Sinensetin Enhances Adipogenesis and Lipolysis by Increasing Cyclic Adenosine Monophosphate Levels in 3T3-L1 Adipocytes

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Abstract

Sinensetin is a rare polymethoxylated flavone (PMF) found in certain citrus fruits. In this study, we investigated the effects of sinensetin on lipid metabolism in 3T3-L1 cells. Sinensetin promoted adipogenesis in 3T3-L1 preadipocytes growing in incomplete differentiation medium, which did not contain 3-isobutyl-1-methylxanthine. Sinensetin up-regulated expression of the adipogenic transcription factors peroxisome proliferator-activated receptor γ, CCAAT/enhancer-binding protein (C/EBP) α, and sterol regulatory element-binding protein 1c. It also potentiated expression of C/EBPβ and activation of cAMP-responsive element-binding protein. Sinensetin enhanced activation of protein kinase A and increased intracellular cAMP levels in 3T3-L1 preadipocytes. In mature 3T3-L1 adipocytes, sinensetin stimulated lipolysis via a cAMP pathway. Taken together, these results suggest that sinensetin enhances adipogenesis and lipolysis by increasing cAMP levels in adipocytes.

Key words 3T3-L1 cells; Sinensetin; Adipogenesis; Lipolysis; Cyclic adenosine monophosphate (cAMP)
INTRODUCTION

Adipose tissue is a dynamic organ that plays an important role in energy balance and changes in mass according to the metabolic requirements of the organism. Many aspects of adipogenesis are regulated by a small set of transcription factors. Transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs) α, β, and δ and peroxisome proliferator-activated receptor (PPAR) γ are the key determinants of terminal adipocyte differentiation. As cells undergo differentiation in response to adipogenic signals, initial expression of C/EBPβ and δ is followed by an increase in PPARγ and C/EBPα expression. PPARγ and C/EBPα promote terminal differentiation by activating transcription of the gene encoding the fatty acid-binding protein aP2, which is involved in establishing and maintaining the adipocyte phenotype. It was reported that sterol-regulatory element-binding protein 1c (SREBP1c) is the earliest transcription factor involved in adipocyte differentiation.

The study of adipogenesis has been greatly facilitated by the establishment of immortal preadipocyte cell lines such as 3T3-L1 preadipocytes, because the adipocytes generated from these cells exhibit most of the key features of adipocytes in vivo. When maintained in culture with fetal calf serum, 3T3-L1 preadipocytes spontaneously differentiate into fat-cell clusters over a period of several weeks. The differentiation of preadipocytes into adipocytes can be accelerated by inducing agents such as dexamethasone and 3-isobutyl-1-methylxanthine (IBMX). High concentrations of insulin have been used in combination with these inducing agents. IBMX is a phosphodiesterase inhibitor that increases intracellular levels of cAMP, a physiological activator of protein kinase A (PKA). An imbalance between adipogenesis and lipolysis leads to obesity. Thus phytochemicals that modulate lipid metabolism may have beneficial effects in the prevention and treatment of metabolic diseases.

Polymethoxyflavones (PMFs), which are unique to citrus plants, are flavones bearing two or more methoxy groups on their basic benzo-c-pyrone (15-carbon, C6–C3–C6) skeleton with a carbonyl group at the C4 position. Concentrations of PMFs in Citrus species are highly variable. Notably, PMF-rich citrus fruit peels, such as those of Citrus depressa Hayata and
C. sunki Hort. ex Tanaka, have anti-obesity properties in high-fat-diet-induced obese mice.\textsuperscript{12, 13} We previously described eight PMFs from C. sunki.\textsuperscript{14} Among these, two common PMFs (nobiletin and tangeretin) have been reported to modulate lipid metabolism. Nobiletin stimulates lipolysis in 3T3-L1 cells via activation of signaling cascades mediated by cAMP pathway.\textsuperscript{15} Tangeretin regulates lipid synthesis in HepG2 cells by suppressing diacylglycerol acetyltransferase (DGAT) and inhibiting microsomal triglyceride transfer protein.\textsuperscript{16} In contrast, the functions of a third PMF, sinensetin, remain largely unknown. We have recently reported that sinensetin had an anti-inflammatory effect in lipopolysaccharide-stimulated RAW 264.7 cells,\textsuperscript{17} and it enhanced fatty acid β-oxidation in mature 3T3-L1 adipocytes.\textsuperscript{18} In the present study, we investigated the effects of sinensetin on adipogenesis in 3T3-L1 preadipocytes and lipolysis in mature 3T3-L1 adipocytes, and characterized the underlying molecular mechanisms.

**MATERIALS AND METHODS**

**Reagents** Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), and penicillin–streptomycin (PS) were obtained from Gibco (Grand Island, NY, USA). Antibodies to PPARγ, fatty acid-binding protein aP2, C/EBPα, C/EBPβ, extracellular signal-regulated kinase 1 and 2 (ERK 1/2), and phospho-Thr204 ERK 1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to cAMP-responsive element-binding protein (CREB), phospho-Ser133 CREB, and phospho-Ser/Thr-cAMP-dependent protein kinase (p-PKA) substrate were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody to SREBP1c was obtained from BD Biosciences (San Jose, CA, USA). Phosphate-buffered saline (PBS) (pH 7.4), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Takara Shuzo Co. (Otsu, Shiga, Japan). Sinensetin was purchased from ChromaDex (San Diego, CA, USA). Sinensetin was dissolved in DMSO to make a 50 mM solution. All other reagents were purchased...
from Sigma Chemical Co. unless otherwise stated.

**Cell culture and differentiation** 3T3-L1 preadipocytes obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured at 37°C in a 5% CO₂ atmosphere in DMEM containing 1% PS (penicillin 100 unit and streptomycin 100 μg) and 10% BCS. To examine the effects of sinensetin on the differentiation of preadipocytes to adipocytes, 2-d post-confluent preadipocytes (designated day 0) were cultured in DI differentiation medium [DMEM containing 1% PS, 10% FBS, 1 μm dexamethasone (D), and 1 μg/mL insulin (I)] that did not contain IBMX (M) for 2 d in the presence of various concentrations of sinensetin. The cells were then cultured for a further 2 d in DMEM containing 1% PS, 10% FBS, and 1 μg/mL insulin. Next, they were maintained in post-differentiation medium (DMEM containing 1% PS and 10% FBS), which was replaced every 2 d. Differentiation, as measured by the expression of adipogenic markers and the appearance of lipid droplets, reached completion between days 4 and 8.

**Cell cytotoxicity** The cytotoxic effects of sinensetin were measured using an LDH cytotoxicity detection kit. Cells were seeded into 96-well plates at a density of 1×10⁴ cells/well. After 24 h, they were treated with various concentrations of sinensetin for 72 h. LDH activity was measured, according to the manufacturer’s protocol, in culture supernatants and cell lysates to evaluate cytotoxicity (amount of LDH released into the medium / maximal LDH release × 100).

**Oil Red O staining and cell quantification** After the induction of differentiation, cells were stained with filtered Oil Red O [6 parts saturated Oil Red O dye (0.6%) in isopropanol plus 4 parts water]. Briefly, the cells were washed twice with PBS, fixed through incubation with 3.7% formaldehyde in PBS for 1 h, washed an additional three times with water, and stained with Oil Red O for 1 h. Excess stain was removed by washing with water, and the stained cells were dried. The stained lipid droplets were dissolved in isopropanol containing 4% Nonidet P-40 and
then quantified by measuring the absorbance at 520 nm. Results are given as the lipid content in each experimental group relative to that of DI-differentiated cells (designated as 1-fold).

**Western blot analysis**  Cells were washed with ice-cold PBS, collected, and centrifuged. The resulting cell pellets were resuspended in lysis buffer [1‘ RIPA (Upstate Biotechnology, Temecula, CA, USA), 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 1 µg/mL aprotinin, 1 µg/mL pepstatin, 1 µg/mL leupeptin] and incubated on ice for 1 h. Cell debris was then removed by centrifugation, and lysate protein concentrations were determined using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were then subjected to electrophoresis on 10–15% polyacrylamide gels containing SDS and transferred to polyvinylidene difluoride membranes. The membranes were blocked through incubation for 1 h at room temperature with a 0.1% solution of Tween 20 in Tris-buffered saline containing 5% nonfat dry milk or 5% BSA. After incubation overnight at 4°C with primary antibody, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Immunodetection was carried out using ECL Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ, USA).

**RNA preparation and quantitative real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) analysis**  Cellular RNA was extracted from 3T3-L1 cells using TRIzol reagent according to the manufacturer’s instructions and treated with DNase (Wako Pure Chemical Industries, Ltd., Osaka, Japan). cDNA was synthesized from 1 µg of total RNA in a 20-µL reaction using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, Kyunggi, Korea). The following primers were used in real-time PCR analysis: adiponectin, 5‘-GAC CGC ACT TTC TCC TC-3‘ and 5‘-GTC ATC TTC GGC ATG ACT GG-3‘; β-actin, 5‘-AGG CTG TGC TGT CCC TGT AT-3‘ and 5‘-ACC CAA GAA GGA AGG CTG GA-3‘. Samples were prepared using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. Adiponectin expression was measured by quantitative real-time RT-PCR using a Chromo4 Real-Time PCR System (Bio-Rad). Formation of a single product was verified by
melting curve analysis. The level of expression of adiponectin was normalized to that of β-actin. Data were analyzed using Opticon Monitor software (ver. 3.1; Bio-Rad).

**Lipolysis assay**  To obtain fully differentiated 3T3-L1 cells, confluent cells were induced to differentiate through incubation in MDI differentiation medium (DMEM containing 1% PS, 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 5 μg/mL insulin) for 2 d. The cells were then cultured for a further 2 d in DMEM containing 1% PS, 10% FBS, and 5 μg/mL insulin. Next, they were maintained in post-differentiation medium, which was replaced every 2 d. Fully differentiated 3T3-L1 cells were incubated with DMEM for 4 h. The cells were then treated with post-differentiation medium containing various concentrations of sinensetin. Culture supernatants were assayed for glycerol levels at 24 and 48 h post-treatment using a free glycerol reagent kit (Sigma-Aldrich).

**Measurement of cellular cAMP levels**  To measure cellular cAMP levels in 3T3-L1 preadipocytes, cell lysates were prepared from 3T3-L1 preadipocytes (day 0) 30 min after treatment with various concentrations of sinensetin. To measure cellular cAMP levels in 3T3-L1 adipocytes, fully differentiated 3T3-L1 cells were incubated with serum-free DMEM (DMEM containing 0.2% BSA) for 16 h. The cells were then treated with serum-free DMEM containing various concentrations of sinensetin for 30 min. cAMP levels were measured using a cAMP Complete ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer’s protocol.

**Statistical analysis**  All qualitative data are representative of at least three independent experiments. Values are expressed as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used for multiple comparisons. Treatment effects were analyzed using the paired *t*-test or Duncan’s multiple range test using the SPSS software package (ver. 12.0; SPSS Inc., Chicago, IL, U.S.A). Differences were considered statistically significant at *p* < 0.05.
RESULTS AND DISCUSSION

Sinensetin enhances adipogenesis in 3T3-L1 preadipocytes in the absence of IBMX

The effect of sinensetin on cytotoxicity of 3T3-L1 cells was first evaluated by LDH assay. Sinensetin (up to 50 µM) did not cause cytotoxicity in 3T3-L1 cells (data not shown). Next, the adipogenic potential of sinensetin was assessed by inducing the differentiation of 3T3-L1 preadipocytes in various combinations of differentiation inducers (-, M, D, I, MD, MI, DI, and MDI). On day 8, the adipocytes were stained using Oil Red O. As shown in Fig. 1A, sinensetin (40 µM) did not affect the cellular lipid accumulation under some incomplete differentiation medium conditions (-, M, I, and MI), but it affected the cellular lipid accumulation under DI incomplete differentiation medium. Thus, we used DI (without IBMX) differentiation medium in the subsequent experiments. When 3T3-L1 cells were treated with sinensetin at various concentration (2, 10, and 40 µM) in DI medium containing dexamethasone and insulin, but with sinensetin in place of IBMX, sinensetin increased cellular lipid accumulation (Fig. 1B) and triglyceride content (Fig. 1C) in a dose-dependent manner, though the effect of sinensetin (40 µM) was less than positive control cells that had been treated with 0.5 mM IBMX. At the molecular level, sinensetin increased the expression of PPARγ1, PPARγ 2, C/EBPα, and aP2 (Fig. 2A). Also, sinensetin dose-dependently increased the expression of adiponectin (Fig 2B). Adiponectin is one of the direct targets of PPARγ. These results indicate that sinensetin enhance adipogenesis of 3T3-L1 preadipocytes by up-regulating the adipogenic transcription factors in the absence of IBMX.

Sinensetin activates 3T3-L1 adipocyte differentiation signals at an early stage

To identify upstream target molecules of sinensetin, we investigated the effects of sinensetin on expressions of SREBP1c and C/EBPβ, which play critical roles in the initial steps of adipogenesis. Like IBMX, sinensetin increased the expression of SREBP1c at the 12 h and 24 h (Fig. 3A) and the expression of C/EBPβ at the 2 h and 6 h (Fig. 3B) compared with DMSO-treated control cells in 3T3-L1 preadipocytes. SREBP1c has been reported to induce the
production of an endogenous ligand that enhances PPARγ transcriptional activity, and it can increase the expression of several genes involved in fatty acid metabolism. C/EBPβ is expressed at an early stage of the differentiation process in response to adipogenic signals and drives the subsequent expression of C/EBPα. C/EBPβ has also been reported to have roles in the stimulation of CDK inhibitor p21 expression and induction of PPARγ2, a key regulator of adipogenesis.

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cAMP-responsive element-binding protein (CREB) is a central transcriptional activator in the adipocyte differentiation program. The expression of C/EBPβ is regulated by CREB, which is activated at an early stage of adipogenesis. CREB only exhibits transcriptional activity when phosphorylated on Ser 133. The activation of C/EBPβ requires the phosphorylation by extracellular signal-regulated kinase (ERK) and glycogen synthase kinase 3β. Thus, we examined the effect of sinensetin on the phosphorylation of CREB and ERK in 3T3-L1 preadipocytes. As shown in Figure 4A, sinensetin increased the phosphorylations of CREB and ERK. In sinensetin-treated early differentiating 3T3-L1 preadipocytes, these proteins were more strongly activated and remained active until 0.25 h compared with DMSO-treated control cells. These results suggest that sinensetin stimulated the activations of CREB and ERK, which are critical for the expression and transcriptional activity of C/EBPβ, thereby enhancing the expression of PPARγ and inducing adipocyte differentiation. It is well known that CREB is activated by a cAMP/PKA pathway. It was confirmed that the phosphorylated forms of PKA in sinensetin-treated 3T3-L1 preadipocytes was significantly increased at 0.25 h and 0.5 h compared with DMSO-treated control cells (Fig. 4B). Sinensetin also significantly and dose-dependently increased cAMP levels at 30 min after treatment (Fig. 4C). These results indicate that sinensetin exerts adipogenic effects via a cAMP/PKA pathway.

Sinensetin stimulates lipolysis in mature 3T3-L1 adipocytes To investigate the effects of sinensetin on lipolysis in mature 3T3-L1 adipocytes, glycerol levels in culture supernatants were measured. Sinensetin significantly increased lipolysis at 24 h and 48 h after treatment (Fig. 5A). Lipolysis in adipocytes is known to be triggered by an increase in the intracellular level of
cAMP, which activates PKA.\textsuperscript{29} PKA mediates phosphorylation of hormone sensitive lipase (HSL), resulting in increased HSL hydrolytic activity and translocation to the surface of a lipid droplet.\textsuperscript{30} It was known that sinensetin increased level of phospho-PKA and phospho-HSL in mature 3T3-L1 adipocytes.\textsuperscript{18} Sinensetin significantly and dose-dependently increased cAMP levels at 30 min after treatment (Fig. 5B). These results suggest that sinensetin promotes lipolysis in mature 3T3-L1 adipocytes by increasing the intracellular cAMP level.

White adipose tissue has the capacity to store triglycerides (TG) and mobilize fatty acids and glycerol according to energetic demands. Approximately 90\% of the adipocyte volume is TG located in lipid droplets that dislocate the nucleus to the periphery and limits the cytosolic space.\textsuperscript{31, 32} Excessive accumulation of TG in adipose tissue results in obesity, as well as related metabolic disorders such as type 2 diabetes, cardiovascular diseases, and hypertension. TG are synthesized and stored in cytosolic lipid droplets during times of energy excess and are mobilized from lipid droplets via lipolysis. Although TG synthesis occurs in other organs, such as the liver in the case of very low-density lipoprotein production, the lipolysis for the provision of fatty acids as an energy source for other organs is a unique function of adipocytes. It was suggested that the phytochemicals which increase the lipolysis in adipocytes, may be a useful therapeutic agents for treating obesity.\textsuperscript{33}

In conclusion, we have shown that sinensetin enhanced not only adipogenesis in preadipocytes growing in incomplete differentiation medium, which did not contain IBMX but also lipolysis in mature adipocytes. Although adipogenesis and lipolysis are intricate processes involving many signaling cascades, we have identified the enhancement of intracellular cAMP levels as a possible mechanism of action of sinensetin. Taken together, these results suggest that sinensetin enhances adipogenesis and lipolysis by increasing cAMP levels in adipocytes.

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Conflict of Interest

The authors declare no conflict of interest.
References

1) Harp JB. New insights into inhibitors of adipogenesis. *Curr. Opin. Lipidol.*, **15**, 303–307 (2004).

2) Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. *Annu. Rev. Cell. Dev. Biol.*, **16**, 145–171 (2000).

3) Camp HS, Ren D, Leff T. Adipogenesis and fat-cell function in obesity and diabetes. *Tren. Mol. Med.*, **8**, 442–447 (2002).

4) Payne VA, Au WS, Lowe CE, Rahman SM, Friedman JE, O'Rahilly S, Rochford JJ. C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. *Biochem. J.*, **425**, 215–223 (2009).

5) Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. *Cell*, **3**, 127–133 (1974).

6) Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. *Physiol. Rev.*, **78**, 783–809 (1998).

7) Martini CN, Plaza MV, Vila Mdel C. PKA-dependent and independent cAMP signaling in 3T3-L1 fibroblasts differentiation. *Mol. Cell Endocrinol.*, **298**, 42–47 (2009).

8) Triscari J, Nauss-Karol C, Levin BE, Sullivan AC. Changes in lipid metabolism in diet-induced obesity. *Metabolism*, **34**, 580–587 (1985).

9) Li S, Pan MH, Lo CY, Tan D, Wang Y, Shahidi F, Ho CT. Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones. *J. Funct. Foods*, **1**, 2–12 (2009).

10) Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M, Ohta H. Flavonoid composition of fruit tissues of citrus species. *Biosci. Biotechnol. Biochem.*, **70**, 178–192 (2006).

11) Choi SY, Ko HC, Ko SY, Hwang JH, Park JG, Kang SH, Han SH, Yun SH, Kim SJ. Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. *Biol. Pharm. Bull.*, **30**, 772–778 (2007).

12) Lee YS, Cha BY, Saito K, Choi SS, Wang XX, Choi BK, Yonezawa T, Teruya T, Naqai K,
Woo JT. Effects of a Citrus depressa Hayata (shiikuwasa) extract on obesity in high-fat diet-induced obese mice. *Phytomedicine*, 18, 648–654 (2011).

13) Kang SI, Shin HS, Kim HM, Hong YS, Yoon SA, Kang SW, Kim JH, Kim MH, Ko HC, Kim SJ. Immature Citrus sunki peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. *Biol. Pharm. Bull.*, 35, 223–230 (2012).

14) Ko HC, Jang MG, Kang CH, Lee NH, Kang SI, Lee SR, Park DB, Kim SJ. Preparation of a polymethoxyflavone-rich fraction (PRF) of Citrus sunki Hort. ex Tanaka and its antiproliferative effects. *Food Chem.*, 123, 484–488 (2010).

15) Saito T, Abe D, Sekya K. Nobiletin enhances differentiation and lipolysis of 3T3-L1 adipocytes. *Biochem. Biophysi. Res. Commun.*, 357, 371–376 (2007).

16) Kurowska EM, Manthey JA, Casachi A, Theriaul AG. Modulation of HepG2 cell net apolipoprotein B secretion by the citrus polymethoxyflavone, tangeretin. *Lipids*, 39, 143–151 (2004).

17) Shin HS, Kang SI, Yoon SA, Ko HC, Kim SJ. Sinensetin attenuates LPS-induced inflammation by regulating the protein level of IκB-α. *Biosci. Biotechnol. Biochem.*, 76, 847–849 (2012).

18) Kang SI, Shin HS, Ko HC, Kim SJ. Effects of sinensetin on lipid metabolism in mature 3T3-L1 adipocytes. *Phytother. Res.*, 27, 131–134 (2013).

19) Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes*, 50, 2094–2099 (2001).

20) Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M, Shimomura I. Induction of adiponectin, a fat-deriver antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes*, 52, 1655–1663 (2003).

21) Kim JB, Spiegelman BM. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.*, 10, 1096–1107 (1996).

22) Christy RJ, Kaestner KH, Geiman DE, Lane MD. CCAAT/enhancer binding protein gene
promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA*, **88**, 2593–2597 (1991).

23) Tang QQ, Otto TC, Lane MD. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proc. Natl. Acad. Sci. USA*, **100**, 850–855 (2003).

24) Hamm JK, Park BH, Farmer SR. A role for C/EBPbeta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *Biol. Chem.*, **276**, 18464–18471 (2001).

25) Niehof M, Manns MP, Trautwein C. CREB controls LAP/C/EBP beta transcription. *Mol. Cell. Biol.*, **17**, 3600–3613 (1997).

26) Zhang JW, Klemm DJ, Vinson C, Lane MD. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. *J. Biol. Chem.*, **279**, 4471–4478 (2004).

27) Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*, **59**, 675–680 (1989).

28) Tang QQ, Grønborg M, Huang H, Kim JW, Otto TC, Pandey A, Lane MD. Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. *Proc. Natl. Acad. Sci. USA*, **102**, 9766–9771 (2005).

29) Maurière P, De Pergola G, Berlan M, Lafontan M. Human fat cell beta-adrenergic receptors: beta-agonist-dependent lipolytic responses and characterization of beta-adrenergic binding sites on human fat cell membranes with highly selective beta 1-antagonists. *J. Lipid Res.*, **29**, 587–601 (1988).

30) Carmen GY, Víctor SM. Signalling mechanisms regulating lipolysis. *Cell. Signal.*, **18**, 401–408 (2006).

31) Thompson BR, Lobo S, Bernlohr DA. Fatty acid flux in adipocytes: the in’s and out’s of fat cell lipid trafficking. *Mol. Cell Endocrinol.*, **318**, 24–33 (2010).

32) Meex RC, Schrauwen P, Hesselink MK. Modulation of myocellular fat stores: lipid droplet dynamics in health and disease. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **297**, R913–
33) Ahmadian M, Wang Y, Sul HS. Lipolysis in adipocytes. *Int. J. Biochem. Cell Biol.*, **42**, 555–559 (2010).
Figure legends

**Fig. 1.** Effect of Sinensetin on Adipogenesis in 3T3-L1 Preadipocytes.

(A) Cell were cultured for 2 days in various combinations of differentiation inducers (-, M, D, I, MD, DI, and MDI) with sinensetin (40 µM). Differentiated adipocytes were stained with Oil Red O on day 8. (B) Cells were cultured for 2 days in DI differentiation medium containing various concentrations of sinensetin or 0.5 mM IBMX. Differentiated adipocytes were stained with Oil Red O on day 8. Oil Red O-stained adipocytes were photographed at 100× and 200× magnification. The data shown are representative of three independent experiments. (C) Lipid accumulation was assessed by measuring the OD$_{520}$ (as described in the Materials and Methods). Control cells were treated with DMSO instead of sinensetin. All values are presented as the mean ± SD (n = 3). Mean separation was performed using Duncan’s multiple range test. $^{(a-d)}p < 0.05$, the values not sharing a common superscript letter are statistically significant in difference between the groups. NT, no treatment; M, IBMX (0.5 mM); D, dexamethasone (1 µM); I, insulin (1 µg/mL).

**Fig. 2.** Effect of Sinensetin on the Expression of Key Adipogenic Regulators in 3T3-L1 Preadipocytes.

Cells were cultured for 2 d in DI differentiation medium containing various concentration of sinensetin or 0.5 mM IBMX. (A) Western blot analysis of PPARγ, C/EBPα, and aP2 (day 6). The data shown are representative of three independent experiments. (B) Total RNA was extracted at day 4, and adiponectin mRNA levels analyzed by real-time PCR. Control cells were treated with DMSO instead of sinensetin. All values are presented as the mean ± SD (n = 3). Mean separation was performed using Duncan’s multiple range test. $^{(a-d)}p < 0.05$, the values not sharing a common superscript letter are statistically significant in difference between the groups.

**Fig. 3.** Effect of Sinensetin on the Adipogenic Signal at Early Stages in 3T3-L1 Preadipocytes.

Cells (day 0) were cultured in DMEM containing 10% FBS in the presence or absence of...
sinensetin (40 μM) or IBMX (0.5 mM). Proteins were harvested at the indicated times. Control cells were treated with DMSO instead of sinensetin. The levels of matured SREBP1c (A) and C/EBPβ (B) were analyzed by Western blot. The data shown are representative of three independent experiments.

**Fig. 4.** Effect of Sinensetin on C/EBPβ Upstream Signal in Differentiating 3T3-L1 Cells.

(A) The activations of CREB, ERK, and PKA were analyzed by Western blot. Cells (day 0) were cultured in DMEM containing 10% FBS in the presence or absence of sinensetin (40 μM). Proteins were harvested at the indicated times. (B) PKA level and relative band intensity was determined by Western blotting and densitometer. Phosphorylated PKA was normalized to the β-actin expression level. All values are presented as the mean ± SD (n = 3; *p < 0.05 vs. sinensetin-free conditions). The Western blotting data shown are representative of three independent experiments. (C) Intracellular cAMP production by sinensetin in 3T3-L1 preadipocytes. Cell lysates were prepared from 3T3-L1 preadipocytes (day 0) at 30 min after treatment with various concentrations of sinensetin. Control cells were treated with DMSO instead of sinensetin. All values are presented as the mean ± SD (n = 3). Mean separation was performed using Duncan’s multiple range test. (a, b)*P < 0.05, the values not sharing a common superscript letter are statistically significant in difference between the groups.

**Fig. 5.** Effect of Sinensetin on Lipolysis in Mature 3T3-L1 Adipocytes.

(A) 3T3-L1 preadipocytes were induced to differentiate as described in the Material and Methods. At day 8, mature 3T3-L1 adipocytes were incubated for 16 h with DMEM containing 0.2% BSA. The cells were treated for 24 or 48 h with post-differentiation medium containing various concentrations of sinensetin. Glycerol content relative to control cells (grown under sinensetin-free conditions and assigned a value of 1) was measured at 24 h. (B) Intracellular cAMP production by sinensetin in mature 3T3-L1 adipocytes. Cell lysates were prepared from mature 3T3-L1 adipocytes 30 min after treatment with various concentrations of sinensetin. Control cells were treated with DMSO instead of sinensetin. All values are presented as the mean ± SD.
± SD (n = 3). Mean separation was performed using Duncan’s multiple range test. \(^{(a,b)} P < 0.05\), the values not sharing a common superscript letter are statistically significant in difference between the groups.
Fig. 1.

A

B

C

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Fig. 2.

(A) Treatment with different concentrations of Sinensetin (0, 2, 10, 40 μM) and IBMX (0, 2, 10, 40 μM) for 24 h resulted in an increase in PPARγ2, PPARγ1, C/EBPα (42 kDa and 30 kDa), aP2, and β-actin expression. The conditions are as follows:

| DI | IBMX | Sinensetin |
|----|------|------------|
|    |      | 0          |
| -  | -    | 0          |
| +  | +    | 0          |
| +  | +    | 2          |
| +  | +    | 10         |
| +  | +    | 40         |
| +  | +    | 0 (μM)     |

(B) Adiponectin expression under the same conditions as above, showing relative gene expression levels.
Fig. 3.

A

| Time (h) | DMSO | Sinensetin | IBMX |
|----------|------|------------|------|
| 0        |      |            |      |
| 12       |      |            |      |
| 24       |      |            |      |

SREBP1c →
β-actin →

B

| Time (h) | DMSO | Sinensetin | IBMX |
|----------|------|------------|------|
| 0        |      |            |      |
| 2        |      |            |      |
| 6        |      |            |      |

C/EBPβ →
β-actin →
Fig. 4.

A

| Time (h) | DMSO  | Sinensetin (40 μM) |
|----------|-------|--------------------|
| 0        | 0.25  | 0.25               |
| 0.25     | 0.5   | 0.5                |
| 0.5      | 1     | 1                  |
| 1        | 2     | 2                  |

B

| Time (h) | DMSO  | Sinensetin (40 μM) |
|----------|-------|--------------------|
| 0        | 0.25  | 0.25               |
| 0.25     | 0.5   | 0.5                |
| 0.5      | 1     | 1                  |
| 1        | 2     | 2                  |

C

- Increase in cAMP (fold)
  - Sinensetin 0: a
  - Sinensetin 2: a
  - Sinensetin 10: b
  - Sinensetin 40: b
Fig. 5.

A

Glyceral release (fold of increase)

Sinensetin 0 2 10 40 (μM)

B

Increase in cAMP (fold)

Sinensetin 0 2 10 40 (μM)