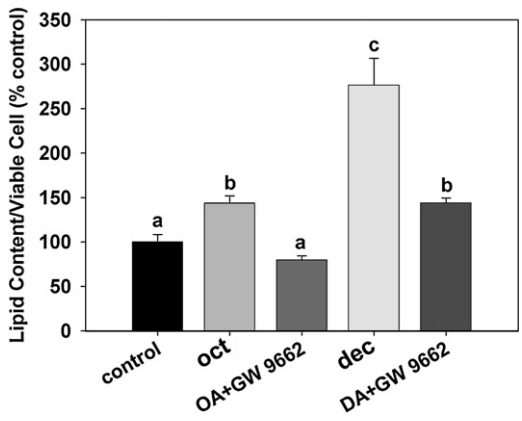

Fig. 5. Effect of GW9662 on octanoate (oct)-induced and decanoate (dec)-induced lipid accumulation. Postconfluent preadipocytes were treated with octanoate or decanoate at 0.5 mmol/L in 10% FBS/DMEM with or without 15 μM GW9662 during Days 0–6. On Day 6, lipid content was measured by AdipoRed assay. [abc]Means that are not denoted with a common letter are different ($P<05$; $n=8$).

In conclusion, we showed that incubation of 3T3-L1 postconfluent preadipocytes with octanoate or decanoate, without standard differentiation medium, rapidly induced differentiation with characteristic marked triglyceride accumulation and increased GPDH activity. We also provide evidence that MCFA alone can up-regulate the expression of adipocyte-specific transcription factors. The opposite effects of octanoate and decanoate on adipogenesis with and without standard hormonal cocktail may be attributed to the possibility that MCFA can act as either PPARγ agonists or PPARγ antagonists, depending on the concentration of exogenous hormones. However, more studies are needed to investigate the dual effects of MCFA on lipid accumulation in the presence and in the absence of standard hormonal cocktail. The results of this study demonstrate that, in adipocytes, MCFA can act as potent activators of the expression of genes encoding proteins directly involved in their differentiation and metabolism.

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