Curcumin Inhibits Adipogenesis Elicited by Clozapine in 3T3-L1 Cells

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Abstract

Although clozapine (CZP), which is used for schizophrenia treatment, causes weight gain, the mechanism remains unclear. We recently reported that the naturally occurring compound curcumin (CUR) suppresses adipogenesis in 3T3-L1 cells. The aims of the present study were to determine the mechanism by which CZP induces adipocyte differentiation of 3T3-L1 cells, and whether CUR reduces CZP-induced adipogenesis. We found that cells grown in the presence of CZP had significantly higher triacylglycerol levels, numbers of lipid-filled adipocytes, and mRNA expression levels of CCAAT-enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) than those grown without CZP. Treatment with CZP plus CUR resulted in major reductions in these four parameters. These results suggest that CZP enhances adipogenesis in 3T3-L1 cells via the C/EBPα-PPARγ pathway and that by interrupting CZP’s effects, CUR might be a potent agent for preventing CZP-induced weight gain.

Keywords

Clozapine, Curcumin, Adipocyte Differentiation, 3T3-L1 Cell, Atypical Antipsychotic Drug

1. Introduction

Clozapine (CZP) is a second-generation atypical antipsychotic drug used to treat many schizophrenia symptoms, such as positive symptoms, negative symptoms, and recognition disorders. The major side effects of CZP are weight gain and diabetes mellitus, which are risk factors for cardiovascular diseases [1]. The probable causes of the metabolic adverse effects are poor satiety and increased food intake due to blockade of receptors such as 5-hydroxytryptamine 2C
(5HT₂C), dopamine D₂ (D₂), and histamine 1 (H₁) in the central nervous system [2]. In the last decade, it has been suggested that second-generation atypical antipsychotic drugs cause body weight gain not only by affecting the receptors of the central nervous system but also by directly affecting peripheral adipogenesis [3]–[8]. However, the mechanism by which second-generation atypical antipsychotic drugs directly induce adipocyte differentiation remains unclear.

Over the years, many phytochemical compounds have been found to have a high capacity to act against various disorders, including metabolic syndrome. One of these substances, curcumin (CUR), a nutraceutical and pharmaceutical, is the active ingredient in turmeric, a well-known Indian spice derived from the dried roots of the Curcuma longa plant. The effects of CUR in metabolic syndrome have been studied in several clinical trials. In these trials, CUR improved the lipid profiles and modified the cholesterol-related parameters of the subjects [9] [10]. Furthermore, CUR can improve anthropometric measurements and body composition when taken as part of the diet together with the implementation of appropriate lifestyle interventions [11].

3T3-L1 preadipocytes are extensively used as an in vitro model for studying adipogenesis. Our laboratory [12] and other groups [13] [14] [15] have reported that CUR suppresses the adipogenic differentiation of 3T3-L1 preadipocytes by downregulating the CCAAT-enhancer binding protein α (C/EBPα)-peroxisome proliferator-activated receptor γ (PPARγ) pathway, which is a major adipogenesis pathway [16] [17]. Thus, the present study was designed to determine whether CZP induces adipocyte differentiation of 3T3-L1 cells via the C/EBPα-PPARγ pathway. Furthermore, to our knowledge, this is the first study to evaluate whether CUR could interrupt CZP-induced adipogenesis.

2. Materials and Methods

2.1. Materials

Mouse 3T3-L1 preadipocytes were obtained from the European Collection of Cell Cultures (Wiltshire, UK). Transcriptor First Strand cDNA Synthesis kit and Light Cycler First Start DNA Master plus SYBR green reagent were obtained from Roche Diagnostics (Indianapolis, IN, USA). TRIzol reagent and the primers for β-actin, PPARγ, and C/EBPα were purchased from Invitrogen (Carlsbad, CA, USA). CZP, CUR, olanzapine (OZP), chlorpromazine (CPZ) and a protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The triglyceride E-test Wako kit was obtained from Wako Pure Chemical Industries, Limited (Osaka, Japan). Lipid Droplets Fluorescence Assay kit was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other reagents used were of analytical grade.

2.2. Cell Culture

3T3-L1 preadipocytes were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cells were maintained in a growth medium containing Dul-
becco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin-streptomycin (growth medium). Cell differentiation was induced according to the protocol received with the 3T3-L1 preadipocytes obtained from the European Collection of Cell Cultures. The procedure was initiated 2 days after confluence and performed for 3 days in a growth medium containing 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 µg/mL insulin (initiation medium). This was followed by 2 days of culture in growth medium containing 1 µg/mL insulin (maturation medium). Thereafter, the cells were cultured in the growth medium for 2 days. Treatment with various drugs during the differentiation of the 3T3-L1 preadipocytes and subsequent Oil Red O staining, determination of triacylglycerol (TG) levels, and mRNA expression levels of PPARγ and C/EBPα were performed according to previously reported methods [12] [18] [19].

2.3. Treatments with CZP and CUR

CZP and CUR were prepared in dimethyl sulfoxide (Me₂SO) and added to the growth, initiation, and maturation media from day 3 (time of addition of dexamethasone, IBMX, and insulin) to day 9 (the end of the experiment). The Me₂SO concentration was maintained up to 0.25% of the total volume of the medium. Preliminary experiments demonstrated no significant effects of 0.25% v/v Me₂SO on cell differentiation.

2.4. Oil Red O Staining

The cells were fixed in 4% formaldehyde-phosphate buffer (pH 7.4) for 1 h, rinsed with water, and stained with 0.3% Oil Red O dye for 1 h. After washing again with water, the cells were monitored using a microscope (10 × 20 magnification).

2.5. Quantification of Mature Adipocytes

Mature adipocytes in the culture were quantified using Lipid Droplets Fluorescence Assay kit, and flow cytometry. The cells were trypsinized carefully and centrifuged at 4°C and 200 ×g for 5 min. The cell pellet was resuspended, fixed, and stained with the lipophilic fluorescent dye Nile Red according to the protocol received with Lipid Droplets Fluorescence Assay kit. The samples were then analyzed with a FACSARia III flow cytometer (Becton Dickinson, Basel, Switzerland). Nile Red fluorescence was measured on the FL2 emission channel through a 585 ± 21 nm bandpass filter following excitation with an argon ion laser source at 488 nm. Using a forward scatter (FSC)/side scatter (SSC) representation of events, the P1 region was defined to exclude cellular debris from the analysis. A selection window called P2 was then established on the FL2/FSC blot of the P1 population to count cells with high FL2 values (mature adipocytes) as described by Sottile and Seuwen [20]. Data analysis was performed using BD FACSDiva Software ver. 8.0 (Becton Dickinson). For each sample, 20,000 events
were collected. The results are expressed as the percentage of cells in the P2 region.

2.6. Determination of TG Levels

The cells were harvested by scraping them from the culture dishes into lysis buffer (1% Triton-100, 150 mM NaCl, 4 mM ethylenediaminetetraacetic acid, and 20 mM Tris-HCl [pH 7.4] containing a protease inhibitor cocktail) and lysed completely using a horn-type sonicator. The TG level, which is an index of lipid accumulation, was determined using Triglyceride E-test Wako kit following normalization of the protein amounts and expressed as TG content (µg/mg protein).

2.7. Determination of the mRNA Expression Levels of β-Actin, PPARγ, and C/EBPα

Untreated cells, as well as those treated with CZP or CZP plus CUR up to day 5, were washed with ice-cold phosphate-buffered saline. Total cellular RNA was prepared using TRIzol reagent. One microgram of total RNA was reverse-transcribed into cDNA by using the Transcriptor First Strand cDNA Synthesis kit. Next, the concentration and quality of the purified total RNA were determined spectrophotometrically at 260 nm and by the OD260:280 ratio. mRNA expression was determined by real-time reverse transcription polymerase chain reaction using Light Cycler First Start DNA Master™ SYBR Green reagent and a Light Cycler instrument. The results are expressed as the target mRNA level relative to that of β-actin mRNA, and the values obtained in the presence or absence of the drugs were expressed relative to the values associated with exposure to the differentiation medium (DM) alone.

The primers used for β-actin, PPARγ, and C/EBPα were as follows: β-actin, 5’-ACACCCCAGCCATGTACG-3’, 5’-TGGTGGTGAAGCTGTAGCC-3’; PPARγ, 5’-GTGAAGCCCATCGAGGACA-3’, 5’-TGGAGCACCTTGGCGAACA-3’; and C/EBPα, 5’-ATGGTTTCGGGTCGCTGGAT-3’, 5’-CCACGGCCTGACTCCCTCAT-3’.

2.8. Statistical Analysis

The results are expressed as the mean ± standard error of the mean values. Significant differences in data between two groups were assessed using Student’s t-test. Differences were considered statistically significant at P < 0.05.

3. Results

3.1. CZP Promotes the Differentiation of 3T3-L1 Cells

Figure 1(a) shows the effect of CZP, OZP and CPZ at concentrations ranging from 1 to 20 µM on TG level during the differentiation of 3T3-L1 preadipocytes into mature adipocytes. Exposure to the second-generation atypical antipsychotics CZP and OZP increased TG levels, whereas exposure to CPZ, a classical neuroleptic drug that primarily acts on dopamine D2 receptors, did not. At 5 µM,
Figure 1. Alterations in triacylglycerol (TG) levels in 3T3-L1 adipocytes treated with clozapine (CZP), olanzapine (OZP), chlorpromazine (CPZ), and curcumin (CUR). (a) Data are presented as mean ± standard error of the mean values (n = 3 - 8); (b) Data are presented as mean ± S.E. values (n = 19 - 23). CZP, 20 µM; CUR, 20 µM. αindicates P < 0.01 compared to the untreated cells (None). βindicates P < 0.05 and γindicates P < 0.01 compared to the cells cultured in the differentiation medium (DM). δindicates P < 0.01 compared to the cells treated with CZP alone.

CZP, but not OZP, promoted TG accumulation with statistical significance. The findings show that second-generation atypical antipsychotic drugs cause body weight gain partially by affecting peripheral adipogenesis directly. This result is in agreement with results of previous studies [3]-[8].

3.2. CUR Interferes with CZP-Induced Adipogenesis in 3T3-L1 Cells

To study the potential inhibitory effects of CUR on CZP-induced adipogenesis, 20 µM CUR was added to the culture medium together with CZP (20 µM) during the differentiation of the 3T3-L1 cells. Changes in differentiation were monitored by determining the TG levels (Figure 1(b)), Oil Red O staining (Figure 2(a)), and determining the percentage of mature adipocytes (Figure 2(b) and Figure 2(c)).

Compared to exposure to DM alone, exposure to both 20 µM CZP and DM resulted in a significant increase in TG levels (1.3 fold in Figure 1(b)), Oil Red O-stained cells (Figure 2(a)), and percentage of lipid-filled adipocytes as shown by Nile Red and flow cytometry (2.4 fold in Figure 2(c)). However, the administration of 20 µM CUR with CZP almost nullified the effects of CZP; the TG levels (Figure 1(b)), number of Oil Red O-stained cells (Figure 2(a)), and percentage of lipid-rich adipocytes (Figure 2(c)) were similar to the respective levels in the presence of DM or CUR alone. The results shown in Figure 1(b) and Figure 2 demonstrate that CUR interferes with the CZP-induced adipogenesis in 3T3-L1 cells.

3.3. CUR Disrupts the Effect of CZP at the Site of the C/EBPα-PPARγ Pathway

As shown in Figure 3(a) and Figure 3(b), compared to exposure to DM alone,
Figure 2. Alterations in results of (a) Oil Red O staining and (b) Nile red staining with flow cytometry, and (c) percentages of cells stained by Nile red (P2), in 3T3-L1 adipocytes treated with clozapine (CZP) and curcumin (CUR). (a) The photographs shown are representative of results from six independent experiments. The cells stained by Oil Red O were visually monitored by microscopic observation (10×20 magnification). CZP, 20 µM; CUR, 20 µM; (b) A representative flow cytometric analysis using Nile red staining; (c) Data are presented as mean ± S.E. values (n = 4). α indicates P < 0.01 compared to the untreated cells (None). β indicates P < 0.01 compared to the cells cultured in the differentiation medium (DM). γ indicates P < 0.01 compared to the cells treated with CZP alone. CZP, 20 µM; CUR, 20 µM.
CZP (20 µM) exposure significantly increased the mRNA expression levels of PPARγ (1.7 fold) and C/EBPα (1.8 fold). In contrast, 20 µM CUR in combination with 20 µM CZP diminished the mRNA levels of PPARγ and C/EBPα compared to the cells treated with CZP alone, and the levels of these two mRNAs reached almost the same levels as those associated with exposure to DM alone. The same tendency was observed when PPARγ levels were determined by western blotting (data not shown). These results indicate that CUR interrupts the effect of CZP at the site of the C/EBPα-PPARγ pathway.

4. Discussion

Generally, patients responding to CZP should continue maintenance treatment on their effective dose beyond the acute episode. Several studies have assessed the relationship between plasma CZP levels and clinical responses [21]. Most studies have found that a threshold plasma level of 1.1 - 2.6 µM CZP is associated with an increased probability of a good clinical response to the drug.
During continuous dosage of CZP, weight gain is a common side effect of this drug [1]. Short-term weight gain leads to poor adherence and other deleterious effects. However, the mechanism underlying CZP-induced weight gain is not completely understood.

The differentiation of preadipocytes into mature insulin-responsive adipocytes involves the exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors, commencing with C/EBPβ and C/EBPδ, and ultimately the induction of C/EBPα and PPARγ expression [22] [23]. PPARγ is a master regulator of adipocyte differentiation. Together with C/EBPα, PPARγ promotes terminal differentiation by activating the expression of downstream adipocyte-specific genes.

In the present results, we showed that CZP at 5 µM or less concentration potentiated TG accumulation in 3T3-L1 cells (Figure 1(a)). Additionally, compared to exposure to DM alone, 20 µM CZP exposure significantly increased the mRNA expression levels of PPARγ and C/EBPα (Figure 3). Thus, the present study confirms that CZP at clinical levels causes body weight gain partially by directly affecting peripheral adipogenesis. It might be also possible that the CZP-induced adipogenesis is partially ascribed to enhancement of the C/EBPα-PPARγ pathway.

Humans can consume up to 8 grams of CUR per day without experiencing toxic effects [24]; this makes CUR a very interesting potential preventive agent against various diseases. CUR has been reported to inhibit adipogenesis in both cellular and animal models of obesity by several research groups, including ours [9]-[15]. In addition, recent clinical trials have demonstrated that CUR can reduce blood TG levels in patients with type II diabetes [25] [26].

In the present study, the level of adipogenesis in cells treated with 20 µM CZP plus 20 µM CUR was almost identical to that in the cells that were not treated with CZP (Figure 1(b) and Figure 2). Furthermore, the results shown in Figure 3 demonstrate that CUR reversed the CZP-induced increments in the mRNA expression of PPARγ, and C/EBPα. These results suggest that because of its ability to inhibit the effects of CZP, CUR might be a potent naturally occurring compound that prevents CZP-induced weight gain, thus preventing obesity and worsening of obesity-related complications.

5. Conclusion

In conclusion, to our knowledge, this is the first study to show that CUR could inhibit CZP-induced weight gain and its complications via inhibition of adipocyte differentiation. Our results suggest that CUR has the potential to prevent the side effects of atypical antipsychotic drugs.

Conflict of Interest

There are no conflicts of interest to declare.
References


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S. Sakuma et al.


