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## [5-(3-Indol-1-ylpropoxy)-1H-indol-3-yl] Acetic Acid Enhances Adipocyte Differentiation and Glucose Uptake in 3T3-L1 Cells

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**Abstract:** The nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) plays an important role in adipocyte differentiation and is the target for anti-diabetic drugs known as thiazolidinediones. Here, we synthesized and characterized a new PPAR $\gamma$  agonist, SPA0432. COS-7 cells treated with SPA0432 showed significantly increased PPAR $\gamma$  transcriptional activity compared to that of vehicle-treated cells. However, its efficacy was less than that of rosiglitazone. Using a standard differentiation protocol, SPA0432 effectively enhanced differentiation of 3T3-L1 preadipocytes as evidenced by increased lipid droplet formation and triglyceride accumulation. Real-time RT-PCR analysis indicated that SPA0432 significantly increased the expressions of adipogenesis-related genes, CAAT/enhancer binding protein  $\alpha$ , PPAR $\gamma$ , fatty acid synthase, aP2 and lipoprotein lipase and significantly decreased the expression of Pref-1, a preadipocyte marker. Moreover, SPA0432 increased insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes. These results suggest that SPA0432 may exert beneficial effects against insulin resistance through its ability to promote adipocyte differentiation and insulin-stimulated glucose uptake.

**Key words:** PPAR $\gamma$ , adipogenesis, glucose uptake, insulin, adipocyte, rosiglitazone

### INTRODUCTION

Adipocyte differentiation is a complex process regulated by various transcription factors such as CAAT/enhancer binding proteins (C/EBP) and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Evans *et al.*, 2004). PPAR $\gamma$  is predominantly expressed in adipose tissue and is an essential regulator of adipocyte differentiation (Rosen *et al.*, 2002). PPAR $\gamma$  promotes the expression of a set of genes involved in maturation of adipocytes such as fatty acid synthase (FAS), lipoprotein lipase (LPL), sterol response element binding protein (SREBP) and adipocyte-specific lipid binding protein (aP2) (Rosen and Spiegelman, 2000). In addition to its regulatory role in adipogenesis, PPAR $\gamma$  activation modulates the expressions of genes associated with insulin signaling, glucose and lipid metabolism and endocrine function in adipocytes (Rosen and Spiegelman, 2000; Picard and Auwerx, 2002). This regulatory effect is

induced by a ligand that binds to and activates the receptor. Well-known natural ligands of PPAR $\gamma$  are fatty acids and eicosanoids (Bocher *et al.*, 2002). Thiazolidinedione derivatives such as pioglitazone and rosiglitazone are synthetic ligands for PPAR $\gamma$  and are widely employed in clinical practice for the treatment of type 2 diabetes (Ferre, 2004). The insulin-sensitizing mechanism of thiazolidinediones is at least in part related to the fact that they promote adipocyte differentiation in adipose tissue. These differentiated adipocytes are more sensitive to insulin and thereby trap fatty acids in adipose tissue, increase the release of adiponectin and stimulate glucose uptake (Fu *et al.*, 2005; Wang *et al.*, 2008; Takahashi *et al.*, 2009). However, adipogenic activity results in unwanted side effects such as fluid retention, hepatotoxicity and weight gain (Lee, 2003; Guan *et al.*, 2005). Therefore, the development of safer PPAR $\gamma$  agonists is necessary for more successful anti-diabetic therapy.

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In an effort to identify new PPAR $\gamma$  agonists, we replaced the critical pharmacophore thiazolidinedione moiety with indolylacetic acid. A series of indolylacetic acids were synthesized and evaluated with respect to their functional PPAR $\gamma$  activities. Among them, SPA0432 revealed promising PPAR $\gamma$  activity and was selected for further biological investigation. The present study was designed to elucidate whether SPA0432 can modulate adipogenesis and insulin signaling.

## MATERIALS AND METHODS

**Chemicals:** SPA0432 (Fig. 1a) was synthesized during the study period June 2009 to December 2010 when we built a chemical library of indole analogs toward PPAR $\gamma$  agonists. *N*-hydroxypropylation of indole gave 3-indol-1-ylpropan-1-ol which was coupled with (5-hydroxy-1*H*-indol-3-yl) acetic acid methyl ester under Mitsunobu conditions to obtain [5-(3-indol-1-ylpropoxy)-1*H*-indol-3-yl] acetic acid methyl ester. Basic hydrolysis of the ester provided [5-(3-indol-1-ylpropoxy)-1*H*-indol-3-yl] acetic acid (SPA0432).

The chemical structures and purities of the prepared compounds were assigned by IR and NMR spectra: 3-indol-1-ylpropan-1-ol-IR (neat,  $\text{cm}^{-1}$ ) 3366, 2940, 2879, 1511, 1464, 1315, 1057, 1015, 742;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.63 (1H, m), 7.37 (1H, dd,  $J = 8.0, 0.8$  Hz), 7.20 (1H, m), 7.11 (1H, m), 7.09 (1H, d,  $J = 8.0, 0.8$  Hz), 4.26 (2H, t,  $J = 6.8$  Hz), 3.57 (2H, t,  $J = 6.0$  Hz), 2.04 (2H, m), 1.45 (OH, bs);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  135.9, 128.5, 127.9, 121.4, 120.9, 119.2, 109.3, 101.1, 59.5, 42.6, 32.6; [5-(3-indol-1-ylpropoxy)-1*H*-indol-3-yl] acetic acid methyl ester-IR (neat,  $\text{cm}^{-1}$ ) 3408, 2950, 1731, 1486, 1463, 1205, 1062, 1015, 743;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.99 (NH, bs), 7.62 (1H, d,  $J = 8.4$  Hz), 7.39 (1H, dd,  $J = 8.4, 0.8$  Hz), 7.24 (1H, dd,  $J = 8.4, 0.8$  Hz), 7.16-7.20 (1H, m), 7.07-7.13 (3H, m), 7.01 (1H, d,  $J = 2.4$  Hz), 6.88 (1H, dd,  $J = 8.4, 2.4$  Hz), 6.46 (1H, dd,  $J = 3.2, 0.8$  Hz), 4.40 (2H, t,  $J = 6.8$  Hz), 3.94 (2H, t,  $J = 5.6$  Hz), 3.70 (2H, d,  $J = 0.8$  Hz), 3.67 (3H, s), 2.29 (2H, m);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.7, 153.4, 136.1, 131.6, 128.8, 128.5, 127.9, 124.1, 121.7, 121.2, 119.5, 113.1, 112.2, 109.6, 108.4, 102.2, 101.3, 65.3, 52.2, 43.1, 31.4, 30.2; [5-(3-indol-1-ylpropoxy)-1*H*-indol-3-yl] acetic acid (SPA0432) - IR (neat,  $\text{cm}^{-1}$ ) 3408, 2925, 1718, 1463, 1313, 1212, 1064, 743;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.97 (NH, bs), 7.62 (1H, d,  $J = 8.0$  Hz), 7.39 (1H, dd,  $J = 8.8, 0.8$  Hz), 7.25 (2H, d,  $J = 8.8$  Hz), 7.15-7.20 (2H, m), 7.03-7.09 (2H, m), 7.00 (1H, d,  $J = 2.4$  Hz), 6.89 (1H, dd,  $J = 8.8, 2.4$  Hz), 6.46 (1H, dd,  $J = 3.2, 0.8$  Hz), 4.39 (2H, t,  $J = 6.4$  Hz), 3.94 (2H, t,  $J = 6.0$  Hz), 3.74 (2H, s), 2.28 (2H, m).

**Cell culture:** 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

Table 1: Sequences and accession numbers for primers (forward, FOR; reverse, REV) used in real-time RT-PCR

Gene	Sequences for primers	Accession No.
PPAR $\gamma$	FOR: GAAAGACAACGGACAAATCACC REV: GGGGGTGATATGTTTGAACCTG	NM_011146
C/EBP $\alpha$	FOR: TTGTTTGGCTTTATCTCGGC REV: CCAAGAAGTCGGTGGACAAG	NM_007678
aP2	FOR: AGCCTTTCTCACCTGGAAGA REV: TTGTGGCAAAGCCCACTC	NM_024406
FAS	FOR: TGATGTGGAACACAGCAAGG REV: GGCTGTGGTGACTCTTAGTGATAA	NM_007988
LPL	FOR: GGACGGTAACGGGAATGTATGA REV: TGACATTGGAGTCAGGTTCTCT	NM_008509
Pref-1	FOR: GCTGGGACGGGAAATCTG REV: TCCAGGTCCACGCAAGTTC	NM_010052
GAPDH	FOR: CGTCCCCTAGACAAAATGGT REV: TTGATGGCAACAATCTCCAC	NM_008084

10% FBS, 100  $\mu\text{g mL}^{-1}$  streptomycin and 100 U  $\text{mL}^{-1}$  penicillin in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air at 37°C. For 3T3-L1 differentiation experiments, confluent cells were treated with differentiation medium (MDI: DMEM, 10% FBS, 1  $\mu\text{M}$  dexamethasone, 10  $\mu\text{g mL}^{-1}$  insulin and 0.5 mM isobutylmethylxanthine) for two days. The medium was then replaced with standard medium containing 10  $\mu\text{g mL}^{-1}$  insulin and was changed every other day for the subsequent four days.

**Oil red O staining:** Cells were washed twice with PBS and fixed with 10% formaldehyde for 1 h at room temperature. After washing once with 60% isopropanol, cells were stained with a filtered Oil red O/60% isopropanol solution for 1 h at room temperature. Adipocytes that stained red were observed using light microscopy. The cellular triglyceride (TG) content was assayed using a TG assay kit (Sigma, St. Louis, MO).

**Quantitative real-time PCR:** Total RNA was extracted from 3T3-L1 cells using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was precipitated with isopropanol and was dissolved in DEPC-treated distilled water. Total RNA (2  $\mu\text{g}$ ) was treated with RNase-free DNase (Invitrogen) and first-strand cDNA was generated using random hexamer primers provided in the first-strand cDNA synthesis kit according the manufacturer's protocol (Applied Biosystems, Foster City, CA). Specific primers for each gene (Table 1) were designed using Primer Express software (Applied Biosystems). The real-time RT-PCR reaction mixture consisted of 10 ng reverse-transcribed total RNA, 167 nM forward and reverse primers and 2 $\times$ PCR master mixture in a final volume of 10  $\mu\text{L}$ . The PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All experiments were performed in triplicate.

**Transfection and luciferase assay:** COS-7 cells were grown to 70-80% confluence in 12-well cell culture dishes. After 24 h, the cells were transfected with 1  $\mu$ g of PPRE-luciferase (tk-PPRE  $\times$ 3-luciferase, Addgene, Cambridge, MA) using the *Trans* IT-LT1 reagent (Mirus, Madison, WI) as described by Kim *et al.* (2007). The PPRE-luciferase construct contains three copies of the PPRE (peroxisomal proliferators response element) found in the thymidine kinase (tk) promoter upstream of the luciferase reporter gene. Twenty-four h after transfection, cells were cultured for another 48 h in a medium containing 10  $\mu$ M SPA0432 or 10  $\mu$ M rosiglitazone. Cells were harvested and lysed in Reporter Lysis Buffer (Promega, Madison, WI) and cell lysates were assayed for firefly and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was reported as relative light units and was normalized to renilla luciferase activity for each condition. Transfection efficiency was determined by cotransfection with the pRL null vector using the same protocol.

**Glucose uptake assay:** Fully differentiated 3T3-L1 cells were starved for 10 h and incubated without or with 10  $\mu$ M SPA0432 for 24 h. Cells were then washed with HEPES buffered saline (140 mM NaCl, 5 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 20 mM HEPES, pH 7.4) and incubated for 30 min in the same buffer in the presence or absence of 100 nM insulin. Glucose uptake was initiated by addition of [<sup>3</sup>H]-labeled 2-deoxy-D-glucose at a final concentration of 2  $\mu$ M for 10 min and the reaction was terminated by separating cells from the mixture. After washing three times in ice-cold PBS, cells were extracted with 0.1% SDS and subjected to scintillation counting.

**Statistical analysis:** Statistical analysis of the data was performed using ANOVA and Duncan's tests. Differences of  $p < 0.05$  were considered statistically significant.

## RESULTS AND DISCUSSION

**SPA0432 acted as a PPAR $\gamma$  agonist:** The chemical structure of SPA0432 ([5-(3-indol-1-ylpropoxy)-1*H*-indol-3-yl] acetic acid) is presented in Fig. 1a. The functional potency of SPA0432 as a PPAR $\gamma$  agonist was evaluated using a reporter gene assay. At a 10  $\mu$ M concentration, SPA0432 and rosiglitazone (RGZ) significantly increased PPAR $\gamma$  transcriptional activity by 7.9- and 14.7-fold of control levels, respectively (Fig. 1b). These results indicate that SPA0432 is a PPAR $\gamma$  agonist.

Surflex docking was performed to understand the protein-ligand interaction (data not shown). SPA0432 was docked to the binding site of PPAR $\gamma$  and the binding

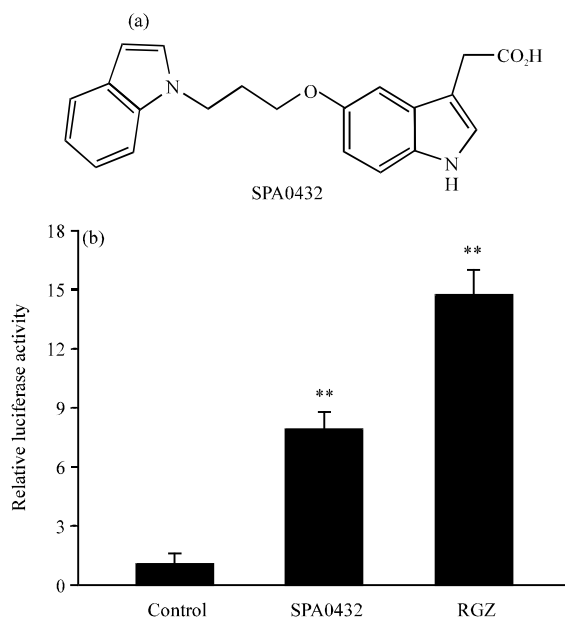


Fig. 1(a-b): (a) Chemical structure of SPA0432 and (b) transcriptional activation of PPAR $\gamma$ . COS-7 cells were transiently transfected with PPRE-containing reporter plasmid and then treated with 10  $\mu$ M concentrations of either rosiglitazone (RGZ) or SPA0432 for 48 h. Luciferase activity was determined after cell lysis and was expressed as fold-increase in activation relative to that of the control (vehicle-treated) cells. Each value is expressed as the Mean  $\pm$  SEM of five independent experiments. \*\* $p < 0.01$  vs. control

mode was compared with that of rosiglitazone. SPA0432 was found to have similar lipophilic and hydrophilic networkings to those of rosiglitazone. Hydrogen bondings of the carboxylic acid head with Tyr473, His449 and His323 were observed. This polar networking is almost the same as that seen in thiazolidinedione derivatives and is thought to stabilize the conformations of the acidic head group and of the participating amino acids (Nolte *et al.*, 1998).

**SPA0432 enhanced adipocyte differentiation in 3T3-L1 cells:** Since PPAR $\gamma$  plays a key role in adipocyte differentiation, we determined whether SPA0432 affects adipocyte differentiation. 3T3-L1 cells were treated with an MDI mixture in the presence or absence of SPA0432 for two days. Six days after differentiation, accumulated lipid droplets were visualized by Oil red O staining. As shown in Fig. 2a, 3T3-L1 cells which were treated with SPA0432

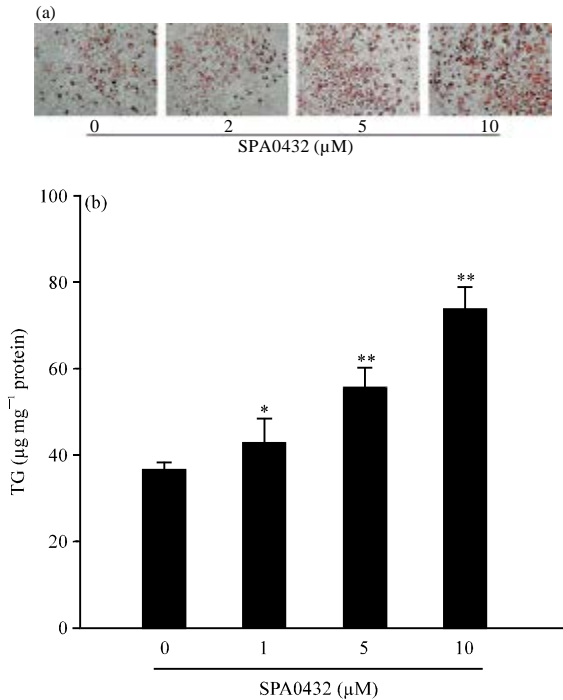


Fig. 2(a-b): Acceleration of adipocyte differentiation by SPA0432. 3T3-L1 preadipocytes were grown in MDI medium for the first two days in the presence or absence of the indicated concentrations of SPA0432. (a) Six days after initiation of differentiation, lipid droplets were visualized by Oil red O staining. (b) On day 6, cells were harvested and TG contents were measured. Each value is expressed as the Mean±SEM of three independent experiments. \*p<0.05, \*\*p<0.01 vs. control

accumulated significantly increased amounts of lipid droplets compared to those of the vehicle-treated cells. Intracellular TG content was measured on day 6. TG accumulation was facilitated with as little as 1 μM SPA0432 and increased in direct relation to the concentration of SPA0432 (Fig. 2b). These results indicate that SPA0432 stimulates differentiation of 3T3-L1 preadipocytes. It should be noted that treatment with the tested concentrations of SPA0432 alone did not affect the viability of 3T3-L1 cells (data not shown).

**SPA0432 induced the expression of adipocyte differentiation markers:** We further investigated the effects of SPA0432 on the expressions of adipocyte markers using quantitative real-time RT-PCR. We compared the expression profiles of 3T3-L1 cells treated with 10 μM SPA0432 with those of vehicle-treated cells. In

SPA0432-treated cells, PPARγ and CEBP/α were significantly elevated on day 3 after differentiation induction (Fig. 3a, b). Expressions of other adipocyte markers such as FAS, aP2 and LPL which are also PPARγ target genes, were also increased compared to that of the control cells (Fig. 3c-e). Preadipocyte factor-1 (Pref-1), a preadipocyte marker that normally disappears during adipocyte maturation (Smas and Sul, 1993; Yu *et al.*, 2009) declined significantly compared to that of control cells (Fig. 3f). These results indicate that SPA0432 induces PPARγ target gene expression in 3T3-L1 cells and suggests that SPA0432 may control the functions of adipocytes through regulation of PPARγ target gene expression.

**SPA0432 stimulated insulin-stimulated glucose uptake in 3T3-L1 adipocytes:** To confirm the effect of SPA0432 on the regulation of adipocyte function, we examined the effect of SPA0432 on insulin-stimulated glucose uptake (Fig. 4). Differentiated 3T3-L1 adipocytes were treated with 10 μM SPA0432 for 24 h, after which insulin was added for an additional 30 min. The presence of insulin led to a 2.0-fold increase in [<sup>3</sup>H]-labeled deoxyglucose uptake compared with that of the control. In addition, 10 μM SPA0432 significantly increased insulin-stimulated glucose uptake (p<0.01). These results indicate that SPA0432 sensitizes adipocytes to insulin.

PPARγ is essential for normal adipocyte differentiation (Rosen *et al.*, 2002) and plays an important role in the regulation of insulin sensitivity and glucose homeostasis (Rosen and Spiegelman, 2000; Picard and Auwerx, 2002). In the current study, SPA0432 enhanced differentiation of 3T3-L1 cells as evidenced by increased lipid droplet formation and intracellular TG content. This was accompanied by upregulated expressions of PPARγ and CEBP/α during the early stage of differentiation. Fatty acid synthase, aP2 and LPL, all of which are critical for maintenance of the adipocyte phenotype, were also upregulated in SPA0432-treated adipocytes. The adipogenesis-promoting activities of PPARγ agonists result in an increase of insulin responsive adipocytes that increase systemic insulin sensitivity (Fu *et al.*, 2005; Wang *et al.*, 2008; Takahashi *et al.*, 2009). The synthetic PPARγ agonists rosiglitazone and pioglitazone have been reported to increase adipogenesis and to improve insulin resistance in obese diabetic mice (Hallakou *et al.*, 1997). We therefore, examined whether SPA0432 would affect glucose uptake accompanied by altered adipocyte differentiation. In parallel to an increase in adipocyte differentiation, SPA0432 significantly increased insulin-stimulated glucose uptake. Taken together, this study provides evidence that SPA0432 is a PPARγ agonist and has insulin-sensitizing effects.

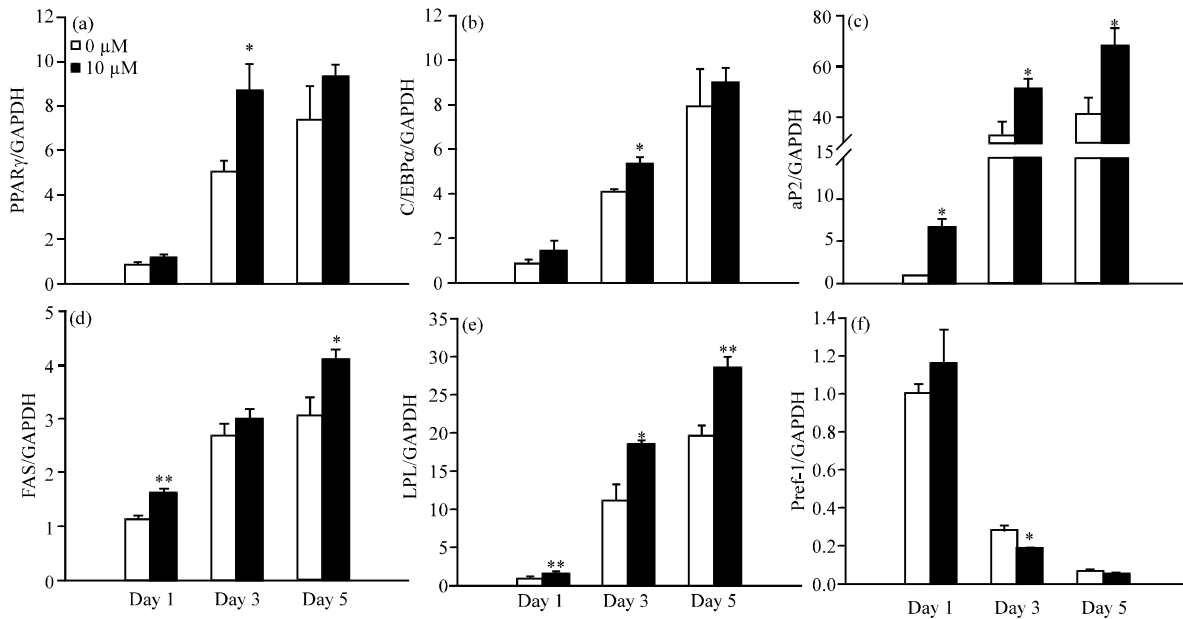


Fig. 3(a-f): Real-time RT-PCR analysis of adipocyte differentiation marker genes. Total RNA from 3T3-L1 cells incubated in the presence or absence of 10 μM SPA0432 was isolated at the indicated time points after induction of differentiation and real-time PCR analysis was performed. (a) PPARγ/GAPDH, (b) C/EBPα/GAPDH, (c) aP2/GAPDH, (d) FAS/GAPDH, (e) LPL/GAPDH and (f) Pref-1/GAPDH. Each value is expressed as the Mean±SEM of three independent experiments. \*p<0.05, \*\*p<0.01 vs. control

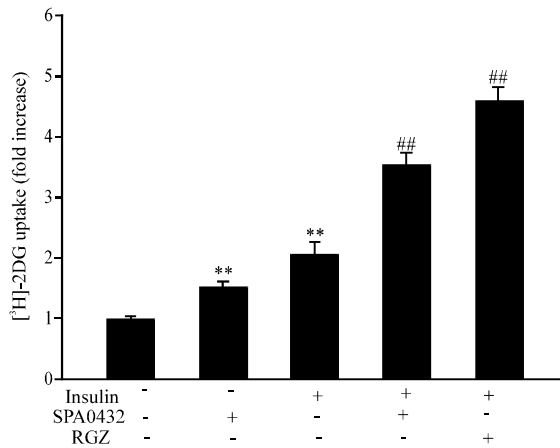


Fig. 4: Effect of SPA0432 on glucose uptake in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with 10 μM SPA0432 for 24 h. After washing, cells were treated with or without 100 nM insulin for 30 min. Uptake of [<sup>3</sup>H]-labeled 2-deoxy-D-glucose was expressed relative to that of the control. Each value is expressed as the Mean±SEM of five independent experiments. \*\*p<0.01 vs. control, ##p<0.01 vs. insulin

## CONCLUSION

SPA0432, a synthetic PPARγ agonist, enhanced adipocyte differentiation and stimulated insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Therefore, SPA0432 might be used as anti-diabetic drug.

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