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Regulation of adipocyte differentiation and metabolism by lansoprazole

Ameena Benchamana[a,c], Hiroyuki Mori[c], Ormond A. MacDougald[c], Sunhapas Soodvilai[a,b,*]

[a] Research Center of Transport Protein for Medical Innovation, Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand
[b] Excellent Center for Drug Discovery (ECDD), Faculty of Science, Mahidol University, Bangkok, Thailand
[c] University of Michigan Medical School, Department of Molecular & Integrative Physiology, Ann Arbor, MI, USA

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ABSTRACT

Aims: Lansoprazole (LPZ) is one of the most commonly prescribed drugs for treatment of acid-related diseases, and it is increasingly recognized for its potential application as an anti-diabetic therapy. Although LPZ target tissues remain poorly understood, possible sites of action include adipose tissue. In this study, we assessed effects of LPZ on adipocyte differentiation and function by using 3T3-L1 preadipocytes and HFD-induced obesity mice as an *in vitro* and *in vivo* model, respectively.

Main methods: Oil red O staining and intracellular triacylglycerol content were used to determine lipid accumulation. Glucose uptake was performed to measure mature adipocyte function. Expression of adipocyte genes was determined by qRT-PCR and immunoblotting.

Key findings: LPZ has dual effects on differentiation of 3T3-L1 cells. At low concentrations, LPZ enhanced adipocyte differentiation via induction of PPARγ and C/EBPα, two master adipogenic transcription factors, as well as lipogenic proteins, ACC1 and FASN. Increasing of adipocyte number subsequently increased basal and insulin-stimulated glucose uptake, and expression of *Glut4* mRNA. Conversely, high concentrations of LPZ strongly inhibited differentiation and expression of PPARγ and C/EBPα, and maintained expression of preadipocytes markers, β-catenin and Pref-1. Inhibition of adipogenesis by LPZ reduced mature adipocyte number, *Glut4* mRNA expression and insulin-stimulated glucose uptake. In addition, treatment with LPZ at 200 mg/kg significantly reduced body weight gain and total fat mass in HFD-induced obese mice.

Significance: These results indicate that effects of LPZ on adipocyte differentiation are dependent on concentration and are correlated with PPARγ and C/EBPα.

1. Introduction

Lansoprazole (LPZ) is an effective proton pump inhibitor (PPI) that is widely used for gastric acid-related disorders because of its ability to reduce acid secretion of parietal cells [1]. While LPZ circulates as a prodrug, upon activation by low pH in the stomach, LPZ binds covalently to gastric H+,K + -ATPase via a disulphide bond [2]. Because it circulates in an inactive form, LPZ has few side-effects, and it has become one of the most commonly prescribed drugs over the past few decades [3,4]. Apart from its acid-suppressing effects, LPZ has been reported to inhibit inflammation, oxidative stress, growth of cancer cells, and cyst growth in polycystic kidney disease [5–9]. Importantly, clinical studies and experiments in animal model have shown that PPIs including LPZ are associated with improved glycemic control, lowered HbA1c levels, and increased circulating insulin concentration [10–14]. Although LPZ is increasingly recognized for its anti-diabetic properties, the tissue targets and mechanisms of action remain poorly understood. There are myriad tissues and organs integral to regulation of whole-body glucose homeostasis including pancreas, liver, and adipose tissue [15–17]. Of these we report herein our evaluation of adipose tissue as a potential target of LPZ.

Adipose tissues play central roles in glucose and lipid homeostasis [18]. Blood glucose is lowered by adipocytes in adipose tissue, which take up excess glucose and store as triacylglycerol until needed. Differentiation of adipocytes from mesenchymal precursors, termed adipogenesis, is an important process for maintaining functional adipose tissues. Disruption of this process can aggravate the hypertrophic expansion of adipocytes, and results in increased inflammation and insulin resistance. At the cellular and molecular level, adipogenesis is a multistep process controlled by a cascade of transcription factors. Of particular importance are CCAAT/enhancer-binding protein alpha (C/EBPα), a member of the C/EBP family, and peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear hormone receptor superfamily, which serve in this context as master

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[*] Corresponding author. Department of Physiology, Faculty of Science, Mahidol University, Rama IV Road, Bangkok, 10400, Thailand.
E-mail address: sunhapas.soo@mahidol.ac.th (S. Soodvilai).

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transcriptional regulators of adipogenesis. C/EBPα and PPARγ positively feedback to regulate the expression of each other, and induce expression of genes that give rise to the mature adipocyte [19–24]. LPZ has been reported to have glucose-lowering effects, [11,13]; however, whether LPZ exerts these effects through a mechanism of action in adipocytes is unknown.

In this study, we have used a drug repositioning strategy [25,26] to determine if new therapeutic opportunities exist for LPZ through effects on adipocyte biology. Thus, we used 3T3-L1 preadipocytes and HFD-induced obesity animal as models to study underlying mechanism of LPZ on adipocyte differentiation, mature adipocyte functions and *in vivo* adipose tissue development.

2. Materials and methods

2.1. Drugs and reagents

Lansoprazole (LPZ), isobutyl-3-methylxanthine (IBMX), dexamethasone (DEX), and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and calf serum (BCS) were purchased from Gibco (Grand Island, NY, USA). Antibodies against PPARγ, C/EBPα, ACC1, FASN, α-Tubulin and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture and 3T3-L1 cell differentiation

Mouse 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were maintained in DMEM containing 4.5 g/l^{-1} glucose supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. Two days after confluence, 3T3-L1 preadipocytes were induced to differentiate by differentiated cocktail (MDI) (DMEM containing 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/ml insulin) for 48 h then cultured in growth medium containing 10 μg/ml insulin for another 48 h. Cells were maintained in growth medium without MDI for an additional six days. Cells were treated with either vehicle (DMSO) or LPZ at the concentrations and times as indicated for each experiment.

2.3. Cell viability assay

3T3-L1 preadipocytes were seeded in a 96 well plate (1 × 10^4 cells/well) and incubated at 37 °C, 5% CO_2 with complete medium for 48 h. Medium was then changed to differentiation medium in the absence or presence of various concentrations of LPZ for 48 h. After incubation, the medium was replaced with 100 μl per well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 mg ml^{-1}) under humidified atmosphere at 37 °C and further incubated for 2 h. The MTT solution was removed, and the formazan salt form was dissolved by 100 μl per well of DMSO. Cell viability was measured at an absorbance of 570 nm by a microplate reader. The data are shown as the percentage of cell viability compared with the control group.

2.4. Oil Red O lipid staining and quantification

Intracellular neutral lipid accumulation was investigated by using Oil Red O staining at day 10 of differentiation. Oil Red O was prepared as a 0.5% stock solution in isopropanol. 3T3-L1 cells were differentiated in the presence of the indicated concentrations of LPZ. After removal of medium, cells were fixed with 10% formalin for at least 1 h. Cells were then stained with working Oil Red O solution (40% water and 60% Oil Red O stock solution, left at least 20 min, filtered and then added to fixed cells) for at least 1 h. After rinsing four times with distilled water, the cells were visualized using phase-contrast microscopy and photographed. To quantify cellular lipid, stained cells were eluted with 100% isopropanol and incubated for 10 min. Absorbance was determined at 500 nm.

Table 1
Oligonucleotide primer sequence used for qRT-PCR.

Gene	Sequence 5′-3′
Pparg	F: TGCCTATGAGCACTTCACAAGA R: ATGCGAGTGGTCTTCCATCA
Cebpa	F: CCAGAGGATGGTTTCGGGTC R: GCGGAAAGTCTCTCGGTCTC
Cebpd	F: GAACCCGCGGCCTTCTAC R: TGTTGAAGAGGTCGGCGAAG
Adipoq	F: GATGGCAGAGATGGCACTCC R: GAGCGATACACATAAGCGGC
Acc1	F: ATGCGATCTATCCGTCGGTG R: AGCAGTTCTGGGAGTTTCGG
Fasn	F: GGCCCCTCTGTTAATTGGCT R: GGATCTCAGGGTTGGGGTTG
Gadph	F: ATGGTGAAGGTCGGTGTGAA R:ACTGGAACATGTAGACCATGTAGT
Srebp1c	F: CACCACTTCGGGTTTCATGC R: CAAGGCCATCGACTACATCCG
Abca1	F: AGTTTCGGTATGGCGGGTTT R: AGCATGCCAGCCCTTGTTAT
Fabp4	F: GTCACCATCCGGTCAGAGAG R: GGTCGACTTTCCATCCCACTT
Glut4	F: GCCCGGACCCTATACCCTATT R: GGGTTCCCCATCGTCAGAG
Cdk4	F: GTGGCTGAAATTGGTGTCGG R:TAACAAGGCACCTCACGAA
p21	F:ATCCAGACATTCAGAGCCACAG R:ACGAAGTCAAAGTTCCACCGT
p27	F:CAGATACGAGTGGCAGGAGG R:ACGAGTCAGGCATTTGGTCC
Cyclin D1	F: GAGCTGCTGCAAATGGAACTG R: AAAGAAAGTGCGTTGTGCGG
Cyclin E1	F: GCTTCGGGTCTGAGTTCCAA R: GGATGAAAGAGCAGGGGTCC

2.5. Intracellular triacylglycerol measurement

The 3T3-L1 preadipocytes were differentiated in the absence or presence of various concentrations of LPZ for the indicated times. Cell pellets were ruptured with 5% NP-40 and the triacylglycerol content of supernatants were assayed using a Triglyceride determination kit (Sigma-Aldrich, MO, USA) according to manufacturer's protocol. The absorbance was measured at 540 nm.

2.6. Glucose uptake

Adipocytes were serum-starved for 2 h before the assay and incubated in 1 ml of KRH buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM HEPES, pH 7.4) at 37 °C for 15 min. Adipocytes were then incubated with 2-deoxy-D-[2,6-^{3}H] glucose (^{3}H-2DG; PerkinElmer, Bangkok, Thailand) for an additional 10 min. Uptake of glucose was stopped by three rapid washes with ice-cold KRH, the cells were solubilized in 200 μl of 0.4 N NaOH for at least 4 h and neutralized with 80 μl of 1 M HCl. The radioactivity was determined by liquid scintillation counting.

2.7. Quantitative real-time PCR analysis

Total RNA from 3T3-L1 cells was isolated using RNA STAT-60 (Tel-Test Inc, Friendswood, TX, USA) reagent according to the manufacturer's protocol. The concentration of RNA was determined using Nanodrop. After treatment with DNase I (Thermo Fisher Scientific, Waltham, MA, USA), first-strand cDNA was synthesized from total RNA following the manufacturer's protocol. qPCR was perform using the MyiQ real time PCR detection system (Bio-Rad Laboratories). The relative amount of each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

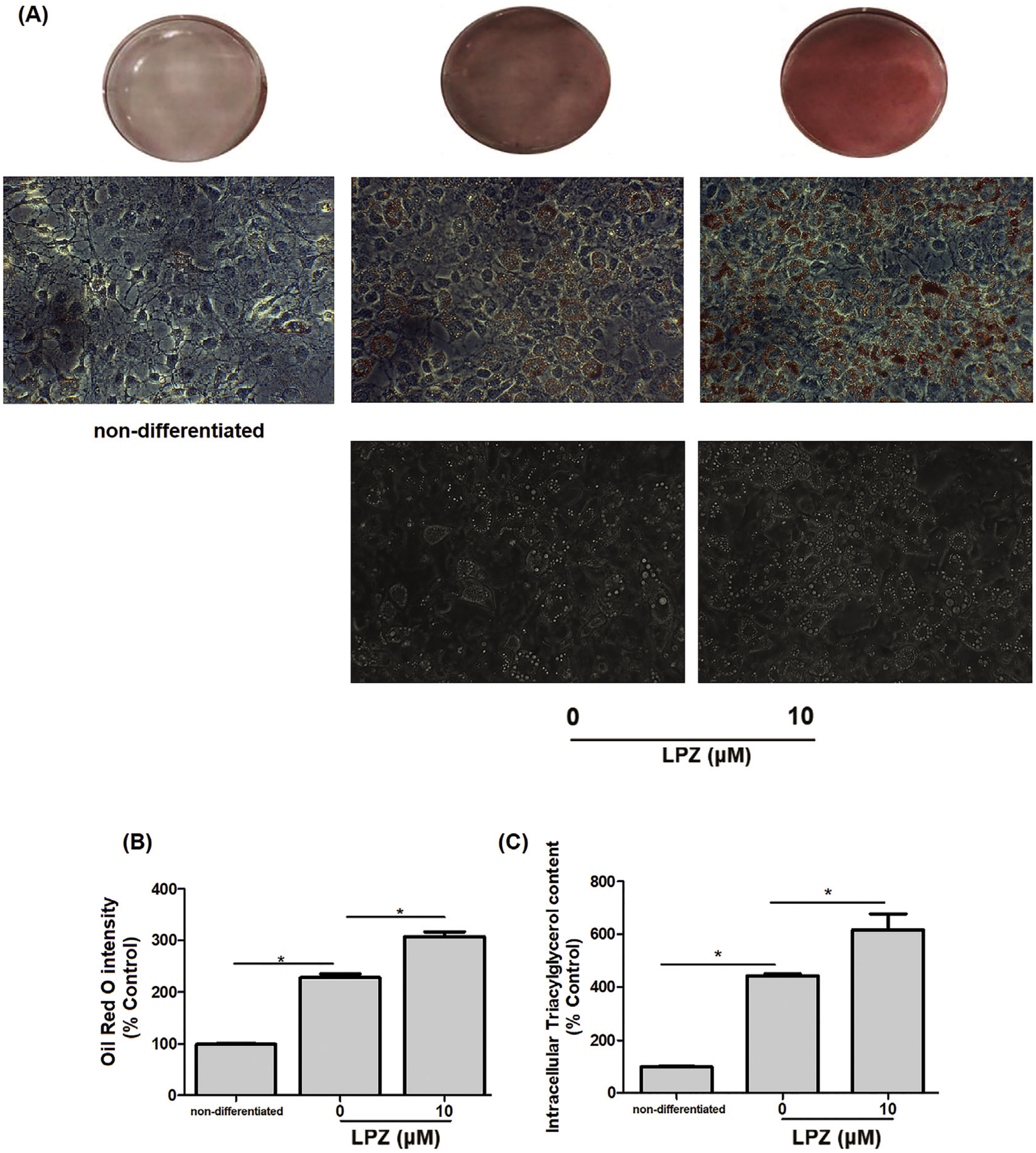

Fig. 1. LPZ stimulates lipid accumulation of 3T3-L1 adipocytes.
3T3-L1 cells were differentiated with MDI adipogenic cocktail in presence or absence of 10 μM LPZ for 10 days followed by (A) observation of differentiated adipocytes by Oil Red O staining and bright field under phase-contrast microscopy, (B) Quantification of Oil Red O staining and (C) cellular triacylglycerol content. The results are the mean ± SEM of at least three independent experiments. $^*P < 0.05$ was regarded as statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Expression of transcripts was normalized using Peptidylprolyl Isomerase A (*Ppia*) or Glyceroldehyde 3-phosphate dehydrogenase (*Gapdh*) as internal standard. Expression of mRNA in control cells was normalized to 1, and expression of gene transcripts in treated cells was presented as relative expression compared to control. The sequence used in this study were shown in Table 1.

2.8. Protein extraction and immunobloting

3T3-L1 preadipocytes were differentiated and treated at the indicated concentrations. Cells were harvested and homogenized in a modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM each of NaF, $NaVO_3$ and PMSF, and 1% of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C and then supernatants were transferred to new tubes and stored at −80 °C. Concentration of protein was measured using BCA assay (Thermo Scientific, Rockford, IL, USA) according to manufacturer's protocol. Protein was run on 4–12% Nupage™ 4–12% Bis-Tris Medi gel (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF. Membranes were blocked in 5% non-fat dried milk, and immunoblotted with primary antibodies and peroxidase-conjugated anti-rabbit IgG antibodies. Detection of secondary antibodies was by chemiluminescence. β-actin or α-tubulin were included as loading controls.

2.9. Animals and experimental protocol

Twenty nine male C57BL/6 J mice (Nomura Siam International Co., Ltd., Bangkok, Thailand) at 6 weeks of age were housed in a controlled environment of 22 ± 2 °C with 12:12 h dark-light cycle. The animals were allowed free access to diet and tap water throughout the experiment. After acclimatization for 1 week, all mice were fed a HFD (60% energy from lard). After 6 weeks of dietary manipulation to induce

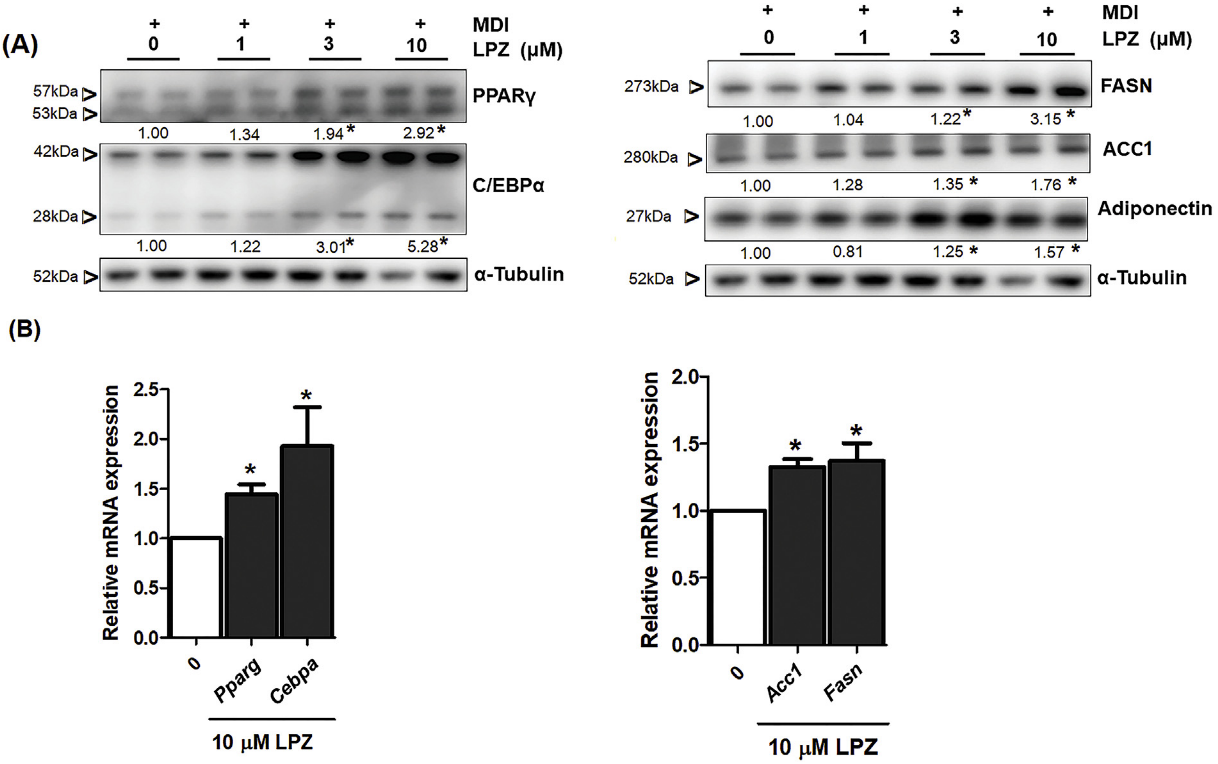

Fig. 2. LPZ up-regulates expression of adipogenic genes and proteins.
A) Confluent 3T3-L1 cells were differentiated in the indicated concentration of LPZ for 10 days. After whole cell lysis, expression of PPARγ, C/EBPα, FAS, adiponectin, and ACC1 were assessed by immunoblot analyses. α-Tubulin is included as a loading control. (B) Expression of mRNAs for *Pparg*, *Cebpa*, *Fasn* and *Acc1* were determined by qRT-PCR. Peptidylprolyl Isomerase A (*Ppia*) was used as internal control. Results are expressed as mean ± SEM of triplicate experiment. $^*P < 0.05$ was statistical significance compared with control.

obesity, the animals were divided into three groups. Mice in each group were either given LPZ (100 mg/kg ($n = 9$) or 200 mg/kg ($n = 10$)) or vehicle alone ($n = 10$) by oral gavage. Food intake, energy consumption and body weight were measured once a week. After 8 weeks of treatment, all mice were sacrificed. All studies were performed under approval of the Faculty of Science, Mahidol University Animal Care and Use Committee (SCMU-ACUC).

2.10. Statistical analysis

A minimum of three independent experiments were performed. Data were analyzed by one-way ANOVA or unpaired *t*-test using GraphPad Prism software. Data were presented as mean ± SEM. A probability level of $^*P$-value < 0.05 was regarded as statistically significant.

3. Results

3.1. Low concentration of LPZ promotes adipocyte differentiation

Incubation 3T3-L1 cells with the adipogenic cocktail induced cell differentiation with a dramatic increase in lipid droplet formation, as observed by Oil Red O staining and by phase contrast microscopy. Interestingly, treatment with 10 μM LPZ further increased staining with Oil Red O compared with control adipocytes. Visualization of cells by phase contrast microscopy suggesting LPZ increased lipid droplet formation compared with control (Fig. 1A). Mature adipocytes increased Oil Red O staining compared with non-differentiated cells, and differentiation in the presence of 10 μM LPZ further increased retention of this neutral lipid stain (Fig. 1B). In addition, intracellular triacylglycerol content was found to increase in mature adipocyte with LPZ treatment (Fig. 1C).

3.2. LPZ increases expression of adipogenic mRNAs and proteins

To evaluate mechanisms by which LPZ potentiated adipocyte differentiation, qRT-PCR and immunoblot analyses were used to estimate expression of adipogenic genes and proteins. Consistent with effects on preadipocyte differentiation, LPZ increased the expression of adipogenic transcription factors, PPARγ and C/EBPα, with significant induction observed at concentrations as low as 3 μM and the highest level of expression observed at 10 μM. LPZ significantly induced protein expression of FASN, ACC1 and adiponectin (Fig. 2A). Not surprisingly, expression of *Pparg* and *Cebpa* mRNAs were also elevated by LPZ. In addition, the level of mRNA expression of these adipogenic markers was also significantly increased by LPZ treatment compared with control adipocytes (Fig. 2B).

3.3. LPZ increases insulin-stimulated glucose uptake in 3T3-L1 adipocytes

Since LPZ potentiated adipocyte differentiation, we further determined whether differentiated cells induced by LPZ supported insulin-stimulated glucose uptake. In the absence of insulin, LPZ treatment for 10 days significantly increased glucose uptake compared with untreated cells, and level of glucose uptake further increased when co-stimulated with insulin (Fig. 3A). Addition of cytochalasin B (CytoB) impaired activity of GLUT4-mediated glucose uptake. As predicted, expression of *Glut4* mRNA, which is downstream of *Pparg* and *Cebpa*, was increased (Fig. 3B). Treatment of mature adipocytes with LPZ for 24 h, however, did not alter basal glucose uptake and insulin-stimulated glucose uptake (Fig. 3C), suggesting that effects of long-term LPZ treatment on glucose uptake are secondary to elevated adipogenesis.

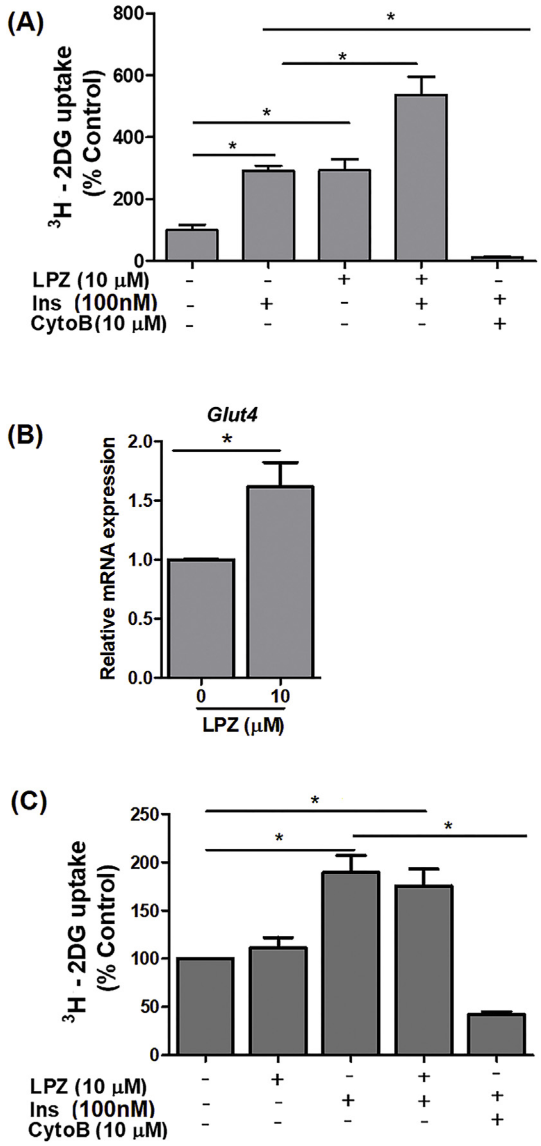

Fig. 3. LPZ increases insulin-dependent glucose uptake in 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiation with adipogenic cocktail, and cells were simultaneously treated with or without 10 µM LPZ for 10 days. Glucose uptake and *Glut4* mRNA expression were measured at day 10 (A and B). Fully-differentiated adipocytes were treated with 10 µM LPZ for 24 h prior to assay of glucose uptake (C). Results are expressed as mean ± SEM of triplicate experiments. $^*P < 0.05$ (compared with basal condition) was considered as significantly difference. Ins and Cyto B represent insulin and cytochalasin B, respectively.

3.4. Adipogenic effects of LPZ appear to be independent of Cebpd and cell cycle regulators

We next tested whether LPZ treatment for 2 days was sufficient to potentiate adipocyte differentiation compared with 10 days treatment. As shown in Figs. 4A and 2 days treatment with LPZ significantly increased Oil Red O staining compared with that of control cells. We then hypothesized that LPZ might potentiate adipocyte differentiation by acting to stimulate mitotic clonal expansion (MCE) in the early phase of adipogenesis. The MCE is regulated by a group of cell cycle regulator genes and *Cebpd* transcription factor. Therefore, we tested whether the effect of LPZ on adipocyte differentiation was via up-regulation of *Cebpd* and cell cycle regulators. The mRNA expression level of cell cycle regulator genes (*Cdk4*, *Cyclin D1*, *CyclinE1, p21*, *and p27*), and *Cebpd* were measured by qRT-PCR after 24 h treatment with LPZ. We found that LPZ treated cells did not alter the expression level of cell cycle regulator genes compared with control cells. The expression level of *Cebpd* mRNA after LPZ treatment was also not changed compared with control cells (Fig. 4B). These finding indicated that the action of LPZ at the early phase of adipogenesis did not require up-regulation of cell cycle regulators or *Cebpd* expression and may involve other signaling mechanisms.

3.5. High concentrations of LPZ suppress adipocyte differentiation

We then investigated whether LPZ at concentrations higher than 10 µM would have effects on adipocyte differentiation. 3T3-L1 cells were differentiated using the MDI adipogenic cocktail in the presence of LPZ (50 and 100 µM) for 48 h. At day 10, as showed in Fig. 5A, treating the cells with 50 µM LPZ had a slight reduction in intensity of Oil Red O staining compared with control adipocytes. However, cells differentiated in the presence of 100 µM LPZ had substantially reduced Oil Red O staining, and number of adipocytes as assessed by phase contrast microscopy (Fig. 5A). After quantification of stained Oil Red O, the reduction in retained dye for 50 and 100 µM LPZ was reduced in dose-dependent manner (Fig. 5B). These results were bolstered by quantification of intracellular triacylglycerol content, which was suppressed even further by high concentrations of LPZ (Fig. 5C). Importantly, treatment of 3T3-L1 preadipocytes with 10–100 µM LPZ for 48 h had no influence on cell viability (Fig. 5D). These results indicate that two days' exposure to 50 or 100 µM LPZ is sufficient to suppress adipocyte differentiation.

3.6. High concentrations of LPZ inhibit expression of adipogenic mRNAs and proteins in 3T3-L1 adipocytes

Since high concentrations of LPZ inhibited adipocyte differentiation, we then investigated whether LPZ inhibited adipocyte differentiation by disrupting expression of adipogenic genes and proteins. Treatment of cells with LPZ at 50 and 100 µM for 48 h significantly reduced protein expression of PPARγ, C/EBPα, Adiponectin, and ACC1. Suppression of FABP4 and FASN protein required higher concentrations of LPZ than that of PPARγ, C/EBPα, Adiponectin, and ACC1 (Fig. 6A). In agreement with protein expression results, the expression of adipogenic mRNA was dramatically reduced in 100 µM LPZ treatment condition (Fig. 6B).

3.7. High concentration of LPZ elevates preadipogenic markers in 3T3-L1 adipocyte cells

We next tested whether impaired adipogenesis was associated with increased expression of preadipocyte markers, and as expected, β-catenin and Pref-1 proteins were expressed higher in LPZ-treated cells (Fig. 6C). Consistent with the tight control of β-catenin protein degradation, the protein was elevated whereas the mRNA was not influenced by LPZ treatment. In addition, the expression of β-catenin target genes, *Axin2,* was also increased, which supports not only high expression of β-catenin, but also high activity.

3.8. High concentrations of LPZ reduced glucose uptake

Since high concentrations of LPZ inhibited adipocyte differentiation, we further determined whether decreasing in adipocyte number by LPZ reduces total glucose uptake in 3T3-L1 cells. As expected, treatment of 3T3-L1 cells with 100 µM of LPZ during differentiation (48 h) reduced basal and insulin-stimulated glucose uptake (Fig. 7). As predicted, LPZ also reduced expression of *Glut4* mRNA. These observations provide further support for the idea that high concentrations of LPZ inhibit adipocyte differentiation.

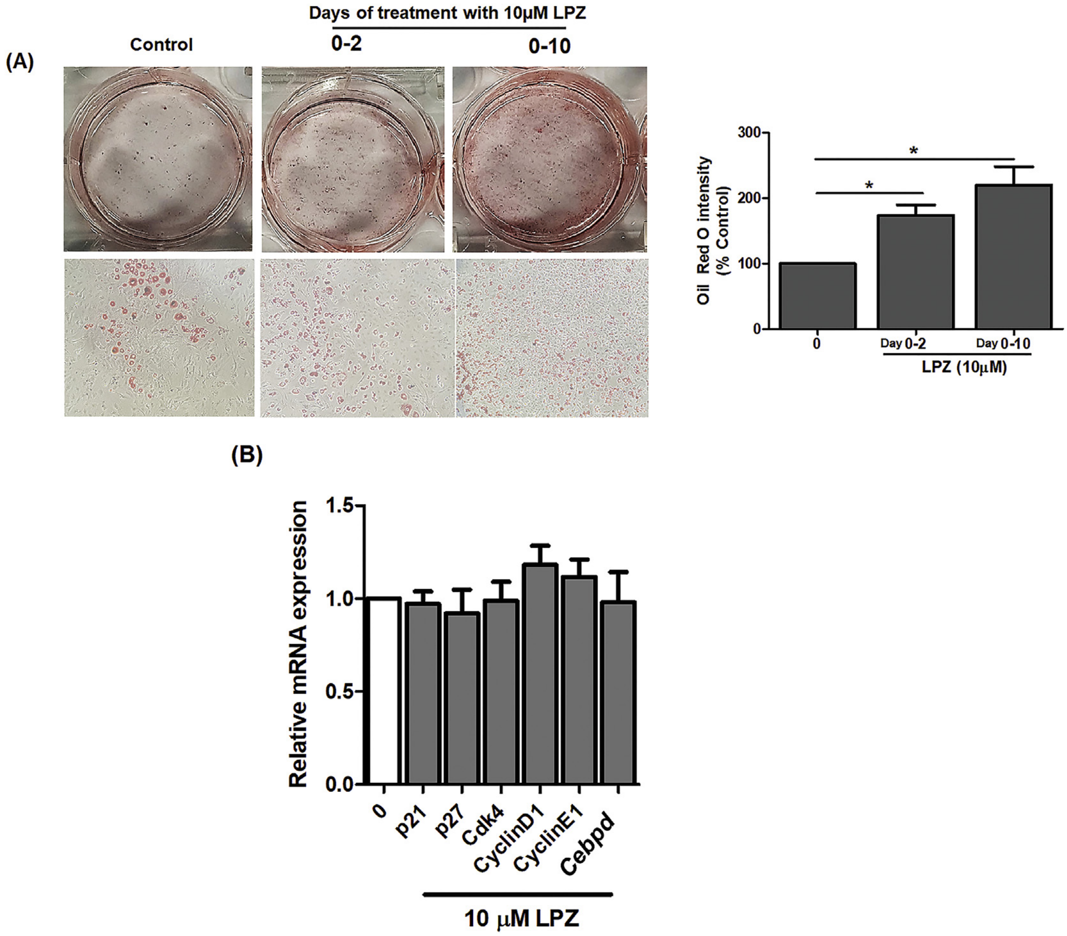

Fig. 4. Up-regulation of *Cebpd* and cell cycle regulator genes was not involved in stimulation of adipogenesis by LPZ.
Confluent 3T3-L1 cells were induced to differentiate in the presence or absence of LPZ. Oil Red O staining and quantification was at day 10 (A). The mRNA expression of *Cebpd* and cell cycle regulator genes following treatment with 10 μM of LPZ for 24 h was measured by qRT-PCR (B). *Gapdh* was used as internal control. Results are expressed as mean ± SEM of triplicate experiment. *$P < 0.05$ was considered as statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.9. *LPZ prevents body weight gain and reduces fat mass of mice with HFD-induced obesity*

Next, we investigated further whether LPZ has inhibitory effects on adipose tissue mass in HFD-induced obese mice. We found that HFD successfully induced obesity in mice with increases from initial weight of approximately 20–25 g to 30–40 g, and significantly increased body weight compared to mice on a Normal diet (ND; data not shown). Importantly, oral administration of 100–200 mg/kg/day LPZ for 8 weeks decreased HFD-induced weight gain (Fig. 8A). This reduction was not due to a reduction in energy consumption. Total body fat accumulation was slightly lower in LPZ treatment at 100 mg/kg/day. However, the reduction of total fat mass was far more significant with an LPZ treatment of 200 mg/kg/day (Fig. 8B). Interestingly, only the subcutaneous adipose depots were significantly reduced by both LPZ treatment groups. In contrast, significant changes in epididymal and visceral adipose depots, liver weight and kidneys weight were not observed (Fig. 8C). These results suggest that LPZ suppresses HFD-induced obesity through a specific reduction of subcutaneous adipose tissues.

4. Discussion

Considerable evidence indicates that LPZ has anti-glycemic effects in patients and animal models with type 2 diabetes mellitus [10–14]. However, the mechanism underlying these effects is not well understood. The present study revealed the pharmacological effect and underlying mechanism of LPZ on adipogenesis. Our results showed that treating 3T3-L1 preadipocytes with 10 μM LPZ during differentiation stimulated adipogenesis, as revealed by elevated triacylglycerol content and number of adipocytes.

Increasing of adipocyte number is predominantly mediated by stimulating the differentiation of existing preadipocytes. Among the transcription factors involved with this complex process, PPARγ and C/EBPα are major regulators [19–24,27]. As expect for an inducer of adipogenesis, LPZ increased expression of PPARγ and C/EBPα mRNA and protein. LPZ enhanced mRNA and protein expression of adipocyte-related genes such as *Fasn* and *Acc1*, which are regulated by *Pparg* and *Cebpa* during adipocyte differentiation. Interestingly, action of LPZ on adipocyte differentiation, function and insulin sensitivity is similar that of thiazolidinediones, which are drugs used to treat type 2 diabetes patients. Thus, effects on adipocyte biology may be a possible mechanism by which LPZ improves glucose homeostasis.

We observed that treatment with LPZ during the early phases of adipogenesis potentiated adipocyte differentiation, and these effects on differentiation were independent from the expression of *Cebpd* and cell cycle regulator genes, *Cdk4, Cyclin D1, CyclinE1, p21, and p27*. However, several signaling cascades such as Akt, MAPK, S6K, and many others also play role in this early phase [28,29]. Our findings cannot rule out effects of LPZ on other signaling pathways.

Another important issue is whether the concentrations of LPZ used in our *in vitro* experiments are pharmacologically relevant. The maximum plasma concentration after oral administration of a single 30 mg

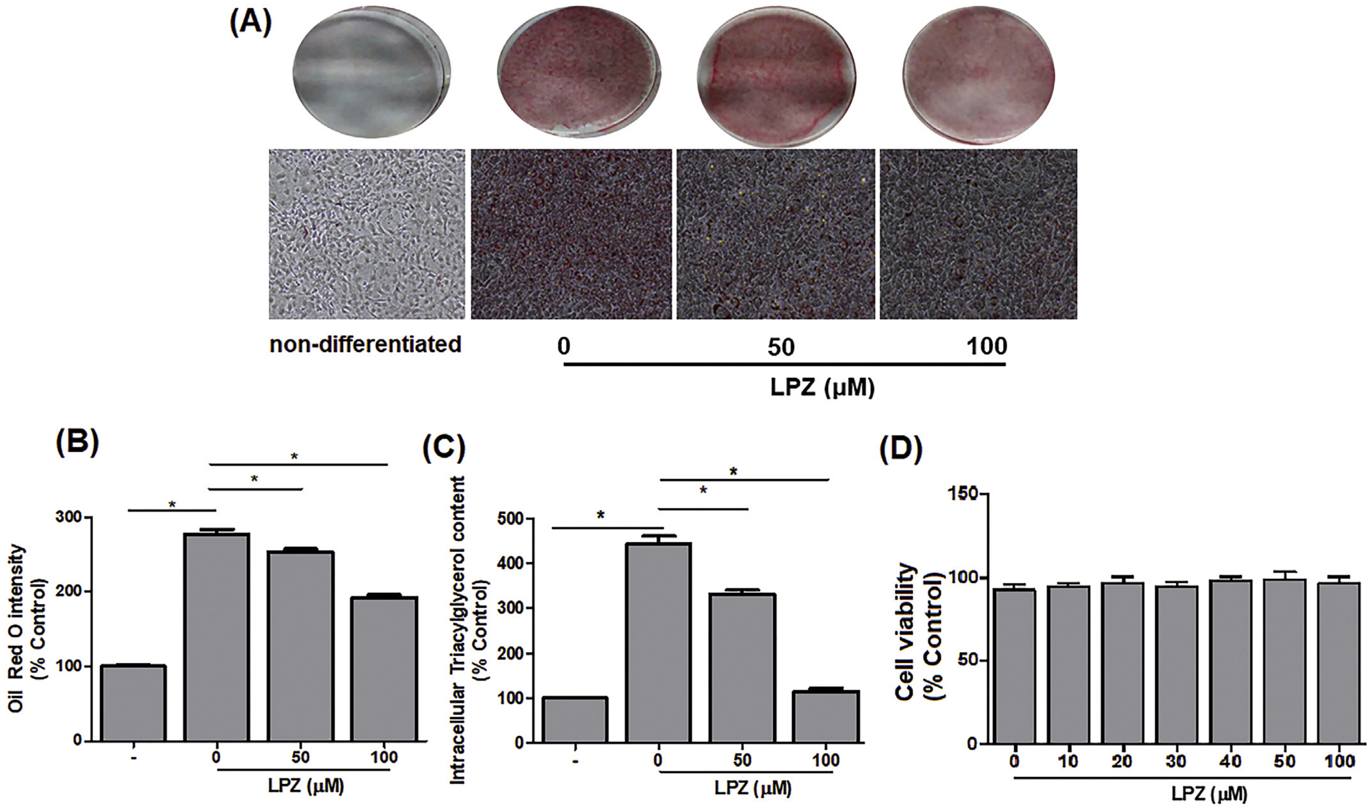

Fig. 5. High concentrations of LPZ inhibit preadipocyte differentiation.
3T3-L1 preadipocytes were stimulated with adipogenic differentiation cocktail in the absence or presence of the indicated concentrations of LPZ for 48 h The cells were further cultured in a differentiation medium for another 8 days. Cellular morphology of cells was observed using phase contrast microscope. Lipid accumulation in adipocytes was assessed by Oil Red O staining (A), quantification of retained Oil Red O dye (B), measurement of intracellular triacylglycerols (C) and measurement of cell viability (D). Data are presented as the mean ± SEM of triplicate experiments. $^{*}P < 0.05$ was regarded as statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dose is approximately 3.25 μM [3,30]. Clinical evidence indicates that patients who take pharmacological doses of LPZ have improved glycemia [10,12,13]. Our study showed that treatment with 3 μM LPZ, the pharmacological dose, was sufficient to induce adipogenesis as assessed by expression of adipogenic proteins.

One of the primary functions of adipose tissue is the uptake and storage of excess energy. Under conditions of positive energy balance, increasing adipocyte number (i.e. hyperplasia) is healthier than adipocyte hypertrophy, which ultimately creates large dysfunctional adipocytes that are associated with insulin-resistance and metabolic syndrome [17,18]. Our study on glucose uptake revealed that low concentrations of LPZ augmented both basal and insulin-mediated glucose uptake. However, treatment with LPZ for 24 h in fully-differentiated adipocytes did not show any significant difference in basal or insulin-stimulated glucose uptake. Thus, it is likely that the increase glucose uptake with LPZ treatment was secondary to an increase in adipocyte number as well as *Glut4* mRNA expression.

On the other hand, our observations revealed that high concentrations of LPZ, above the pharmacological range, inhibited adipogenesis. Although repressive effects of LPZ on adipocyte differentiation could have resulted from cell toxicity; in 3T3-L1 cells, 50 and 100 μM LPZ for 48 h had no effect on cell viability. Instead, the inhibitory effect was mediated via suppressing the expression of key transcription factors, *Pparγ* and *C/ebpα*, which then further inhibited other adipogenic related genes. In agreement with our findings, we also observed that 50 and 100 μM LPZ maintained expression of preadipocyte markers, β-catenin and Pref-1 protein. Interestingly, the mRNA of β-catenin was not altered, suggesting a posttranscriptional mechanism for effects on β-catenin by LPZ. A relationship between PPARγ and β-catenin degradation has been previously reported [31,32]. It is also possible that LPZ inhibited PPARγ activity, which resulted in cellular accumulation of β-catenin. In our study, we found that high concentrations of LPZ reduced both basal and insulin-stimulated glucose uptake, and *Glut4* mRNA expression. We also observed that insulin can further stimulate glucose uptake in adipocytes treated with high concentrations of LPZ. These results indicated that high concentrations of LPZ likely reduce glucose uptake secondary to a reduction of mature adipocyte number but without altering insulin-stimulated glucose uptake in adipocytes. Consistent with *in vitro* results, this study also presented evidence that LPZ has anti-obesity effects in HFD-fed mice. LPZ reduced weight gain and reduced total fat mass, which reflected growth of adipose tissue in obese animal. The reduction of body weight and total adipose tissue were not the result of reduced food intake, suggesting that energy expenditure may be stimulated. However, the underlying mechanisms by which LPZ exerted these effects, as well as selective loss of the posterior subcutaneous adipose will require further study.

5. Conclusion

In summary, the present study demonstrates, for the first time, the dual pharmacological effects of LPZ on 3T3-L1 preadipocyte conversion, with effects of LPZ on adipogenesis highly correlated with expression of adipogenic proteins including PPARγ, C/EBPα, Adiponectin, FASN, and ACC1. In addition to providing fundamental knowledge of how LPZ influences differentiation and function of cultured adipocytes, our current study also demonstrates that LPZ has anti-obesity effects in a HFD-induced obesity model. This study supports drug repositioning of LPZ as an alternative agent for either lowering blood glucose or obesity

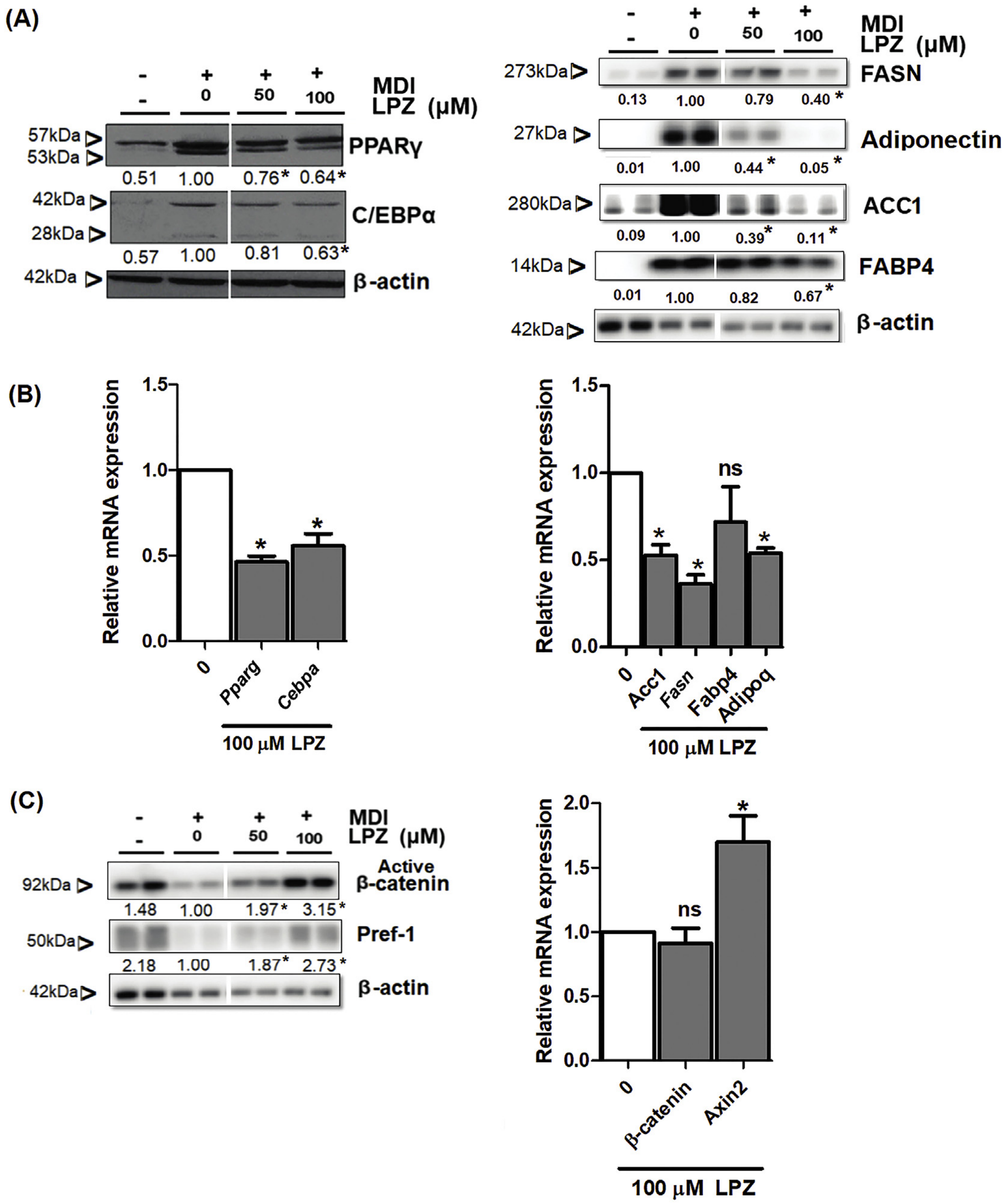

Fig. 6. High concentration of LPZ inhibits adipogenesis via suppression of adipogenic factors.

3T3-L1 preadipocytes were stimulated with the MDI differentiation cocktail in the presence or absence of the indicated concentrations of LPZ for 48 h. The cells were further cultured in a differentiation medium for another 8 days. Cells were lysed for evaluation of mRNA and protein expression. Expression of protein (A) and mRNA (B) of the adipogenic transcription factors, *Pparg, Cebpa, Fas, Fabp4, Adiponectin, and Acc1*. Expression of preadipogenic markers, active β-catenin and Pref-1 protein and mRNA expression level of β-catenin, and *Axin2* were analyzed (C). β-actin and *Ppia* were used as internal control for protein expression and mRNA expression, respectively. $^*P < 0.05$ was regarded as statistically significant compared with control.

treatment.

Author contributions

AB, SS, and OAM conceived the studies and planned the experimental design. AB performed the experiments analyzed the data. AB, SS, OAM, and HM interpreted the data. AB, SS and OAM wrote manuscript. SS edited and proved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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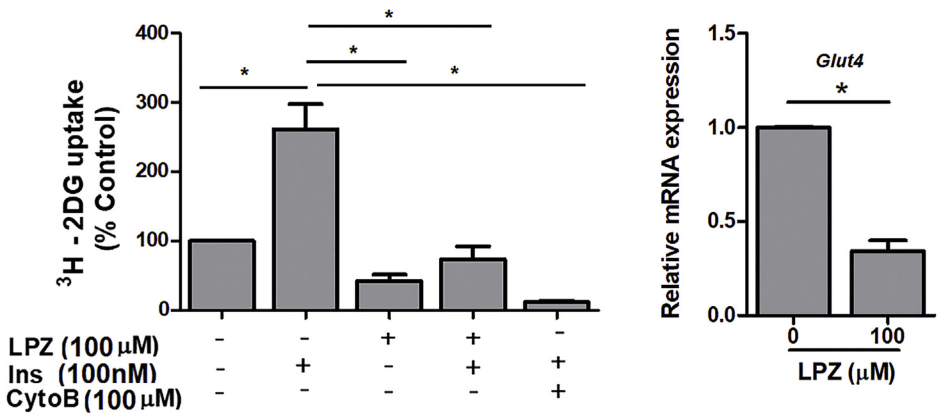

Fig. 7. Inhibition of adipogenesis by high concentration of LPZ subsequently reduced basal and insulin-stimulated glucose uptake.

3T3-L1 preadipocytes were stimulated with the MDI differentiation cocktail in the presence or absence of the indicated concentrations of LPZ for 48 h

. Basal and insulin-stimulated glucose uptake (A), and expression of *Glut4* mRNA (B) were investigated. Results are representative of at least three independent experiments. Data are represented as mean ± SEM. $^*P < 0.05$ was regarded as statistically significant.

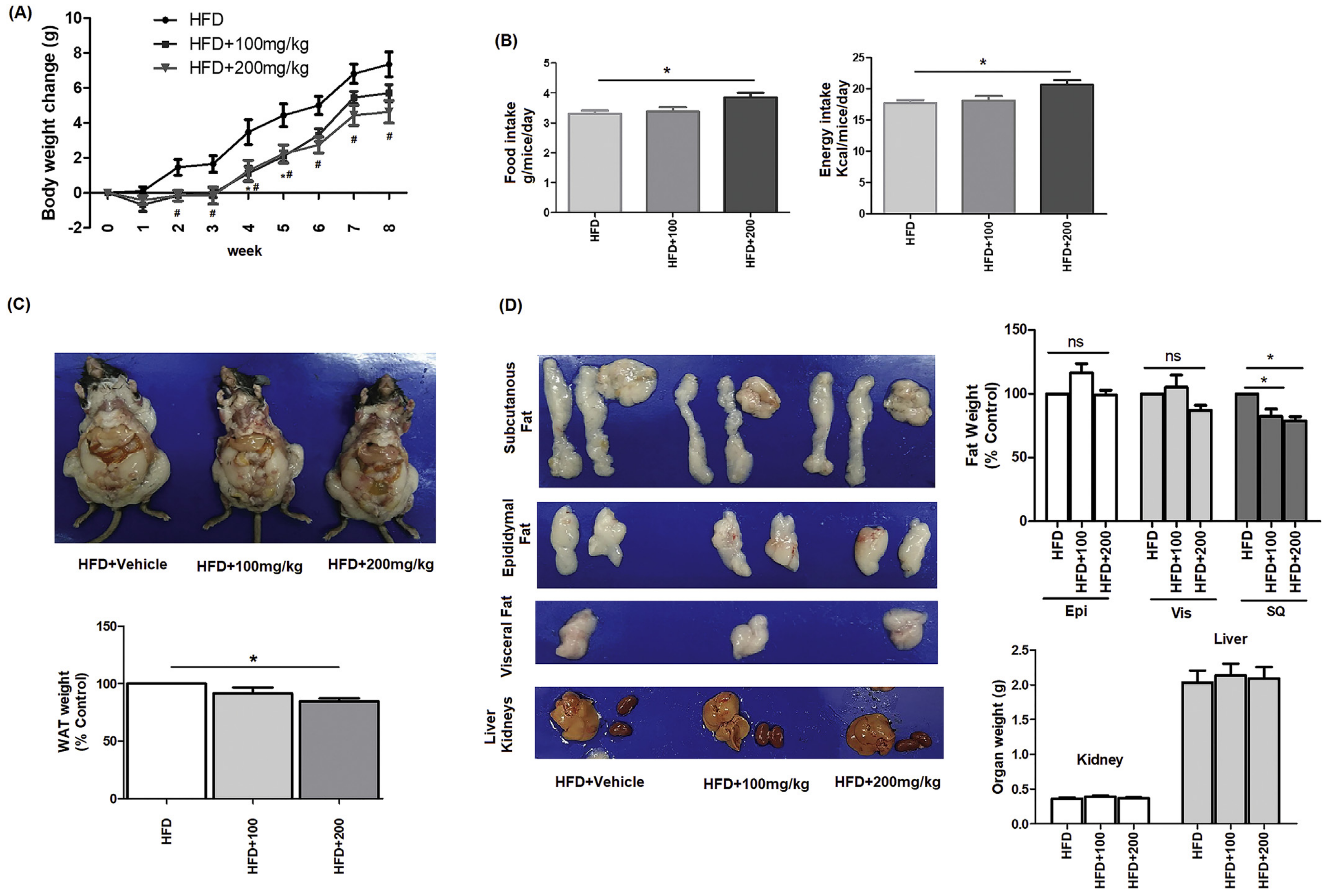

Fig. 8. LPZ reduces gain of body weight and subcutaneous adipose tissue in HFD-induced obese mice.

Mice received LPZ or vehicle daily by oral gavage for 8 weeks. (A) Body weight gain, food intake and energy consumption were observed once a week ($^*P < 0.05$ HDF vs HFD + 100, $^\#P < 0.05$ HFD vs HFD + 200). At 8 weeks of treatment, mice were sacrificed and photographed. (B and C) Total fat mass, subcutaneous adipose tissue, epididymal adipose tissue, visceral adipose tissue, liver and kidneys were weighed and photographed. Data are represented as mean ± SEM (n = 6–9). $^*P < 0.05$ was regarded as statistically significant.

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