Olanzapine enhances adipogenesis and suppresses lipolysis in 3T3-L1 adipocytes under low-glucose and weak differentiation/maturation conditions

TAISUKE MATSUO[1], YUZUKI OMORI[1], TAKASHI TOMITA[2] and YASUYUKI SADZUKA[1]

[1]Division of Advanced Pharmaceutics, Department of Clinical Pharmaceutical Sciences, School of Pharmacy, Iwate Medical University, Yahaba-cho, Shiwa-gun, Iwate 028-3694; [2]Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Nakano-ku, Tokyo 164-8530, Japan

Abstract. Olanzapine, a second-generation antipsychotic used in the treatment of schizophrenia, is classified as a multi-acting receptor-targeted antipsychotic. Abnormal weight gain is one of the most common side effects of this drug, along with an increased appetite and food intake. However, weight gain has also been reported in patients taking olanzapine without an increase in appetite. Olanzapine has been reported to be directly associated with enhanced adipogenesis; however, whether olanzapine increases lipid content in adipocytes under weak stimulus conditions, such as low glucose concentrations and weak differentiation and/or maturation conditions, is poorly understood. The present study examined the stimulatory effect of olanzapine during the differentiation and maturation of 3T3-L1 pre-adipocytes under low-glucose and weak stimulation conditions by evaluating the expression levels of PPARγ by western blotting and oil red O staining. Western blotting revealed that olanzapine suppressed perilipin phosphorylation, which is an important lipolysis step in adipocytes. The findings of the present study provide novel insights to explain weight gain in patients taking olanzapine but not presenting with increased food intake.

Introduction

Second-generation antipsychotics (SGAs) are widely used for the treatment of schizophrenia. These agents are antagonists of dopamine D_2 and various neuroreceptors, such as 5-hydroxy-tryptamine receptors (5-HT$_{2A}$ and 5-HT$_{2C}$) and adrenaline receptors (α1 and α2) (1,2). Extrapyramidal side effects of SGAs are less frequent than those observed in patients taking first-generation antipsychotics (FGAs) (2). However, SGAs frequently cause metabolic dysfunctions, such as abnormal weight gain, hyperglycemia, and dyslipidemia (1). Olanzapine is an SGA classified as a multi-acting receptor-targeted antipsychotic (MARTA). Although olanzapine is widely used for the treatment of schizophrenia, the frequency of abnormal weight gain associated with its administration is the highest among SGAs (1,3). Abnormal body weight gain increases the risk of hyperlipidemia and type 2 diabetes and reduces patient compliance (4). Although an increase in food intake is a major cause of weight gain during olanzapine therapy (5), some patients still become obese while taking the drug even if they do not increase their food intake.

Obesity is caused by enhanced energy uptake that is not balanced by energy expenditure. Energy sources, such as glucose and lipids, are stored as triacylglycerols in adipocytes. During this process, preadipocytes differentiate into adipocytes. Thereafter, adipocytes accumulate triacylglycerols as lipid droplets via maturation (6). 3T3-L1 murine preadipocytes are an established *in-vitro* model for exploring various facets of adipogenesis (6,7). In this model, the differentiation of preadipocytes into adipocytes is induced by stimulation with dexamethasone (DEX), isobutyl-methyl-xanthine (IBMX), and insulin; subsequently, the differentiated cells are cultured in insulin-supplemented culture medium for maturation (6). Peroxisome prolifer-ator-activated receptor γ (PPARγ) is a crucial regulator of these processes (6,8,9). Perilipin is expressed during adipocyte maturation, and it localizes to the surface of lipid droplets (10). Under normal conditions, perilipin suppresses triacylglycerol hydrolysis catalyzed by adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in lipid droplets (10-14). Stimulation of the adrenaline β receptor expressed in adipocytes leads to perilipin phosphorylation mediated by cyclic AMP-dependent protein kinase (PKA), which then accelerates ATGL activation and induces HSL translocation into lipid droplets (10,11,13). It is assumed that patients administered olanzapine who do not increase food intake have a low glucose (LG) concentration and weak

Correspondence to: Dr Taisuke Matsuo, Division of Advanced Pharmaceutics, Department of Clinical Pharmaceutical Sciences, School of Pharmacy, Iwate Medical University, 1-1-1 Idaidori, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan
E-mail: tmatsuo@iwate-med.ac.jp

adipocyte differentiation and maturation stimulation conditions compared with patients with increased food intake. In this study, we investigated the effects of olanzapine on adipogenesis and lipolysis in 3T3-L1 cells under low-glucose and weak differentiation and maturation conditions.

Materials and methods

Materials. Murine preadipocytes (3T3-L1 cells) were purchased from the Japanese Cancer Research Resources Bank (JCRB; Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), insulin (I6634), DEX (D4902), and IBMX (I5879) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Olanzapine (150-03071) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Isoprenaline hydrochloride (I0260) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The Phosphatase Inhibitor Cocktail (EDTA-free; 07575-51) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Antibodies against β-actin (Sigma-Aldrich, A5441), PPARγ [Cell Signaling Technology (CST), Massachusetts, USA, 2435], perilipin (D1D8) XP (CST, 9349), and p-perilipin 1 (Vala Science, CA, USA, 4856; specificity/target: Recognizes human perilipin 1 phosphorylated at serine 522; equivalent to serine 517 of the murine sequence) were used as primary antibodies. Anti-mouse IgG-HRP (Santa Cruz Biotechnology, Texas, USA, sc-2005) and anti-rabbit IgG-HRP (CST, 7074) were used as the secondary antibodies. Block Ace powder was obtained from DS Pharma Biochemical Co., Ltd (Osaka, Japan). The ECL Prime Western Blotting Detection Reagent was purchased from Cytiva (Tokyo, Japan, RPN2232).

Adipogenesis of 3T3-L1 cells. 3T3-L1 cells were maintained in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere. The cells were devoid of *Mycoplasma* contamination based on results of the Takara PCR *Mycoplasma* Detection Set (Takara Bio Inc., Shiga, Japan). The cells were seeded at a density of $1.0x10^5$ cells/mL in a 24-well plate. After 5 days, the medium was changed to DMEM supplemented with 1.6 μM insulin, 1 μM DEX, and 500 μM IBMX, and cells were cultured for 48 h to induce differentiation. Next, the medium was changed to DMEM containing 1.6 μM insulin every 48 h, and the cells were cultured for 8 days for maturation.

Olanzapine was dissolved in dimethyl sulfoxide (100 mM) and added to cells at concentrations ranging between 2.5 and 10 μM when the culture medium for differentiation and/or maturation was changed. Glucose was either added at 5.5 mM (LG) or 25 mM (high glucose, HG). To examine the effects of olanzapine on adipogenesis under conditions of LG and weak stimulation during differentiation and maturation (1/10-fold conditions), cells were treated with 10 μM olanzapine every 2 days for 10 days. When the effects of olanzapine on differentiation were observed, differentiation was induced under 1′ and 1/10′ conditions. In this case, maturation was induced in the presence of insulin alone, without olanzapine. However, when the effects of olanzapine on maturation were assessed, maturation was induced under 1x or 1/10 and 1/100′ conditions. In this case, differentiation was induced in the presence of insulin, DEX, and IBMX, without olanzapine.

Oil red O staining. Ten days after differentiation, the 3T3-L1 cells were stained with oil red O to check the lipid content. Briefly, the cells were fixed with 10% formaldehyde for 1 h. After washing with water, they were immersed in 0.3% oil red O solution prepared in 60% isopropanol for 1 h. The non-specific staining was removed by washing with 60% isopropanol and images were acquired.

Effects of olanzapine on lipolysis. 3T3-L1 cells were differentiated and matured for 10 days under HG conditions. The medium was changed to LG medium without insulin, and the cells were treated with 10 μM olanzapine for 1 h. The cells were then treated with 10 μM isoprenaline for 1 h. The cells were lysed in sodium dodecyl sulfate (SDS) sample buffer [25 mM tris-HCl (pH 6.8), 0.8% SDS, 5% glycerol] in the presence of the Phosphatase Inhibitor Cocktail and boiled. Perilipin phosphorylation was examined by western blot analysis.

Western blotting. Western blotting was performed using previously described methods (15,16). Briefly, the samples were separated using acrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 4% Block Ace solution for 1 h. Subsequently, the membrane was incubated with the primary antibodies specific to β-actin (1:5,000), PPARγ (1:1,000), perilipin (1:5,000), and p-perilipin (pS517) (1:1,000). Next, the membrane was incubated with the relevant HRP-linked secondary antibodies, and immunoreactive signals were detected using the ECL Prime Western Blotting Detection Reagent.

Statistical analysis. Each experiment was repeated twice, and thus, statistical analysis was not performed.

Results

Olanzapine enhances lipid droplet accumulation in 3T3-L1 adipocytes under low-glucose conditions. To verify whether olanzapine promotes the accumulation of lipid droplets in adipocytes under LG conditions, 3T3-L1 cells were differentiated and matured under high- and low-glucose conditions in the presence of olanzapine (0-10 μM). Oil red O staining showed that olanzapine enhanced lipid droplet accumulation in adipocytes under both HG and LG conditions (Fig. 1A). Although lipid accumulation was lower under LG conditions than under HG conditions, olanzapine increased adipogenesis under LG conditions compared with observations made in the absence of olanzapine (Fig. 1B). The expression of PPARγ also increased in response to olanzapine under both conditions (Fig. 1C). In addition, adipogenesis with olanzapine at 10 μM was increased compared with that with olanzapine at 2.5 μM. Therefore, olanzapine at 10 μM was used in the following experiments.

Olanzapine induces adipogenesis under weak differentiation and maturation stimulation conditions. To examine the effects of olanzapine on adipogenesis under LG and weak stimulation conditions, differentiation and maturation were induced by weak stimulation (reduced by 1/10-fold) (Fig. 2A). However, olanzapine treatment led to enhanced oil red O staining and PPARγ expression under all conditions
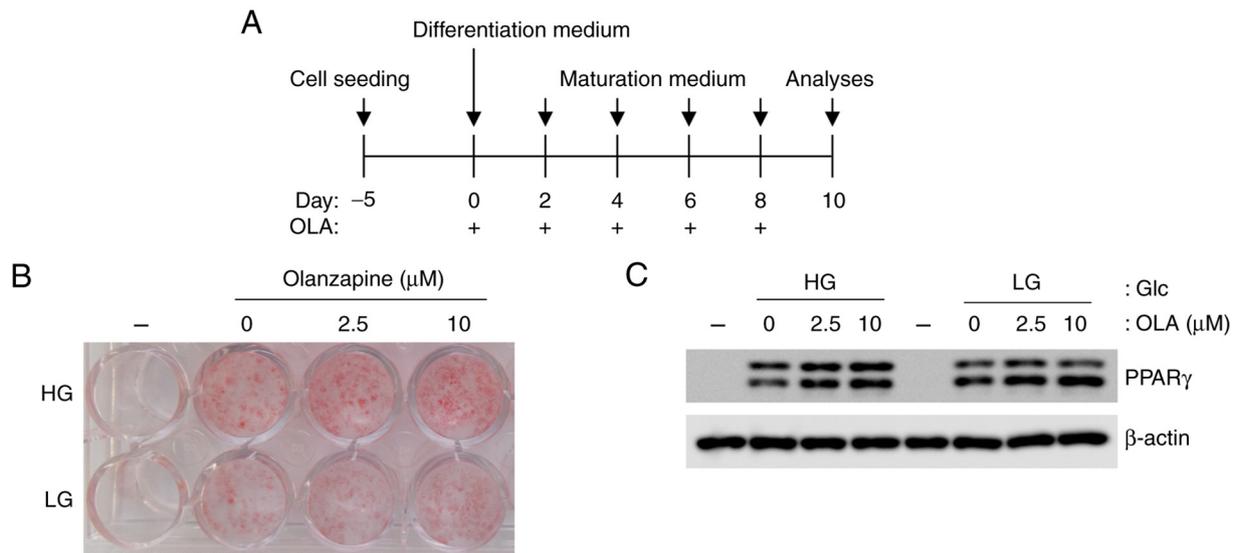
Figure 1. Effects of OLA on adipogenesis under HG and LG conditions. (A) Schematic experimental design. At 10 days after differentiation, 3T3-L1 cells were evaluated using (B) oil red O staining and (C) western blot analysis. PPARγ includes both PPARγ1 (lower band) and PPARγ2 (upper band). The results were confirmed by independent experiments (n=2). -, non-stimulation of differentiation and maturation; OLA, olanzapine; HG, high glucose; LG, low glucose; Glc, glucose; PPAR, peroxisome proliferator-activated receptor.
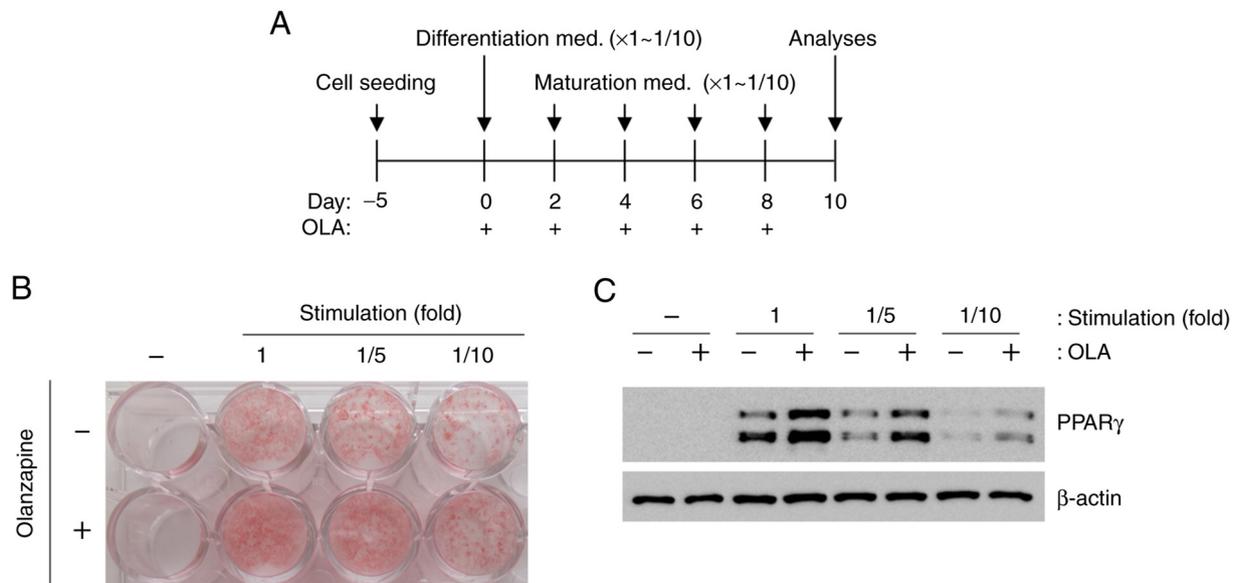

Figure 2. Effects of OLA on adipogenesis under low-glucose and weak differentiation and maturation stimulation conditions. 3T3-L1 cells were cultured under low-glucose conditions. (A) Both differentiation and maturation were induced at a reduced (1/10th) level. At 10 days after differentiation, the cells were examined by (B) oil red O staining and (C) western blot analysis. The results were confirmed by independent experiments (n=2). -, non-stimulation of differentiation and maturation; med., medium; OLA, olanzapine; PPAR, peroxisome proliferator-activated receptor.

(Fig. 2B and C), although these changes were not robust in response to weak stimulation conditions. When olanzapine was added to stimulated 3T3-L1 cells only during differentiation, oil red O staining and PPARγ expression increased under all conditions (Fig. 3A-C). In addition, adipogenesis increased following olanzapine stimulation during maturation (Fig. 3D-F).

Effect of olanzapine on lipolysis. To determine the effect of olanzapine on lipolysis, we analyzed perilipin phosphorylation in mature 3T3-L1 cells stimulated with isoprenaline. Our

results showed that olanzapine treatment suppressed perilipin phosphorylation (Fig. 4).

Discussion

The number of patients with psychiatric disorders continues to increase owing to various reasons. Schizophrenia is a psychiatric disorder that typically occurs in late adolescence and early adulthood (1). The symptoms of the condition are classified as either positive symptoms, including hallucinations and delusions following an increase in dopamine levels,
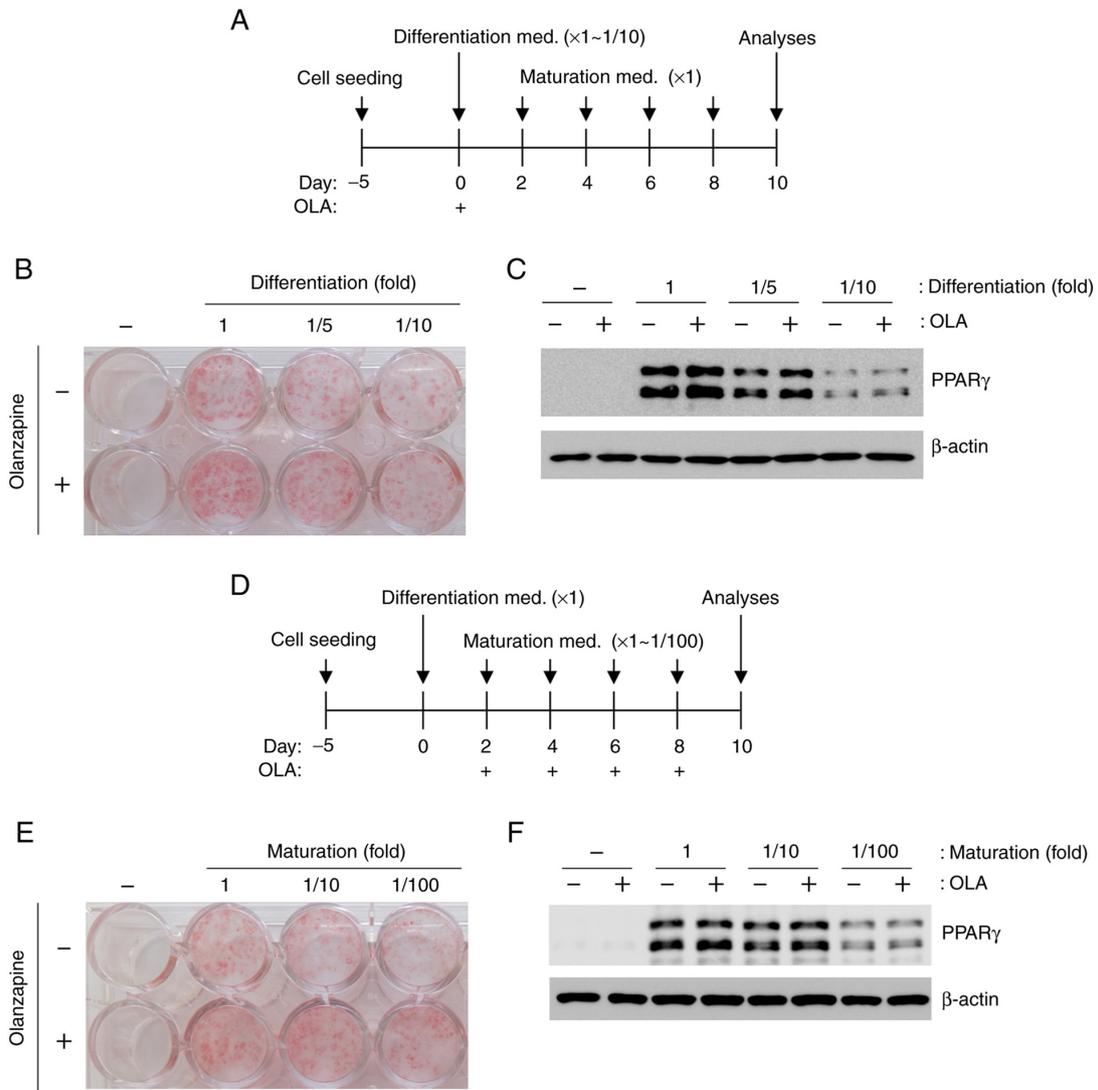
Figure 3. Effects of OLA on adipogenesis under low-glucose and weak differentiation or maturation stimulation conditions. 3T3-L1 cells were cultured under low-glucose conditions. (A) When differentiation was induced at a reduced level (1/10th fold), the maturation was not reduced. At 10 days after differentiation, the cells were examined by (B) oil red O staining and (C) western blot analysis. (D) When maturation was induced at a reduced level (1/100 fold), differentiation was not reduced. At 10 days after differentiation, the cells were examined by (E) oil red O staining and (F) western blot analysis. The results were confirmed by independent experiments (n=2). -, non-stimulation of differentiation and maturation; med., medium; OLA, olanzapine; PPAR, peroxisome proliferator-activated receptor.
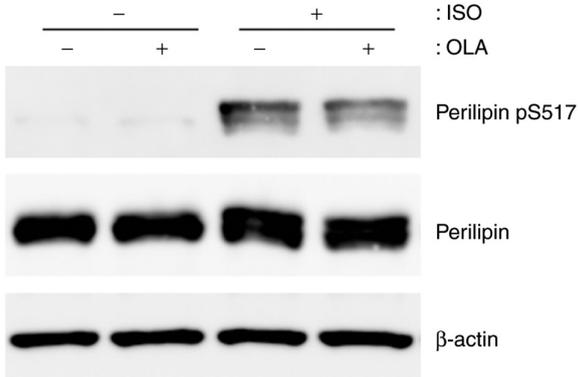

Figure 4. Effects of OLA on lipogenesis under low-glucose conditions. Mature 3T3-L1 cells were treated with OLA (10 μM) for 1 h prior to stimulation with ISO (10 μM). After 1 h of ISO treatment, the cells were used to analyze perilipin phosphorylation (S517) using western blot analysis. The results were confirmed by independent experiments (n=2). -, non-stimulation of isoprenaline or olanzapine; OLA, olanzapine; ISO, isoprenaline.

or negative symptoms, such as apathy and avolition owing to functional decline of the glutamine acid nerve (1). Medications for schizophrenia are classified as first- or second-generation antipsychotics. FGAs strongly inhibit dopamine D_2 receptors and are useful for treating positive symptoms; however, extrapyramidal disorders frequently result from using these agents (1). SGAs inhibit not only the dopamine D_2 receptor but also other neuroreceptors, such as the serotonin 5-HT_2 receptor and noradrenalin receptors (α1 and α2) (1,2). SGAs are classified as serotonin dopamine antagonists, MARTAs, and dopamine partial agonists. Although the extrapyramidal effects caused by these medications are less frequent than those caused by FGAs, SGAs induce metabolic dysfunctions, such as abnormal weight gain (1,2). One reason for this is the increase in food intake following increased appetite (1). However, some patients without enhanced appetite are also prone to weight gain, and the mechanism underlying this effect remains unknown.

Although lack of quantification (owing to n=2) was a limitation of this study, olanzapine showed the tendency to promote adipogenesis in 3T3-L1 cells, even under LG and weak differentiation and maturation conditions. LG (5.5 mM glucose) represents the global age-standardized mean fasting plasma glucose concentration (17). Accumulated triacylglycerols in adipocytes are hydrolyzed by ATGL and HSL (10,11,13). Perilipin is located on the surface of lipid droplets and binds to comparative gene identification-58 (CGI-58), an ATGL activating factor, and regulates ATGL activity (11,14). Perilipin also prevents HSL from approaching lipid droplets. When adipocytes are stimulated by β-adrenaline, perilipin is phosphorylated by PKA (10,11,13). CGI-58 detaches from phospho-perilipin and binds to ATGL, which then accelerates the activation of ATGL. Furthermore, HSL can then approach lipid droplets and hydrolyze triacylglycerols. Olanzapine reportedly suppressed isoprenaline-induced lipolysis in 3T3-L1 cells (18). In our study, olanzapine suppressed perilipin phosphorylation, which might decrease triacylglycerol hydrolysis. These results possibly explain the weight gain observed in patients who do not present with an increased appetite during olanzapine therapy. However, the olanzapine concentrations used in this study were higher than those present in the blood *in vivo*. Previous studies reported that 5 μM olanzapine increased the viability of 3T3-L1 cells by 10%. On other hand, the growing rates were inhibited by olanzapine treatments from 10 to 20 μM (19). They showed that 5 μM olanzapine induced apoptosis (<1%) and increased cell growth. Lv *et al* (19) demonstrated the phenomenon is one of the reasons of olanzapine-induced obesity, but they did not show the direct effects of olanzapine on adipogenesis. Moreover, the glucose concentration of medium was not mentioned, making it difficult to compare our results with their findings (19). Additionally, adipogenesis are regulated by various transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs), signal transducers and activators of transcriptions (STATs) (20,21), and Yanjie *et al* (22) reported olanzapine induced AMP-activated protein kinase-α (AMPKα)-Sterol regulatory element binding protein (SREBP) pathway, which is involved in lipogenesis and cholesterogenesis, in 3T3-L1 cells. Therefore, further studies, including *in vivo* experiments and analyses of various factors are necessary to clarify the detailed mechanisms underlying abnormal weight gain in patients who do not present with olanzapine-induced increased appetite.

In conclusion, this study demonstrated that olanzapine enhances adipogenesis and reduces lipolysis in adipocytes, even when the cells are cultured under LG and weak differentiation and maturation stimulatory conditions. These results provide new insights to elucidate the mechanism underlying abnormal weight gain without increased appetite in patients taking olanzapine.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TM designed the study, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. YO conducted the experiments and analyzed and interpreted the data. Data interpretation was performed by TT and YS. TM and TT confirmed the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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