Evidence for an effect of clozapine on the regulation of fat-cell derived factors

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1. Introduction

Clozapine belongs to one of atypical antipsychotic drugs and it is widely used in the treatment of clinical mental diseases. However, many severe side effects including weight gain and deficiency in granulocyte are observed during the therapeutic proceeding. Among these side effects, weight gain was thought to be a major risk factor of the development of diabetes mellitus, hyperlipidemia, and cardiovascular disease after the patients with psychiatric disease were treated with clozapine for a long time\cite{1}. Up to date, the mechanism of weight gain induced by clozapine is considered to be complicated and remained unknown. Many studies suggest that the possible mechanisms of weight gain are due to poor satiety and increased food intake after the administration of antagonist of 5-HT\textsubscript{2C} (5-Hydroxytryptamine\textsubscript{2C}) receptors\cite{2}. Atypical antipsychotic drugs also increased food intake in animal model\cite{3}. At present, most of the studies focused on the contribution of adipocytokines to weight gain. However, the direct pharmacological evidence for weight gain induced by atypical antipsychotic drugs was less understood.

Obesity is a major public health problem in the population. In rodents, obesity is the consequence of an enlargement of adipocyte due to increase of triglyceride accumulation and is characterized by an additional increase of many mature adipocytes\cite{4}. Adipocytes are

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Length (bp)</th>
<th>T\textsubscript{m} (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward: 5′-CACAGATGAAAGGCGCCGACTCATC-3′  Reverse: 5′-TAAAGACTCTATGCGACAACAGT-3′</td>
<td>241</td>
<td>50.5</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Forward: 5′-TTATGGTGGAACACTCTTGGGA-3′  Reverse: 5′-AATCGAACGCTGTAAGGCGG-3′</td>
<td>210</td>
<td>50.5</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Forward: 5′-CCAGFAGACGACGATATACAG-3′  Reverse: 5′-GCTGACATCTGCTTGGC-3′</td>
<td>112</td>
<td>49</td>
</tr>
<tr>
<td>SREBP\textsubscript{1C}</td>
<td>Forward: 5′-TAGACGATCATTCCCAGGAGTGC-3′  Reverse: 5′-GGTACGGGCGCAAGAAGAATA-3′</td>
<td>245</td>
<td>53</td>
</tr>
<tr>
<td>LPL</td>
<td>Forward: 5′-CTCTGGCGCTAGAGGAGATG-3′  Reverse: 5′-GGGATAGACGCTTGGC-3′</td>
<td>231</td>
<td>55.7</td>
</tr>
<tr>
<td>DGAT1</td>
<td>Forward: 5′-TACAGCCGAAGATGCTAGCT-3′  Reverse: 5′-CACAAGTAGGAGCCAAGATG-3′</td>
<td>105</td>
<td>46</td>
</tr>
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</table>

Abbreviation: PPARγ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT-enhancer binding protein; ADD1/SREBP\textsubscript{1C}, adipocyte determination and differentiation factor 1/sterol regulatory element binding protein; DGAT1, diacylglycerol acyltransferase1; LPL, lipoprotein lipase; PVDF, polyvinylidene difluoride.

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Fig. 1. The photography of the undifferentiated and differentiated 3T3L1 cells stained by red oil. (×100). (A) Cells were stained on day 0; (B) Cells were stained on day 4; (C) Cells were stained on day 8.

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derived from mesenchymal precursor cells. Rosen and coauthors found that three key transcriptional factors including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT-enhancer binding factor 1/sterol regulatory element binding protein (ADD1/SREBP1C) and adipocyte determination and differentiation factor (SREBP1) play a key role in the adipocyte differentiation [5]. The requirement of PPARγ and C/EBPα in adipose tissue development has been demonstrated by a targeted gene knockout strategy in mice. Homozygous knockout of either gene provokes embryonic lethality and fail to develop normal adipose tissue [6–8]. It was proposed that ADD1/SREBP1c enhances adipose conversion by inducing PPARγ expression [9] and controlling the generation of PPARγ ligands that in turn activate the transcriptional activity of PPARγ [10]. However, the mechanism for ADD1/SREBP1c modulating adipocyte differentiation was not clear.

In the process of adipocyte differentiation and maturation, preadipocyte could be induced into adipocyte after a period of differentiation and some adipocyte-specific proteins related to triglyceride metabolism including diacylglycerol acyltransferase1 (DGAT1) and lipoprotein lipase (LPL) were observed. LPL promotes triglyceride metabolism including diacylglycerol acyltransferase1 (DGAT1) and lipoprotein lipase (LPL) were observed. LPL promotes triglyceride metabolism including diacylglycerol acyltransferase1 (DGAT1) and lipoprotein lipase (LPL) were observed.

### 2. Materials and methods

#### 2.1. Cell culture

3T3-L1 cells purchased from ATCC (American Type Culture Collection) (Manassas, VA) were maintained in Dulbecco’s modified eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) before initiating differentiation. Two days after confluence (defined day 0), cells were treated with DMEM containing 10% fetal bovine serum, 1 μg/ml insulin (Sigma, Louis, MO), 0.5 μmol/l dexamethasone (Sigma, Louis, MO, USA), and 1 μmol/l isobutylmethyl xanthine (Sigma, Louis, MO) for 48 h. On day 2, cells were fed with DMEM containing 10% fetal bovine serum and 1 μg/ml insulin for 48 h. On day 4, cells were fed with DMEM containing 10% fetal bovine serum alone and then culture medium was changed every 48 h. We added 1, 5, 10, 50, 100 μmol/l of clozapine and 10 μmol/l rosiglitazone to medium to investigate the effect of clozapine and rosiglitazone on the expression of PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1. The primer pairs of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1 are summarized in Table 1. Polymerase chain reactions (PCR) were performed as described previously to determine the mRNA levels of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1. The primer pairs of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1 are summarized in Table 1. Polymerase chain reactions (PCR) were performed as described previously to determine the mRNA levels of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PPARγ (%)</th>
<th>C/EBPα (%)</th>
<th>ADD1/SREBP1c (%)</th>
<th>LPL (%)</th>
<th>DGAT1 (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>58</td>
<td>42</td>
<td>95</td>
<td>20</td>
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<tr>
<td>Clozapine</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>DMSO</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

### 2.2. Oil Red O staining

Differentiated 3T3-L1 cells were washed three times with phosphate-buffered saline (PBS) on days 0, 4, 8. Then cells were fixed on dishes with 10% formaldehyde for 1 h. After rinsing with PBS three times, cells were incubated with Oil Red O solution (60% of 0.6% Oil Red O dye in isopropanol and 40% water) for 2 h. Cells were extensively washed with PBS to remove unbound dye. Stained cells were dried on air overnight and then observed under light electron microscope.

### 2.3. MTT assay

MTT assay is used to test proliferation and viability of culture cells. Clozapine with different concentrations (including 0, 1, 5, 10, 50 and 100 μmol/l) was added to the medium. After 2 and 4 days’ treatment, 200 μl MTT solution was added to each well and incubated at 37 °C for 4 h. The incubation was continued for 6 h at 37 °C. The formazan was solubilized by adding dimethyl sulfoxide and the absorbance was measured at 490 nm in Multiskan Ascent 354 microplate reader (Thermo Labsystems).

### 2.4. Determination of triglyceride in treated cells

Triglyceride concentration was determined by using Triglyceride GPO-POD assay Kit (Applygen, Beijing, China). Briefly, 5–10×10^6 cells/ml PBS was collected and crushed by ultrasonic. 10 μl standard triglyceride or samples and 200 μl working fluid I were mixed together for 5 min at 37 °C, 70 μl working fluid II was added to above mixture solution and then solution was kept at 37 °C for 10 min. Total triglyceride concentration was detected at 490 nm by using multisikan ascend (Thermo, MA).

### 2.5. RNA isolation and Semi-quantitative RT-PCR

Total RNA was extracted by using trizol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA concentration was determined spectrophotometrically at 260 nm. Genomic DNA from total RNA was removed by using DNasel (Promega, Madison WI) before reverse transcription. Semi-quantitative RT-PCR was performed as described previously to determine the mRNA levels of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1. The primer pairs of β-actin, PPARγ, C/EBPα, ADD1/SREBP1c, LPL and DGAT1 are summarized in Table 1. Polymerase chain reactions (PCR) were carried out using 2 μl cDNA, 2.5 μl 10× standard enzyme buffer (Takara, Dalian, China), 2 μl dNTPs (Takara, Dalian, China), 1 μl of each primer (Biosun, Shanghai, China), and 0.2 units Taq DNA polymerase.
3.3. Effect of clozapine on viability of differentiated 3T3-L1 adipocytes

After 2 and 4 days treatment with clozapine at different concentrations, cells were collected to investigate the effect of drug on viability of cells. We found that clozapine does not seem to affect the viability of 3T3-L1 adipocytes, but cell viability of differentiated adipocytes in clozapine treatment group was higher than that in rosiglitazone treatment group (Fig. 2), and there are no different between with DMSO treatment group and control. Finally, we found that triglyceride concentration in clozapine treatment group was much more than that in rosiglitazone treatment group and DMSO control.

3.4. Effects of clozapine on the mRNA expressions of PPARγ, C/EBPα, ADD1/SREBP1c, LPL, and DGAT1

In this study we found that clozapine within the concentration of 1 to 100 µmol/l increased the expression level of ADD1/SREBP1c mRNA with a dose-dependent manner and reached a peak value at 10 µmol/l concentration of clozapine in adipocytes. However, clozapine at the concentrations of 10, 50, and 100 µmol/l significantly decrease LPL mRNA expression in adipocytes (Fig. 4). There were significant differences in the expression level of LPL mRNA between clozapine treatment group and control (10 µmol/l, P = 0.049; 50 µmol/l P = 0.044; 100 µmol/l, P = 0.037, respectively). No significant differences in the effects of clozapine in mRNA expression levels of PPARγ, C/EBPα, and DGAT1 were found in this study. Moreover, we found that clozapine at 10 µmol/l slightly increased the mRNA expression levels of PPARγ (Fig. 5A) and ADD1/SREBP1c (Fig. 5B), then extension at 72 °C for 5 min.

2.7. Statistical analysis

All data are expressed as mean ± SD. One-way ANOVA followed by Bonferroni post-tests for multiple comparisons were used to compare means. LSD-t test was used to evaluate differences between groups. Statistical significance was accepted when P<0.05. The SPSS software package (Version 13.0 for Windows; SPSS, Chicago, IL) was used for statistical calculation.

3. Result

3.1. Effect of clozapine on 3T3-L1 cells differentiation

3T3-L1 preadipocytes could be differentiated into mature adipocytes after induction. In this study, we observed the effects of 10 µmol/l clozapine and 10 µmol/l rosiglitazone on 3T3-L1 cell differentiation when preadipocytes were incubated at the different days (Fig. 1, Table 2). No lipid droplets were found on day 0 (Fig. 1A). However, we found that there were a lot of cellular lipid droplets while 3T3-L1 preadipocytes were treated with 10 µmol/l clozapine and 10 µmol/l rosiglitazone at day 4 (Fig. 1B) and day 8 (Fig. 1C). The number of differentiated adipocytes in clozapine treatment group was much more than that in rosiglitazone treatment group and DMSO control.

3.2. Effect of clozapine on cellular triglyceride accumulation

After 3T3-L1 cells were treated with 10 µmol/l clozapine and 10 µmol/l rosiglitazone as well as DMSO for 2, 4, 6, and 8 days, respectively, we found that the triglyceride concentrations in cells are significantly increase while cells were incubated for a longer days. Meanwhile, the concentration of cellular triglyceride in clozapine and rosiglitazone treatment groups were marked higher than that in DMSO treatment group and control (P<0.001) (Fig. 2), and there are no different between with DMSO treatment group and control group. Finally, we found that triglyceride concentration in clozapine treatment group was higher than that in rosiglitazone treatment groups after cells were treated for 4 days (P<0.01).

3.3. Effect of clozapine on viability of differentiated 3T3-L1 adipocytes

After 2 and 4 days treatment with clozapine at different concentrations, cells were collected to investigate the effect of drug on viability of cells. We found that clozapine does not seem to affect the viability of 3T3-L1 adipocytes in vitro after 2 and 4 days treatment at concentrations of 1 to 10 µmol/l, but in 50 and 100 µmol/l, there are statistical significance compare with 10 µmol/l (Fig. 3).
we observe that clozapine induced the expression of ADD1/SREBP1C mRNA and reached a peak value for 8 days incubation ($P<0.05$) (Fig. 5C). Meanwhile, rosiglitazone at 10 μmol/l significantly increased the expression of PPARγ mRNA (Fig. 5A) reached a peak value for 6 days incubation ($P<0.05$) and decreased the expression of ADD1/SREBP1C(Fig. 5B). Finally, we found that clozapine had no effect on the mRNA expression levels of C/EBPα and DGAT1 in adipocytes. There are no difference between with DMSO treatment group and control group (Fig. 5).

3.5. Effect of clozapine on the protein expression of LPL and DGAT1

As shown in Fig. 6A, we found that clozapine dose-dependently inhibited the expression of LPL protein in adipocytes. There were significant differences in the expression level of LPL protein between clozapine treatment group and control (10 μmol/l, $P=0.001$; 50 μmol/l, $P=0.000$; 100 μmol/l, $P=0.000$, respectively). However, there was no significant difference in DGAT1 protein among different concentrations of clozapine treatment groups.

Clozapine inhibited the expression of LPL protein with a time-dependent manner (Fig. 6B) and the inhibitory magnitude reached a peak value at day 8 ($P=0.01$). However, no significant difference in the expression of LPL protein in adipocytes treated with different concentrations of rosiglitazone was observed in the current study.

4. Discussion

In the previous study, it was shown that several antipsychotic drugs stimulate lipogenesis in cultured cells [15]. In the present study, we observe that clozapine induced the expression of ADD1/SREBP1C mRNA in differentiated adipocytes (Fig. 4A), and also find that clozapine decreased LPL mRNA and protein expression (Figs. 4B and 6A), which suggests that clozapine might induces fat synthesis in adipocytes. These data suggest that clozapine may induce metabolic adverse effects during antipsychotic drug treatment at least in part through direct disturbances of lipid homeostasis in the adipocytes.

Clozapine is an atypical antipsychotic drug exhibiting a low incidence of extrapyramidal side effects and more effective in clinical treatment, so it has been recommended as the first-line drug for the treatment of schizophrenia. Unfortunately, a common side effect of clozapine, namely weight gain, has also been observed. A retrospective analysis showed that the degree of weight gain ranged from 0.04 kg for ziprasidone to 4.45 kg for clozapine was estimated by the mean difference in the weight at 6 months compared with two days treatment group (10 μmol/l, $P<0.05$ compared with two days treatment group). Lanes 1 to 4, 15 to 18, L9 to L12, and L13 to L16 showed that cells were collected on days 2, 4, 6, and 8, respectively. Lanes 1, 5, 9, and 13 represented that cells were treated without any medicines (control); 2, 6, 10, and 14 represented that cells were treated with clozapine; L3, 5, 11, and 15 showed that cells were treated with rosiglitazone; L4, 8, 12, and 16 showed that cells were treated with DMSO.

In conclusion, data from this study suggest that clozapine could induce the expression of ADD1/SREBP1C mRNA and decrease the metabolic syndrome.

ADD1/SREBP1C is known to interact and cross-talk with other important mediators of metabolic control, such as the transcription regulators PPARs. It has been supposed that ADD1/SREBP1C-enhanced triglycerides by inducing PPARγ expression and by controlling the generation of PPARγ ligands [27]. However, our results showed that clozapine had no effect on the expression of PPARγ mRNA, which suggested that the influence of ADD1/SREBP1C on the differentiation of adipocytes was probably not due to the activation of PPARγ or PPARα ligands. So the effect of clozapine on the expression level of ADD1/SREBP1C protein is necessary in our further study.

The adipocyte plays a crucial role in metabolic regulation, serving as a storage depot for fatty acids and as an endocrine cell to manage energy utilization and feeding behavior [28,29]. The mass of adipose tissue is maintained by a well-controlled balance of cell proliferation (hyperplasia) and increase in fat-cell size (hypertrophy). Increases in adipocyte hypertrophy result from the uptake and assimilation of extracellular fatty acids into cytosolic triacylglycerol-rich lipid droplets. Lipoprotein lipase plays a critical role in the fat metabolism and it is a key enzyme for triglyceride hydrolysis and the transport of free fatty acids [30]. Naturally occurring LPL gene mutations have also been reported in the human population that lead to severe hypertriglyceridemia [31]. In the present study, we found that clozapine could increase the concentration of triglyceride in adipocytes and add additional information is required for the control group (Fig. 2). Meanwhile, we first found that clozapine inhibited the expression of LPL mRNA and LPL protein with a dosage-dependent manner and time-dependent manner (Figs. 4B, 5C, and 6B). These results suggested that an increase in the accumulation of cellular triglyceride was probably due to the inhibition of expression levels of LPL mRNA and LPL protein.

In conclusion, data from this study suggest that clozapine could induce the expression of ADD1/SREBP1C mRNA and decrease the metabolic syndrome.
expression of LPL at the transcriptional and translational levels. The mechanism of weight gain and/or obesity induced by clozapine were possibly due to the suppression of expression levels of LPL mRNA and LPL protein, reduction in triglyceride hydrolysis, and enhancement in lipid droplet aggregation. So we think that clozapine not only affects cell lipogenesis in peripheral but also lipid homeostasis in the whole body and results in weight gain and lipid abnormality.

Acknowledgement

This work was supported by the National Natural Science Foundation of China Grants 30572230, 30873089, by the Hunan Provincial Natural Science Foundation of Grant 08J3058, and by the Supported Project for Scientific Technology and Plan of Changsha Government, No. K0802148-31.

References


