ARTICLE

Journal of Cellular Biochemistry 117:39–48 (2016)

Journal of Cellular Biochemistry

Exogenous Sodium Pyruvate Stimulates Adipogenesis of 3T3-L1 Cells

Ji-Sun Hwang,[1] Song-Yi Kim,[1] Eun-Hye Jung,[1] Mi-Youn Kwon,[1] Kyoung-Hong Kim,[1] Hyeongjin Cho,[2] and Inn-Oc Han[1]*

[1]*Department of Physiology and Biophysics, College of Medicine, Inha University, Incheon, Korea*
[2]*Department of Chemistry, Inha University, Incheon, Korea*

ABSTRACT

We investigated the effects of exogenous sodium pyruvate (SP) on adipocyte differentiation, lipid accumulation, and the mRNA expression levels of adipogenesis-related genes in 3T3-L1 pre-adipocytes. Differentiation of pre-adipocytes was induced by MDI (3-isobutyl-1-methylxanthine: IBMX, dexamethasone: DEX, and insulin), in the presence or absence of SP. Adipogenesis was stimulated by SP in a concentration-dependent manner. SP also induced the expression of genes encoding aP2, GLUT4, and adiponectin, but had no effect on cell proliferation. Exogenous glucose did not promote adipogenesis or lipid accumulation. 2-deoxy-D-glucose inhibited adipogenesis initiated by MDI, but failed to influence the effects of SP on adipogenesis, whereas 3-bromopyruvate inhibited adipogenesis regardless of whether SP was present. The pro-adipogenic properties of SP were limited to the early events of adipogenesis. To determine whether SP mimics the adipogenic action of dexamethasone or insulin, we examined the effects of SP on adipogenesis with combinations of IBMX, DEX, and insulin. SP did not improve incomplete lipid accumulation observed in cells grown under IBMX-, DEX-, or insulin-free conditions. Insulin-stimulated ERK1/2 phosphorylation was diminished by SP, while phosphorylation of Akt was increased, correlating with increased glucose uptake in response to insulin. We also observed that SP stimulated immediate early expression of C/EBPβ and C/EBPδ. The PPARγ antagonist GW9662 inhibited adipogenesis. Our findings highlight the adipogenic function of exogenous SP by stimulating early events of adipogenesis. J. Cell. Biochem. 117: 39–48, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: SODIUM PYRUVATE; ADIPOGENESIS; 3T3-L1

Adipogenesis is the process by which pre-adipocytes differentiate into adipocytes [Otto and Lane, 2005]. This process and the extent of subsequent fat accumulation are closely related to the occurrence and progression of diseases such as insulin resistance and obesity. The number of mature adipocytes determines the number of fat cells that exist in an individual, whereas the size of fat cells is dependent on lipid accumulation in adipocytes. The pre-adipocyte 3T3-L1 cell line is a convenient model for investigating adipogenesis and the physiology of mature adipocytes.

During the differentiation of pre-adipocytes, many key transcription factors are involved, including CCAAT-enhancer binding protein delta (C/EBPδ) and C/EBPβ. Together, these two transcription factors induce the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and C/EBPα [White and Stephens, 2010]. The C/EBP transcription factors play a critical role in the differentiation of fat cells in vitro. The differentiation of adipocytes is defective in transgenic mice lacking either C/EBPα or both C/EBPβ and C/EBPδ [Wang et al., 1995; Tanaka et al., 1997]. In the early phase of adipocyte differentiation, expression of PPARγ is induced by C/EBPβ and C/EBPδ, which leads to the expression of C/EBPα. The PPAR group of proteins is a subfamily of nuclear hormone receptors that regulate transcription by interacting with the corresponding ligand; they are predominantly expressed in adipose tissue [Brun and Spiegelman, 1997; Rangwala and Lazar, 2004]. The role of PPARγ in adipocyte differentiation has been studied extensively in vitro using pre-adipocytes. PPARγ and C/EBPα cross-regulate each other to maintain their gene expression levels

J. S. Hwang and S. Y. Kim equally contributed to this work.
There is no potential conflict of interest in this paper.

Grant sponsor: National Research Federation of Korea; Grant number: NRF-2013R1A1A2058244; Grant sponsor: Inha University Research.

*Correspondence to: Inn-Oc Han, Ph.D., Department of Physiology and Biophysics, College of Medicine, Inha University, 7-241, Shinheung-Dong, Jung-Ku, Incheon 400-712, Korea.
E-mail: iohan@inha.ac.kr

Manuscript Received: 21 April 2015; Manuscript Accepted: 26 May 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 6 June 2015
DOI 10.1002/jcb.25244 • © 2015 Wiley Periodicals, Inc.

and critically regulate the expression of adipose-specific genes that are involved in developing the adipose phenotype during differentiation [Gregoire et al., 1998]. In addition, they also regulate the expression of genes associated with insulin signaling and glucose and lipid metabolism in mature adipocytes [Tafuri, 1996; Rosen and Spiegelman, 2000].

Pyruvic acid (PA) is a simple three-carbon alpha-keto monocarboxylic acid. Under physiological conditions, PA is largely present in cells and extracellular fluids as its conjugate anion, pyruvate. Pyruvate is mainly formed from glucose through glycolysis. As the final product of glycolysis and the starting substrate for the tricarboxylic acid (TCA) cycle, pyruvate plays a central role in intermediary metabolism. In adipocytes, pyruvate plays a versatile role in energy production, biosynthesis, and redox balance. The metabolic fate of pyruvate is mainly determined by three reactions: efflux from the cell via its reduction to lactate; complete oxidation via decarboxylation to acetyl-CoA; and anaplerosis via carboxylation to oxaloacetate [Yang et al., 2014]. Carboxylation or decarboxylation reactions produce ATP via the TCA cycle and oxidative phosphorylation and supply the carbon substrate (acetyl-CoA) for de novo fatty acid synthesis and NADPH generation (via the malate cycle) [Ballard and Hanson, 1967]. Carboxylation and decarboxylation activity is low in undifferentiated 3T3-L1 cells, but increases significantly upon their differentiation [Freytag and Utter, 1980]. Exogenous sodium pyruvate (SP) has been considered a candidate for treating hypoxic-ischemic brain injury because it is a metabolic substrate [Mongan et al., 2001] and an antioxidant [Pan et al., 2012] that can readily pass the blood-brain barrier.

The effect of exogenous SP on early stage of adipogenesis has not been examined. We sought to assess the effects of exogenous SP on adipocyte differentiation and lipid accumulation in 3T3-L1 cell cultures. We found that SP stimulate differentiation and increases glucose uptake in adipocyte, suggesting that adipocyte differentiation and triglyceride accumulation require coordinated adjustments in the flux distribution around pyruvate.

MATERIALS AND METHODS

CELL CULTURE FOR 3T3-L1 AND THEIR DIFFERENTIATION

The 3T3-L1 pre-adipocytes were purchased from ATCC (Manassas, VA). Adipocytes differentiation induction was carried out by following [Chung et al., 2010] with some modification. Briefly, 3T3-L1 pre-adipocytes were seeded in the 24-well plates and expanded in pre-adipocyte growth medium consisting of DMEM supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. Three days post-confluence, 3T3-L1 pre-adipocytes differentiation induced by the addition of adipogenic cocktail (0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, and 1 μg/ml insulin) to DMEM containing 10% FBS. Three days later, the induction medium was replaced with preadipocyte culture medium supplemented with 1 μg/ml insulin. Every two days, the medium was again changed to the original preadipocyte culture medium containing 10% FBS in the absence of any differentiating reagents.

QUANTIFICATION OF LIPIDS USING OIL RED O STAINING

After differentiation, the cells were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h at room temperature and washed three times with PBS. Cells were then stained for 1 h with filtered Oil Red O (0.5% in 60% isopropanol) and washed three times with distilled water. Lipid and Oil Red O were extracted using isopropanol, and absorbance was measured using a microplate reader at a wavelength of 520 nm.

GLUCOSE UPTAKE ASSAY

Differentiated 3T3L-1 adipocytes grown on 48-well plates were washed twice and incubated with serum-free DMEM. After 3 h, cells were incubated with or without 100 nM insulin in KRB buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2% BSA, 0.5 mM glucose, 25 mM $NaHCO_3$, pH 7.4). Glucose uptake measurement was initiated by the addition of [^{14}C]2-deoxyglucose (specific activity: 6 Ci/mmol, 0.5 μCi/well) to each well and, after 15 min the supernatant was removed. Plates were then rinsed with ice-cold PBS and the radioactivity of the cell lysates was determined using a liquid scintillation counter.

RT-PCR AND QUANTITATIVE PCR

Total RNA from 3T3-L1 cells was extracted with Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and total RNA (1 μg) was reverse transcribed. PCR was performed using primers for aP2 (forward, F: TTTGCCACAAGGAAAGTGGC, reverse, R: TTCCATCCCACTTCTGCACC), adiponectin (F: ATCCACACGTGTACTCAC, R: AGCATGGTCTACTTCCAG), PPARγ (F: AAAGACCCAGCTCTACAACA, R: TCGTAGATGACAAATGGTGA), GLUT4 (F: CTGTGCTTGGCTCCCTTCAG, R: CGATGGCCAGTTGGTTGAGT), C/EBPα (F: TCACCTGGAAGACAGCTCCT, R: AATCCCCATTTACGCTGATG), and GAPDH (F: TCATTGACCTCAACTACATGGT, R: CTAAGCAGTTGGTG TGCAG). Analysis of final PCR products on 1% agarose gels with expected sizes. mRNAs were quantitatively detected by measuring incorporation of fluorescent SYBR green into double-stranded DNA (iCycler iQ, Bio-Rad). Relative DNA levels were calculated from the PCR profiles of each sample using the threshold cycle (Ct), corresponding to the cycle at which a statistically significant increase in fluorescence occurred. Ct is considered the amount of template present in the starting reaction. To correct for differences in the amount of total cDNA in the starting reaction, Ct values for an endogenous control (glyceraldehyde 3-phosphate dehydrogenase: GAPDH) were subtracted from those of the corresponding sample.

WESTERN BLOTTING

Whole cell protein lysates were prepared in lysis buffer (10 mM Tris, 140 mM NaCl, 1% NP-40, 0.5% SDS, and protease inhibitors, pH 8.0). Protein samples (20–30 μg for each) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% BSA or skim milk in TBST solution and incubated with antibodies (Santa Cruz Biotechnology, otherwise noted) against p-ERK1/2, ERK1/2, pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), C/EBPβ, C/EBPδ or GAPDH (Cell Signaling Technology). After

washing with TBST, secondary antibodies (Amersham Biosciences) (1:10,000) were applied and detected by the Enhanced Chemiluminescence system (Amersham Biosciences). Densitometric quantification of protein bands were detected using Image J (NIH).

STATISTICAL ANALYSIS

The data are expressed as the mean ± SEM and analyzed for statistical significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparisons and paired Student's *t* test for comparing two. A *P* value <0.05 was considered significant.

RESULTS

EFFECTS OF SP ON ADIPOGENESIS OF 3T3-L1 CELLS

We cultured 3T3-L1 cells in basal media supplemented with the differentiation cocktail MDI (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin) as controls, or in basal media with MDI and 25 mM SP, for 72 h. Cell cultures were then maintained in pre-adipocyte culture media supplemented with insulin (Fig. 1). After 7 days, each culture was visually inspected following Oil red O staining. Cells in the control cultures exhibited a

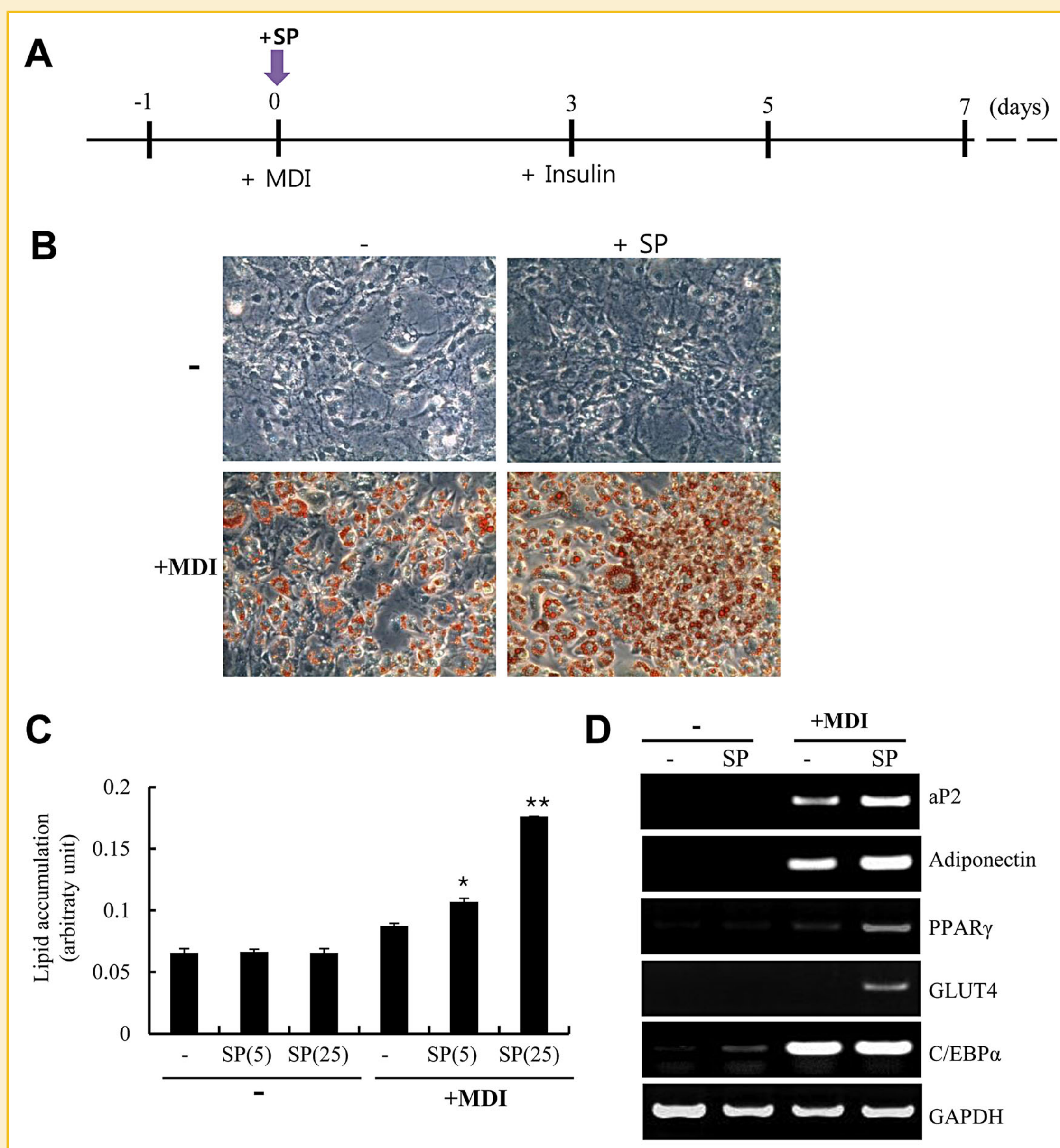

Fig. 1. Modulation of 3T3-L1 adipocyte differentiation by extracellular sodium pyruvate (SP). (A) Post-confluent 3T3-L1 pre-adipocytes were differentiated in medium containing MDI (1 μM dexamethasone [DEX], 1 μg/ml insulin, and 0.5 mM isobutylmethylxanthine [IBMX]) with or without 25 mM SP (B, D), or SP at the indicated concentration (C), at day 0. Culture media were exchanged to medium containing 1 μg/ml insulin at days 3, 5, and 7. (B) Representative images of Oil Red O staining (100× magnification) are shown. (C) Isopropyl alcohol extracts after Oil Red O staining were analyzed with a spectrophotometer at 520 nm to assess the differentiation of 3T3-L1 cells. (D) Total RNA was extracted and mRNA expression of adipocyte-specific genes (aP2, adiponectin, PPARγ, GLUT4, C/EBPα, and GAPDH) were analyzed by RT-PCR. All values are presented as means ± SE (n = 3). The data shown are representative of results from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. the MDI-only group.

differentiated phenotype with increased presence of lipid droplets. Cells that were exposed to 25 mM SP exhibited stimulation of lipid droplet accumulation (Fig. 1B and C). The optical density of the eluted Oil Red O solution was significantly increased in 3T3-L1 pre-adipocytes treated with 5 and 25 mM SP (Fig. 1C). Our results suggest that SP strongly promotes the differentiation of 3T3-L1 pre-adipocytes into adipocytes. Adipogenesis is accompanied by altered expression of various transcription factors and the expression of adipocyte-specific genes [Spiegelman, 1998]. Therefore, we examined the mRNA expression levels of fatty acid binding protein (aP2), adiponectin, PPARγ, C/EBPα, and glucose transporter type 4 (GLUT4) by reverse transcriptase polymerase chain reaction. The expression levels of the aforementioned genes were increased in the presence of MDI and further increased when SP was present (Fig. 1D).

EFFECTS OF SP ON PROLIFERATION AND EFFECTS OF GLUCOSE ON THE ADIPOGENESIS OF 3T3-L1 CELLS

Proliferation of pre-adipocytes is associated with adipocyte differentiation. We cultured pre-confluent 3T3-L1 fibroblasts in the presence of SP for 24, 48, and 72 h, and then determined the number of cells in culture. We observed that proliferation of 3T3-L1 cells was not markedly altered by SP (Fig. 2A).

Given that PA is the final product of glycolysis from glucose, we investigated the effects of glucose on adipogenesis. 3T3-L1 cells were incubated in MDI-containing media supplemented with 25 mM SP or glucose. Morphological examination after 7 days revealed that SP stimulated adipogenesis, while glucose failed to stimulate adipogenesis. Oil Red-O staining indicated that lipid accumulation was promoted in 3T3-L1 cells in the presence of SP. However, extracellular glucose did not significantly affect MDI-induced lipid accumulation (Fig. 2B). This finding indicates that although glucose and pyruvate were sufficient energy sources for adipogenesis, SP specifically promoted lipid accumulation.

EFFECTS OF 2-DEOXYGLUCOSE (2-DG) AND 3-BROMOPYRUVIC ACID (3-BrPA) ON ADIPOGENESIS

Glycolysis can be inhibited by 2-DG and 3-BrPA, via their actions on hexokinase at the rate-limiting step of glycolysis [Hulleman et al., 2009]. Differentiation of 3T3-L1 pre-adipocytes was induced by MDI in the presence or absence of 2-DG, 3-BrPA, or SP. After 7 days, adipogenic differentiation was assessed using Oil red O staining. We found that 25 mM 2-DG inhibited adipogenesis, except when SP was present (Fig. 3). Use of 25 μM 3-BrPA resulted in the inhibition of adipogenesis in all cases (Fig. 3).

SP STIMULATES EARLY PHASE OF ADIPOGENESIS IN THE PRESENCE OF DIFFERENTIATION COCKTAIL

To understand the molecular basis of SP-stimulated adipogenesis, we investigated key phases of adipogenesis. We classified the adipogenic process as having early (day 1), intermediate (day 3), and late (day 5) phases (Fig. 4A). We differentiated 3T3-L1 cells with MDI, with or without 25 mM SP, at the various designated phases of adipogenesis. After 7 days, cultures were stained with Oil red O staining and assessed. Treatment with SP at the early phase stimulated greater levels of lipid accumulation than those in control cultures. Treatment with SP at the intermediate or late phases did not result in any significant increase in lipid accumulation (Fig. 4A). Our results suggest that the stimulatory effects of SP on adipogenesis only occur during the early phase of adipogenesis.

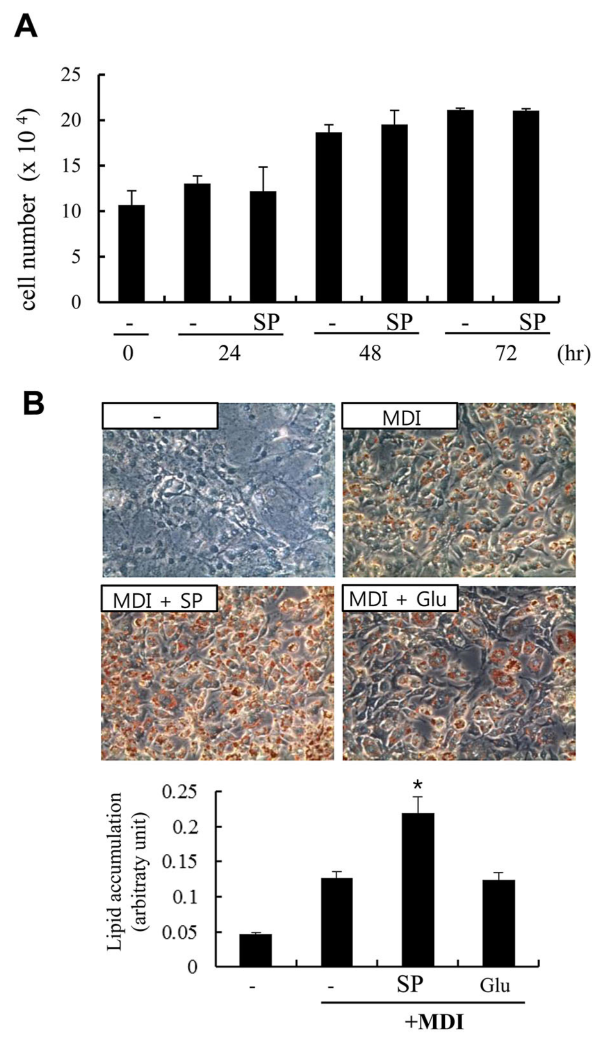

Fig. 2. Effects of SP on 3T3-L1 pre-adipocyte proliferation, and effects of glucose on pre-adipocyte differentiation. (A) We seeded 3T3-L1 cells in 48-well plates (1×10^5 cells/well) in the absence or presence of 25 mM SP. Cell proliferation was monitored at 24, 48, and 72 h. (B) The 3T3-L1 pre-adipocytes were differentiated in medium containing MDI, with or without 25 mM SP or 25 mM glucose for 7 days. Representative images of Oil Red O staining (100× magnification) are shown. Isopropyl alcohol extracts of stained cells were analyzed with a spectrophotometer. Values are presented as the means ± SE (n = 3). Data shown are representative of results from three independent experiments. $^*P < 0.005$ vs. the MDI-only group.

We examined the effects of SP on adipogenesis in the presence of various combinations of isobutylmethylxanthine (IBMX), dexamethasone (DEX) and insulin. We exposed 3T3-L1 cells to MDI in which different components were systemically omitted in the

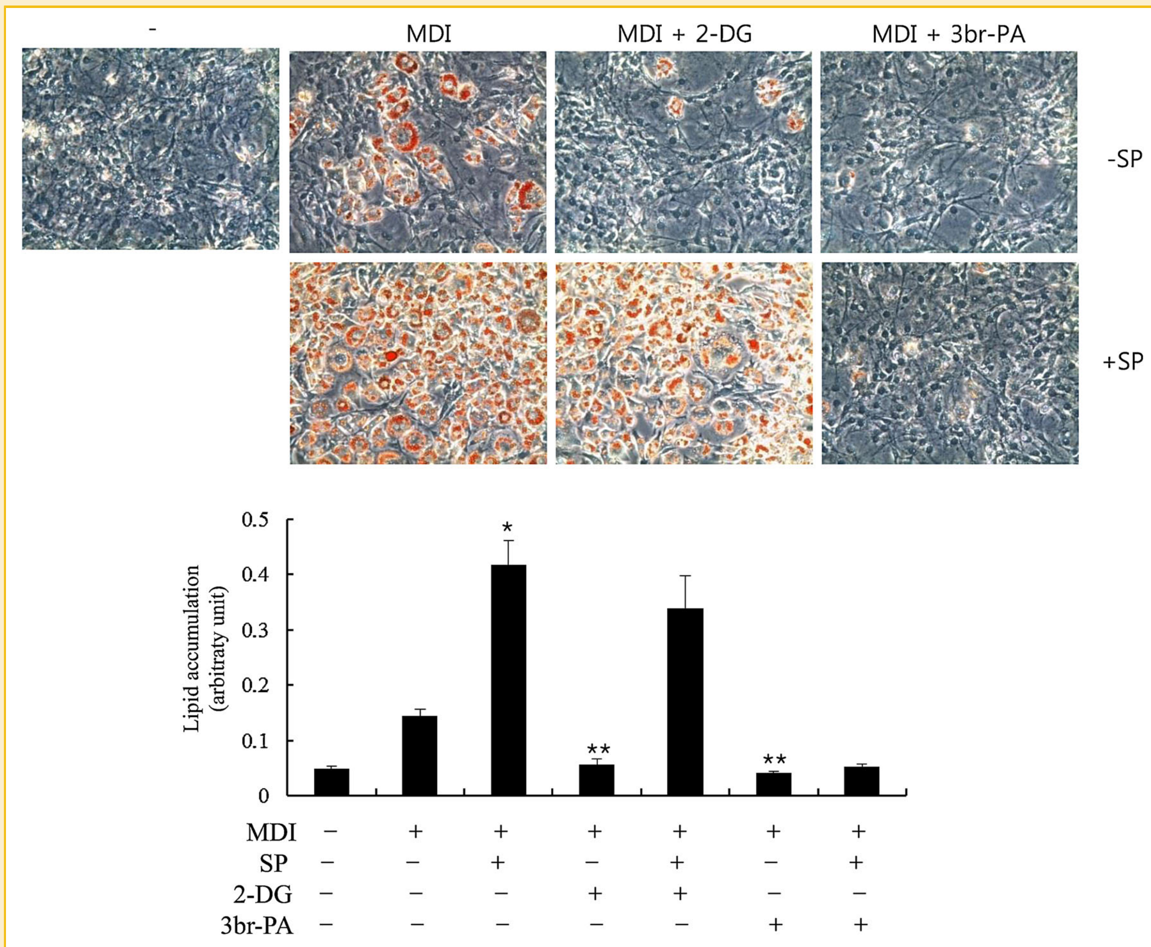

Fig. 3. Effects of 2-deoxyglucose (2-DG) or 3-bromo-pyruvic acid (3br-PA) on the adipogenesis of 3T3-L1 cells. 3T3-L1 cells were differentiated in medium containing MDI, with or without SP. In some cultures, 25 mM 2-DG or 25 μM 3br-PA was added to differentiation media. At day 7, lipid accumulation was determined by Oil Red O staining (100× magnification), with representative images shown. Isopropyl alcohol extracts of stained cells were analyzed by spectrophotometry. Values are presented as means ± SE (n = 3). Data shown are representative of results from three independent experiments. $^{*}P < 0.001$, significantly increased in comparison with the MDI-only group; $^{**}P < 0.05$, significantly decreased in comparison with the MDI-only group.

presence or absence of SP. Adipogenesis was assessed by Oil red O staining. Cultures deprived of insulin differentiated at a slower rate than those where insulin was included (Fig. 4B). We observed minimal evidence of differentiation in cultures deprived of DEX or IBMX. SP stimulated adipogenesis in the presence of insulin, IBMX, and DEX, however if one of these components was missing, adipogenesis was not enhanced (Fig. 4B).

SP UPREGULATES THE INSULIN-STIMULATED Akt SIGNALING PATHWAY IN 3T3-L1 CELLS AND GLUCOSE UPTAKE IN DIFFERENTIATED 3T3-L1 ADIPOCYTES

The immediate induction of the insulin signaling pathway is one of the key events during the early phase of adipogenesis. We investigated the effects of SP on the ERK1/2 and Akt signaling pathways, as they are both associated with initiating adipogenesis. Insulin induced phosphorylation of ERK1/2 and Akt at 15 min and 60 min (Fig. 5A and B). The presence of SP (25 mM) inhibited phosphorylation of ERK1/2 at 15 min and to a greater extent at 60 min. However, SP stimulated insulin-activated Akt phosph orylation at 15 and 60 min (Fig. 5A and B).

Fully differentiated 3T3-L1 adipocytes were stimulated with insulin in the presence or absence of SP for 2 h, and then glucose uptake was assessed. Our results show that basal glucose uptake was not influenced by SP, but insulin-stimulated glucose uptake was significantly increased by SP (Fig. 5C).

SP UPREGULATES mRNA EXPRESSION OF ADIPOGENESIS-ASSOCIATED GENES

The transcription factors C/EBPβ and C/EBPδ are the first to be expressed following exposure of pre-adipocytes to differentiation medium, and were therefore thought to be involved in directing adipogenesis. After the treatment of 3T3-L1 pre-adipocytes with

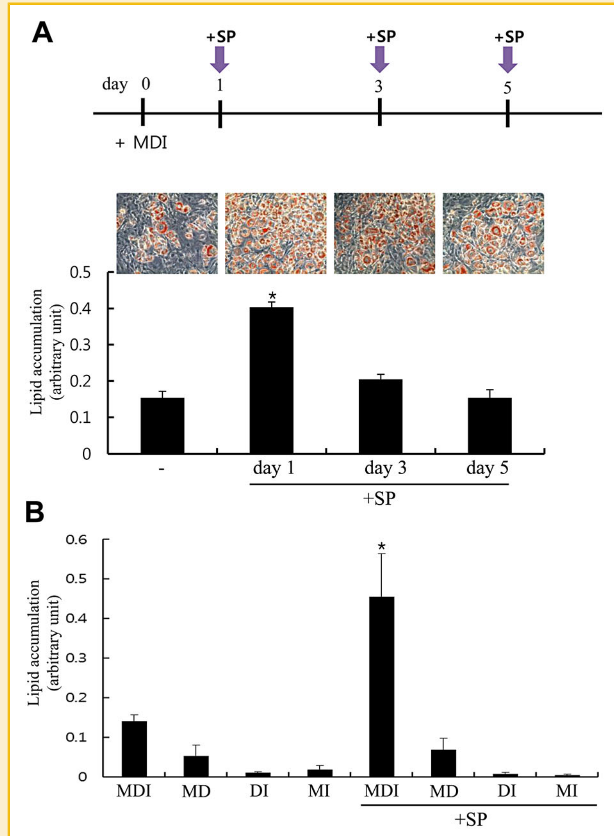

Fig. 4. Effects of SP at different stages of adipogenesis. (A) Post-confluent 3T3-L1 cells were differentiated with MDI, with exogenous SP (25 mM) added at day 1, 3, or 5. Fully differentiated 3T3-L1 cells at day 7 were stained with Oil Red O (100× magnification). Representative images are shown. Isopropyl alcohol extracts of stained cells were analyzed using a spectrophotometer. (B) Post-confluent 3T3-L1 cells were incubated in media containing combinations of differentiation agents, with or without SP. Isopropyl alcohol extracts of Oil Red O-stained cells were analyzed and compared. Values are presented as the means ± SE (n = 3). Data shown are representative of results from three independent experiments. $^{*}P < 0.005$ vs. the MDI-only group.

MDI, a rapid increase in the expression of genes encoding C/EBPβ and C/EBPδ at 3 h was observed, with C/EBPβ expression sustained for a further 21 h (Fig. 6A). The presence of SP resulted in increased expression levels of C/EBPβ and C/EBPδ at 3 h after induction with MDI (Fig. 6A). We also examined mRNA expression levels of C/EBPβ and C/EBPδ at 3 h after MDI induction using quantitative PCR. The addition of MDI resulted in upregulated expression of C/EBPβ and C/EBPδ, with SP further upregulating the expression of C/EBPβ and C/EBPδ (Fig. 6B).

SP UPREGULATES PPARγ ACTIVITY

Studies have shown that C/EBPβ can stimulate adipogenesis in noncommitted fibroblasts by activating expression of PPARγ. In order to assess the effect of SP in the regulation of PPARγ activity, the effect of GW9662, a strong PPARγ inhibitor on the adipogenesis and adipogenic gene expression were examined. 3T3-L1 cells were differentiated with MDI in the presence or absence of SP and/or GW9662. GW9662 inhibited MDI-induced adipogeneiss in the presence or absence of SP (Fig. 7A). GW9662 inhibited SP-mediated increase of aP2, C/EBPα, PPARγ, GLUT4, and adiponectin mRNA expression (Fig. 7B).

DISCUSSION

Adipocytes play a vital role in lipid homeostasis and energy balance by regulating TG storage and free fatty acid release. Adipocyte differentiation is regulated via complex interactions of external and internal events. Although terminal adipocyte differentiation has been studied in great detail, in particular the sequential action of different transcription factors culminating in the expression of adipocyte-specific genes [Farmer, 2006; Hansen and Kristiansen, 2006], studies investigating the early phase of adipogenesis are relatively rare.

Pyruvate plays a versatile role in energy production, biosynthesis, and redox balance in the adipocyte, but its effects on adipocyte differentiation have not been examined. SP was shown to specifically perturb pyruvate metabolism and affect adipocyte TG accumulation [Si et al., 2009]. In that study, SP was added to 3T3-L1 cell cultures over an extended period following induction at day 4, which was when lipid droplets were apparent and lipid accumulation was stimulated. Those researchers focused on the effects of SP at the later stages of adipogenesis and on lipid accumulation, rather than the effect of SP on cellular proliferation or early phase differentiation events. In a different study, the addition of SP or glucose as extracellular energy sources during adipogenesis increased terminal differentiation to mature adipocytes [Temple et al., 2007]. Glucose and pyruvate exert similar effects upon the expression of adipocyte marker genes, which subsequently results in the stimulation of terminal adipogenesis and lipid accumulation. However, in our current study, we found that the addition of exogenous SP enhanced adipogenesis by stimulating early signaling events of adipogenesis. Furthermore, SP enhanced the expression of adipogenic-related genes and lipid accumulation, while glucose did not significantly enhance adipogenesis. During the early phase of adipogenesis, through mechanisms that are yet to be elucidated, SP increases lipid accumulation levels and upregulates the expression of genes related to adipogenesis.

Glucose was required at low concentrations (5 mM) in the differentiation media as it is essential for the differentiation of 3T3-L1 cells (data not shown), however additional extracellular glucose (25 mM) failed to enhance differentiation further. This finding indicates a strong correlation between the initiation of lipogenic activity and pyruvate-associated reaction fluxes in glycolysis, the pentose phosphate pathway, or the malate cycle. The potential involvement of glycolysis in SP-mediated adipogenesis was investigated using 2-DG and 3-BrPA, specific inhibitors of glycolysis. It is known that 2-DG is a competitive

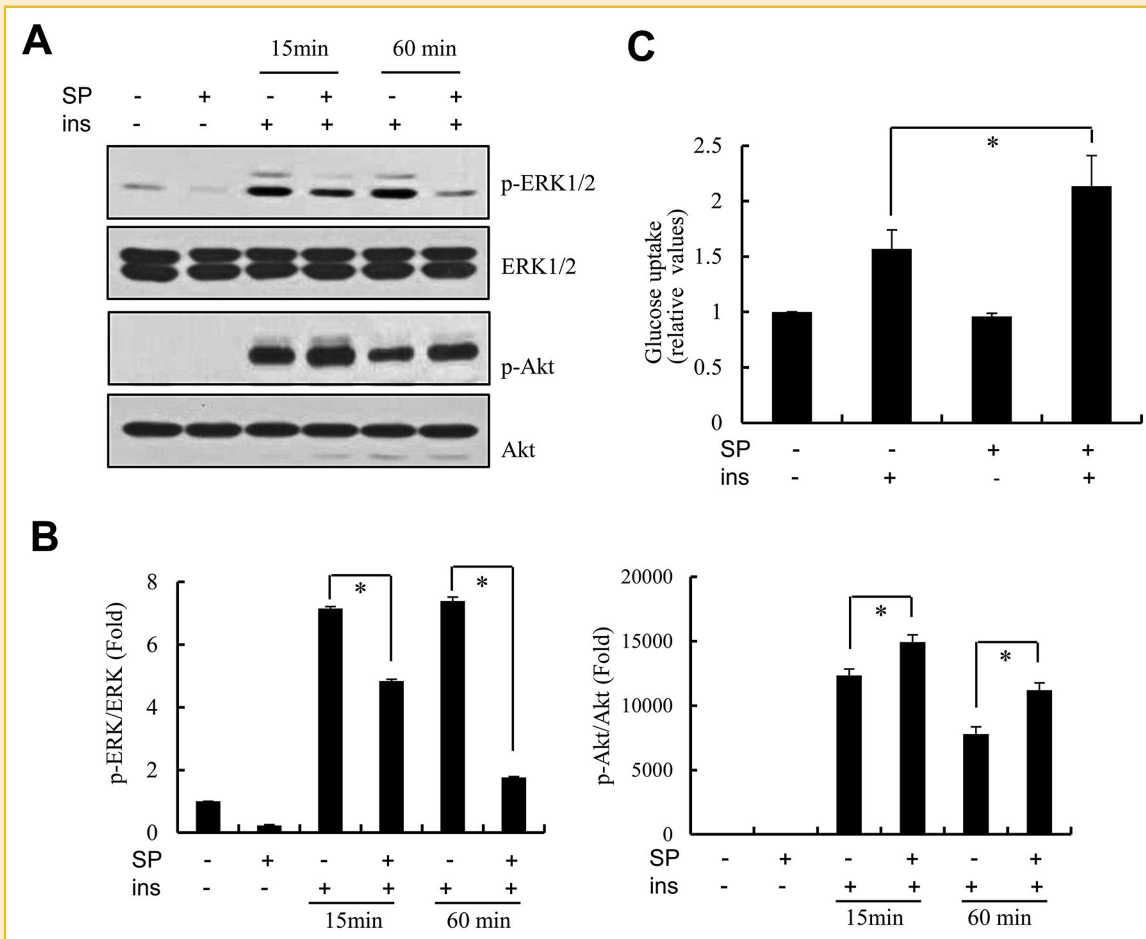

Fig. 5. Effects of SP on insulin signaling and glucose uptake in 3T3-L1 cells. (A,B) Confluent 3T3-L1 pre-adipocytes were incubated with 25 mM SP for 24 h and stimulated with 1 μg/ml insulin for 15 or 60 min. Insulin signaling was determined by western blotting (A) and densitometric quantification (C) using antibodies against phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated Akt (p-Akt). As controls, levels of ERK1/2 and Akt were measured. (C) Confluent 3T3-L1 pre-adipocytes were treated with 25 mM SP for 24 h, and glucose uptake was determined in the absence or presence of 100 nM insulin for 15 min. Values are presented as the means ± SE (n = 3). The western blotting data are representative of results from three independent experiments. $^{*}P < 0.05$.

inhibitor of hexokinase, while 3-BrPA is a direct inhibitor of hexokinase. Therefore, treatment with 2-DG and 3-BrPA completely inhibits glycolysis [Hulleman et al., 2009]. Our results demonstrated that 3-BrPA inhibited differentiation induced by MDI in the presence or absence of SP. However, 2-DG inhibited MDI-induced differentiation in the absence of SP, but was ineffective when SP was present. This result indicates that the enhancing effects of SP upon adipogenesis are not directly associated with increased flux due to glycolysis. It has been proposed that GAPDH is a selective preferred target of 3-BrPA, and that GAPDH increases the intracellular level of reactive oxygen species (ROS), resulting in endoplasmic reticulum (ER) stress. Our results show that the ER stress inducers thapsigargin and tunicamycin moderately inhibited 3T3-L1 adipogenesis (data not shown). In addition, mRNA expression levels of GAPDH were not significantly altered by SP before and after differentiation of 3T3-L1 cells (Fig. 1D). The mechanism by which 3-BrPA inhibits the effects of SP upon adipogenesis remains to be elucidated.

SP was found to upregulate insulin signaling and glucose uptake, suggesting that SP exerts insulin-sensitizing effects. The adipogenic actions of insulin are mediated by the insulin signaling pathway, and the binding of insulin to the insulin receptor (IR) at the cell surface. This event activates the β-subunit of the IR and several downstream signaling molecules. Two major downstream pathways are the Akt and ERK1/2 pathways [White and Kahn, 1994; Xu and Liao, 2004]. Akt is an important signal mediator in the insulin-like growth factor 1 receptor signal cascade, which is involved in the induction of adipocyte differentiation [Xu and Liao, 2004]. Ectopic expression of activated Akt induces the differentiation of 3T3-L1 pre-adipocytes into adipocytes [Kohn et al., 1996; Magun et al., 1996]. In contrast, the role of ERK1/2 signaling in adipogenesis

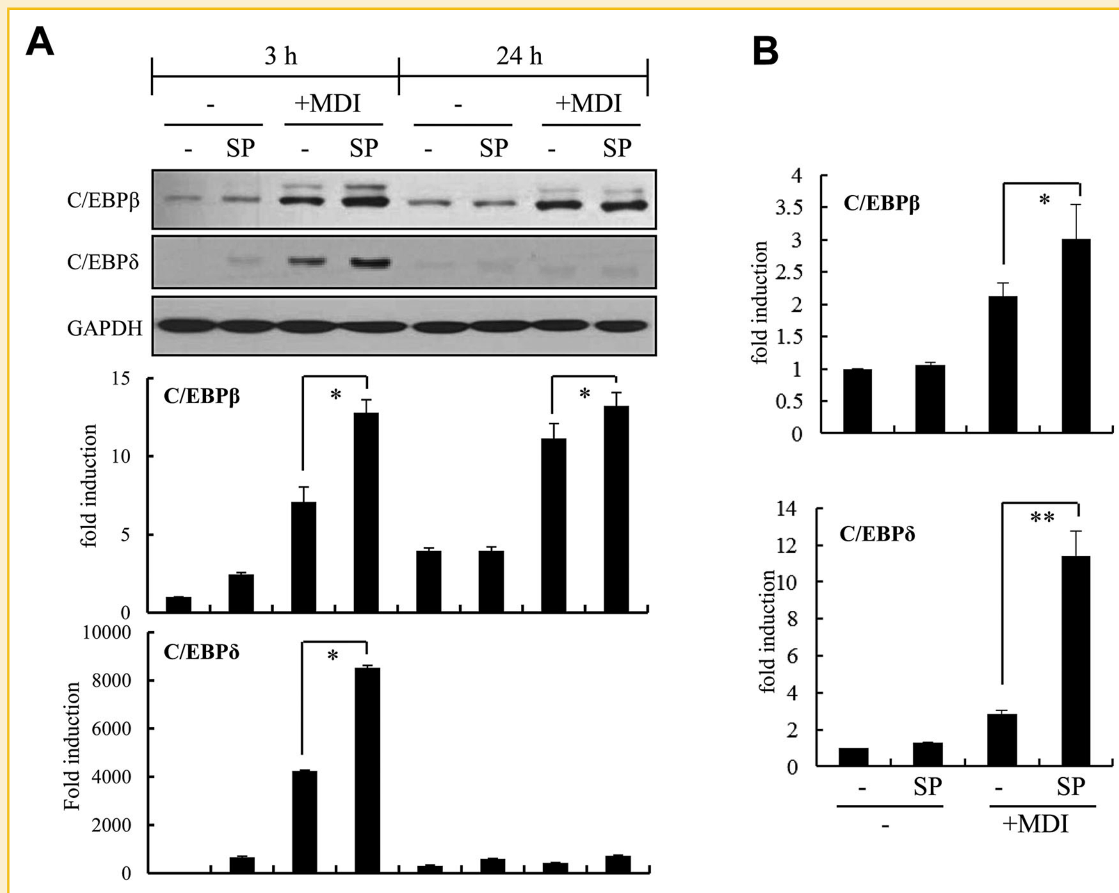

Fig. 6. Effects of SP on C/EBPβ and C/EBPδ expression in 3T3-L1 cells. (A) Post-confluent 3T3-L1 pre-adipocytes were incubated with MDI in the absence or presence of 25 mM SP for 3 or 24 h. Total cell lysate was prepared and analyzed by Western blotting using C/EBPβ and C/EBPδ antibody. Representative Western blot analysis (upper panels) and quantification (lower graphs) of expression was shown. Expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls. (B) Post-confluent 3T3-L1 pre-adipocytes were incubated with MDI in the absence or presence of 25 mM SP for 3 h. The mRNA levels of C/EBPβ and C/EBPδ were determined by quantitative PCR, with GAPDH used as the internal control. Values presented are the mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.001$.

has yet to be clarified. Some researchers have asserted that activation of mitogen-activated protein kinase (MAPK) by various effectors blocks adipogenesis [Font de Mora et al., 1997]. It has also been claimed that MAPK promotes adipocytedifferentiation [Zhang et al., 1996; Prusty et al., 2002; Xu and Liao, 2004]. In our study, SP inhibited insulin-stimulated ERK1/2 phosphorylation, but stimulated insulin-activated Akt phosphorylation in 3T3-L1 cells, thereby promoting adipogenesis.

The expression levels of two important immediate-early genes for adipogenesis C/EBPβ and C/EBPδ were increased in the presence of SP. This would suggest that SP stimulates early events of adipogenesis, rather than sustained lipid accumulation. Production of C/EBPβ and C/EBPδ then stimulates the expression of several other transcription factors, including PPARγ and C/EBPα, and genes strongly associated with adipogenesis, such as aP2, adiponectin, and GLUT4. Inhibition of PPARγ by GW9662 inhibited SP-induced stimulation of differentiation, indicating that SP enhanced the normal adipogenic program as opposed to stimulating additional pathways.

In conclusion, the inclusion of SP in differentiation media results in increased expression levels of all genes examined that are normally increased during 3T3-L1 differentiation, in conjunction with an increased level of lipid accumulation. SP promotes early events of adipogenesis in 3T3-L1 cells by upregulating the expression of C/EBPβ and C/EBPδ. SP also augmented insulin signaling and insulin-mediated glucose uptake. Although the mechanism(s) responsible for the insulin-sensitizing effects of SP remain to be elucidated, an exogenous increase in pyruvate might be effective in treating type 2 diabetes, as it leads to increased adipogenesis. Furthermore, our results suggest a potential strategy for the intervention of adiposity at the cellular metabolism level.

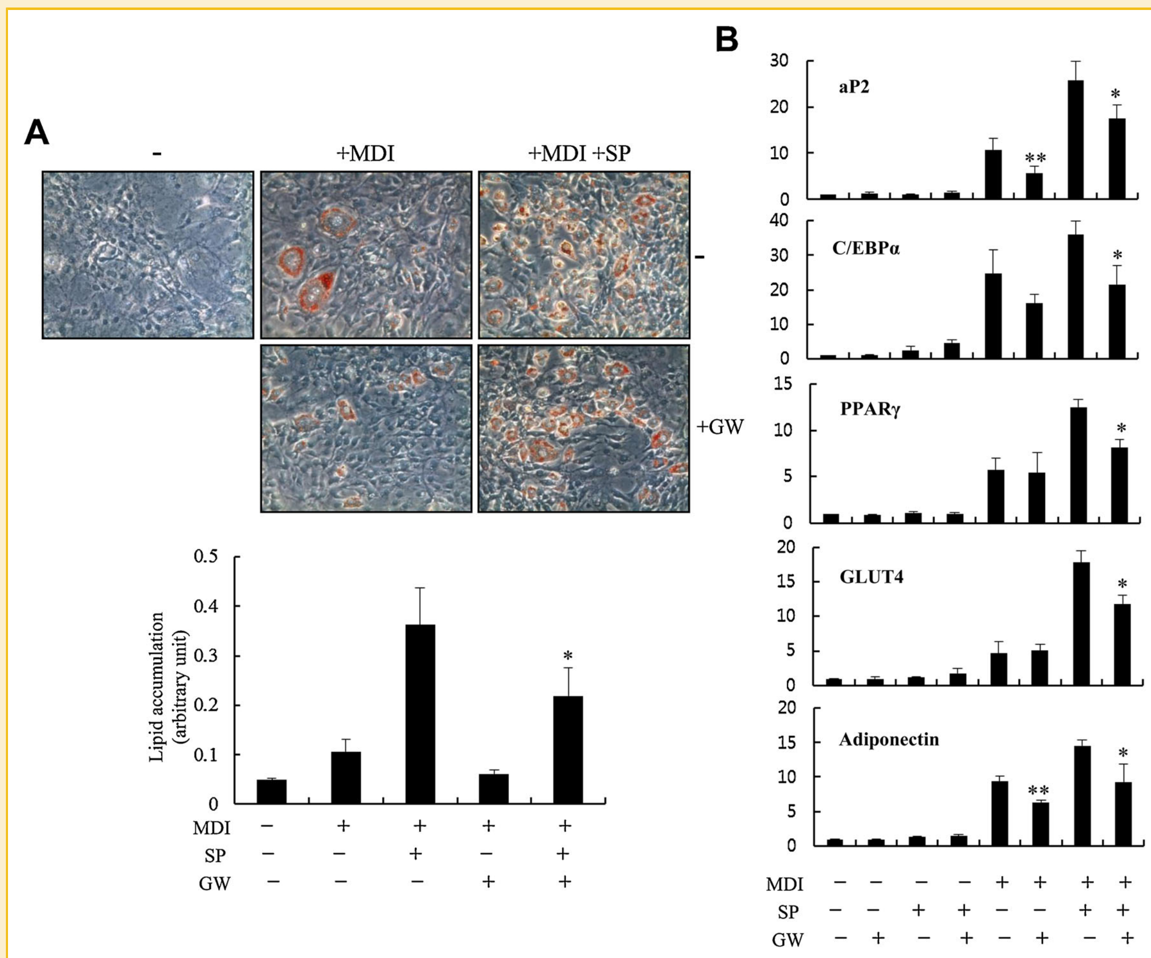

Fig. 7. Effects of GW9662 on SP-stimulated adipogenesis. Post-confluent 3T3-L1 pre-adipocytes were differentiated by MDI, with or without 25 mM SP for 3 days. In some cultures 10 μM GW9662 (GW) was added. After 3 days, cells were placed into medium containing 1 μg/ml insulin and incubated for a further 4 days. (A) Lipid accumulation was determined by Oil Red O staining (top panel), with representative images shown (100× magnification). Isopropyl alcohol extracts of Oil Red O-stained cells were analyzed (bottom panel). (B) Total RNA was subjected to quantitative PCR to determine mRNA levels of aP2, C/EBPα, PPARγ, GLUT4, and adiponectin, with GAPDH used as an internal control. Values presented are the mean ± SE. $^{*}P < 0.05$.

ACKNOWLEDGMENTS

This study was supported by a grant of National Research Federation of Korea (NRF-2013R1A1A2058244) and Inha University Research Grant. There is no potential conflict of interest in this paper.

REFERENCES

Ballard FJ,Hanson RW. 1967. Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. Biochem J 104:866–871.

Brun RP, Spiegelman BM. 1997. PPAR gamma and the molecular control of adipogenesis. J Endocrinol 155:217–218.

Chung SS, Ahn BY, Kim M, Choi HH, Park HS, Kang S, Park SG, Kim YB, Cho YM, Lee HK, Chung CH, Park KS. 2010. Control of adipogenesis by the SUMO-specific protease SENP2. Mol Cell Biol 30:2135–2146.

Farmer SR. 2006. Transcriptional control of adipocyte formation. Cell Metab 4:263–273.

Font de Mora J, Porras A, Ahn N, Santos E. 1997. Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. Mol Cell Biol 17:6068–6075.

Freytag SO, Utter MF. 1980. Induction of pyruvate carboxylase apoenzyme and holoenzyme in 3T3-L1 cells during differentiation. Proc Natl Acad Sci USA 77:1321–1325.

Gregoire FM, Smas CM, Sul HS. 1998. Understanding adipocyte differentiation. Physiol Rev 78:783–809.

Hansen JB, Kristiansen K. 2006. Regulatory circuits controlling white versus brown adipocyte differentiation. Biochem J 398:153–168.

Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJ, Evans WE, Pieters R, Den Boer ML. 2009. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood 113:2014–2021.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372–31378.

Magun R, Burgering BM, Coffer PJ, Pardasani D, Lin Y, Chabot J, Sorisky A. 1996. Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology 137:3590–3593.

Mongan PD, Capacchione J, Fontana JL, West S, Bunger R. 2001. Pyruvate improves cerebral metabolism during hemorrhagic shock. Am J Physiol Heart Circ Physiol 281:H854–H864.

Otto TC, Lane MD. 2005. Adipose development: From stem cell to adipocyte. Crit Rev Biochem Mol Biol 40:229–242.

Pan R, Rong Z, She Y, Cao Y, Chang LW, Lee WH. 2012. Sodium pyruvate reduces hypoxic-ischemic injury to neonatal rat brain. Pediatr Res 72: 479–489.

Prusty D, Park BH, Davis KE, Farmer SR. 2002. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. J Biol Chem 277:462 26–46232.

Rangwala SM, Lazar MA. 2004. Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. Trends Pharmacol Sci 25:331–336.

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

Si Y, Shi H, Lee K. 2009. Impact of perturbed pyruvate metabolism on adipocyte triglyceride accumulation. Metab Eng 11:382–390.

Spiegelman BM. 1998. PPAR-gamma: Adipogenic regulator and thiazolidinedione receptor. Diabetes 47:507–514.

Tafuri SR. 1996. Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3T3-L1 adipocytes. Endocrinology 137:4706–4712.

Tanaka T, Yoshida N, Kishimoto T, Akira S. 1997. Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. EMBO J 16:7432–7443.

Temple KA, Basko X, Allison MB, Brady MJ. 2007. Uncoupling of 3T3-L1 gene expression from lipid accumulation during adipogenesis. FEBS Lett 581:469–474.

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR, Darlington GJ. 1995. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269:1108–1112.

White MF, Kahn CR. 1994. The insulin signaling system. J Biol Chem 269:1–4.

White UA, Stephens JM. 2010. Transcriptional factors that promote formation of white adipose tissue. Mol Cell Endocrinol 318:10–14.

Xu J, Liao K. 2004. Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. J Biol Chem 279:35914–35922.

Yang C, Harrison C, Jin ES, Chuang DT, Sherry AD, Malloy CR, Merritt ME, DeBerardinis RJ. 2014. Simultaneous steady-state and dynamic 13C NMR can differentiate alternative routes of pyruvate metabolism in living cancer cells. J Biol Chem 289:6212–6224.

Zhang B, Berger J, Zhou G, Elbrecht A, Biswas S, White-Carrington S, Szalkowski D, Moller DE. 1996. Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor gamma. J Biol Chem 271:31771–31774.