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Epigenetic histone modulations of PPAR γ and related pathways contribute to olanzapine-induced metabolic disorders

Abstract

The antipsychotic drug olanzapine is widely used in the treatment of schizophrenia, bipolar and other mental disorders; however, it causes serious metabolic disorders, including dyslipidemia. Our previous studies have identified that olanzapine activated expression of the sterol regulatory element binding transcription factor 1 (SREBP-1) gene, a key transcriptional factor for lipogenesis in the liver and adipocytes. SREBP-1 has been reported to positively regulate the peroxisome proliferator-activated receptor gamma (PPAR γ), a master regulator in the process of adipogenesis. This study aimed to investigate epigenetic modulations of the hepatic PPAR γ pathway in olanzapine-induced lipid dysfunctions. Olanzapine led to significant increases of body weight gain, white adipose tissue, fasting triglyceride, and fat accumulation in the liver. A significant upregulation of PPAR γ was observed in olanzapine-treated rats. ChIP-deep sequencing showed the increase of H3K4me2 binding on the whole gene loci of key regulators of adipogenesis and lipogenesis, the Pparg, Srebp-1, Cebps families (Cebpa, Cebpb and Cebpd), the Signal transducer and activator of transcription 5 families (Stat5a and Stat5b) and Klf families (Klf9 and Klf15), as well as muscarinic M3 receptor (Chrm3). ChIP-qPCR revealed that H3K9me3 binding on the promoter of Pparg2 was significantly decreased. Consistently, KDM4B, KDM1A and PHF2, the three histone demethylases responsible for site-specific erasure of H3K9me, was increased in olanzapine-treated rats. These results suggested that olanzapine acted as stimuli to trigger the cascade of adipogenesis and lipogenesis through modulating hepatic histone modifications and subsequently upregulating key transcriptional factors. These findings provided new insight into effective strategies for the prevention and treatment of metabolic side-effects induced by antipsychotic medication.

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1 **Epigenetic histone modulations of PPAR γ and related pathways**
2 **contribute to olanzapine-induced metabolic disorders**

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Abstract

The antipsychotic drug olanzapine is widely used in the treatment of schizophrenia, bipolar and other mental disorders; however, it causes serious metabolic disorders, including dyslipidemia. Our previous studies have identified that olanzapine activated expression of the sterol regulatory element binding transcription factor 1 (SREBP-1) gene, a key transcriptional factor for lipogenesis in the liver and adipocytes. SREBP-1 has been reported to positively regulate the peroxisome proliferator-activated receptor gamma (PPAR γ), a master regulator in the process of adipogenesis. This study aimed to investigate epigenetic modulations of the hepatic PPAR γ pathway in olanzapine-induced lipid dysfunctions. Olanzapine led to significant increases of body weight gain, white adipose tissue, fasting triglyceride, and fat accumulation in the liver. A significant upregulation of PPAR γ was observed in olanzapine-treated rats. ChIP-deep sequencing showed the increase of H3K4me2 binding on the whole gene loci of key regulators of adipogenesis and lipogenesis, the *Pparg*, *Srebp-1*, *Cebps* families (*Cebpa*, *Cebpb* and *Cebpd*), the Signal transducer and activator of transcription 5 families (*Stat5a* and *Stat5b*) and *Klfs* families (*Klf9* and *Klf15*), as well as muscarinic M3 receptor (*Chrm3*). ChIP-qPCR revealed that H3K9me3 binding on the promoter of *Pparg2* was significantly decreased. Consistently, KDM4B, KDM1A and PHF2, the three histone demethylases responsible for site-specific erasure of H3K9me, was increased in olanzapine-treated rats. These results suggested that olanzapine acted as stimuli to trigger the cascade of adipogenesis and lipogenesis through modulating hepatic histone modifications and subsequently upregulating key transcriptional factors. These findings provided new insight into effective strategies for the prevention and treatment of metabolic side-effects induced by antipsychotic medication.

44

45 **Keywords:** Olanzapine, Adipogenesis, Dyslipidemia, Transcriptional factors, Histone
46 modification

47

48 **Abbreviations:**

49 Acc1, CoA carboxylase1

50 BAT, brown adipose tissue

51 AMPK, AMP-activated protein kinase

52 b.i.d, bis in die (twice a day)

53 C/EBPs, CCAAT/ enhancer-binding proteins

54 ChIP, Chromatin Immunoprecipitation

55 EZH2, Enhancer of Zeste Homolog 2

56 H3K4me, histone H3 methylations at 4 lysine residue

57 H3K9me, histone H3 methylations at 9 lysine residue

58 KLFs, Krüppel-like family of transcription factors

59 NEFA, non-esterified fatty acid

60 ORO, Oil-Red-O

61 PPAR γ , peroxisome proliferator-activated receptor gamma

62 SGAs, second-generation antipsychotic drugs

63 SREBP-1, Sterol regulatory element binding transcription factor 1

64 STAT5, signal transducer and activator of transcription 5

- 65 TC, cholesterol
- 66 TF, transcriptional factor
- 67 TG, Total triglyceride
- 68 TSS, transcriptional start sites
- 69 WAT, white adipose tissue

1. Introduction

Second-generation antipsychotic drugs (SGAs), such as olanzapine, are widely used in the treatment of schizophrenia, bipolar and other mental disorders; however, they cause weight gain/obesity and other serious metabolic disorders, including dyslipidemia (1, 2). Accumulated evidence showed that SGAs could directly impair lipid metabolisms that may further lead to insulin resistance, glucose intolerance and even type II diabetes (3-5). Therefore, a better understanding of the molecular mechanisms underlying antipsychotic-induced dysregulation of lipid metabolisms is critical for developing effective strategies for prevention and treatment of these detrimental side-effects (5).

Both clinical and animal studies have reported that olanzapine caused hepatic lipid and adipose accumulations (6-8). Lipogenesis and adipogenesis are regulated by a complex transcriptional cascade that requires a highly orchestrated and sequential activation of a large variety of transcriptional factor (TF) families (9, 10). Sterol regulatory element binding transcription factor 1 (SREBP-1) is a key TF in regulating the link between genes and lipid and cholesterol production (10). It has also been reported that SREBP-1 promotes peroxisome proliferator-activated receptor γ (PPAR γ) expression through directly binding on the promoter of *PPARg* (11), as well as synthesizing endogenous PPAR γ ligands (12). PPAR γ is a master regulator in modulating the process of adipogenesis and lipid storage (13). Besides PPAR γ and SREBP-1, CCAAT/ enhancer-binding proteins (C/EBPs; the basic leucine zipper family TFs characterized as a highly conserved bZIP domain at the C-terminus), and the Krüppel-like family of transcription factors (KLFs; a kind of protein containing one or more zinc motifs), are also associated with activation of the adipogenesis network (9, 14). In addition, the signal transducer and activator of transcription 5 (STAT5,

including *STAT5A* and *STAT5B*) has been reported to enhance transcription of PPAR γ in promoting adipogenesis, and is involved in lipid metabolism (15, 16). Previous reports from our laboratory and other laboratories have shown that olanzapine induced the upregulation of hepatic SREBP-1 expression (7, 17-19), but it is still unclear how hepatic PPAR γ , and related C/EBPs, KLFs and STAT5 pathways are modulated by SGAs.

Epigenetic regulation, such as histone modification, has also been reported to play a key role in progression of hepatic lipogenesis and adipogenesis (20-22). Histone modification, particularly acetylation and methylation on the special site of histone tails, dynamically regulates specific gene expression by altering the chromatin structure for facilitating (“open chromatin”) or restricting (“close chromatin”) TFs binding on DNA (21). For example, the histone 3 lysine 4 methylation of the 2 or 3 methyl groups (H3K4me2 or H3K4me3) is a mark of open chromatin promoting *PPAR γ* and *C/EBPs* expression, while H3K9-methylation (H3K9me2 or H3K9me3) is commonly restricted by closed chromatin modification (22). Furthermore, a list of position and cell type-specific histone methyltransferases (such as EHMT2 and KMT2C) and histone demethylases (such as KDM1A, KDM4B, PHF2) have been reported to regulate adipogenesis (21, 23). To date, however, histone modification of hepatic lipogenesis and adipogenesis associated with SGA-induced dysfunctions of lipid metabolism remain unexplored, and has therefore been investigated in this study. Furthermore, olanzapine is known to elicit its therapeutic effects predominantly through strong antagonistic affinities with dopaminergic D2 and serotonin 5-HT2A receptors, and its metabolic side-effects through strong antagonistic affinities with 5-HT2C and muscarinic M3 receptors (2, 24, 25). However, D2 and M3 receptors, but not 5-HT2A/2C receptors, are expressed in the rat liver (26, 27); therefore, histone

modification of hepatic dopaminergic D2 and muscarinic M3 receptors has also been investigated.

2. Materials and methods

2.1 Animal housing and treatment

Twenty-four female Sprague-Dawley rats weighing from 200g to 220g were purchased from the Animal Resource Centre (Perth, WA, Australia). After one week of environmental familiarization, all animals were housed in individual cages under environmentally controlled conditions (22 °C, with light cycle from 07:00 am–7:00 pm and dark cycle from 7:00 pm–7:00 am), with free access to water and standard laboratory chow diet (3.9 kcal/g; 10% fat, 74% carbohydrate and 16% protein) throughout the experiment period. The rats were trained to eat cookie dough pellets (0.3g, including 30.9% cornstarch, 30.9% sucrose, 6.3% gelatin, 15.5% casein, 6.4% fiber, 8.4% minerals and 1.6% vitamins) without any drug twice a day for 1 week (28, 29). They were then treated orally with Olanzapine in 0.3g cookie dough mixed with water (n=12; 3 mg/kg, b.i.d, Zyprexa, Eli Lilly, Indianapolis, USA), or vehicle (0.3g cookie dough mixed with water; Control, n=12) for nine weeks. It has been previously reported that, at this dosage, the drug reached 60–80% D2 receptor occupancy rates in the rat brain, and was effectively to induce metabolic side-effects in rats (7, 30, 31). Since olanzapine has a shorter half-life in rats than in humans (32), the drug was administrated twice a day. Food intake, water consumption and body weight were measured once a week, respectively. All of these procedures have been approved by the Animal Ethics Committee, University of Wollongong, Australia (AE12/26), in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes (2004).

2.2 Liver and adipose tissue analysis

After 9 weeks' treatment, rats were randomly chosen to sacrifice after 2 hours (n=6 for each treatment group) or overnight drug-washout (15±1 hours of the final treatment) (n=6 for each treatment group). Livers, white adipose tissue (WAT; including perirenal, periovary, inguinal and mesentery adipose tissue), and brown adipose tissue (BAT) were immediately dissected, weighed, and frozen in liquid nitrogen, and then stored at -80 °C until used for further analysis. The livers were cut at 14 µm in a Cryostat and stained by Oil-Red-O (ORO) and H&E staining. Images of ORO staining in the liver sections were measured using ImagerJ software (33).

2.3 Blood collection and analysis of metabolic parameters

Plasma was separated from 4-5 ml EDTA (20ml of a 5% solution) anticoagulated fasting blood after centrifuging at 4000 g for 10 min at 4 °C. Separated plasma was stored at -80 °C until further analysis. Total triglyceride (TG), total cholesterol (TC) and non-esterified fatty acid (NEFA) concentration were measured using a Thermo Scientific Kit on a KoneLab 30i biochemistry analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland).

2.4. RNA isolation and gene expression analysis by real time qPCR

Total RNA from liver tissue was prepared using the Aurum TM RNA Mini Kit (Bio-RAD, USA). cDNA was synthesized from 1 µg purified RNA using the iScriptTMRT Supermix Kit (Bio-RAD, USA). The mRNA expression of *Pparg* (Rn00440945_m1), *Kdmla* (Rn01181029_m1), *Ehmt2* (*G9*; Rn01525918_m1), *Phf2* (Rn01435384_m1), *Kdm4b* (Rn01527809_m1), and *Kmt2c* (*Mll3*; Rn01410347_m1) was determined by qRT-PCR in duplicate using TaqMan[®]Gene Expression Assays (Thermo Fisher, USA) on a Quant Studio 5 Real-Time PCR System (Thermo Fisher, USA). The cycling parameters were 95

⁰C for 10 minutes followed by 40 cycles (95 ⁰C 15 s, 60 ⁰C 1 min). All mRNA expression levels were normalized relative to four endogenous control genes, *β-Actin* (Actb) (Rn00667869 ml), *Rplp0* (Rn03302271_gH), *Hprt1* (Rn01527840_m1) and glyceraldehyde-3-phosphatedehydrogenase (*Gapdh*) (Rn01775763_gl). The 2- $\Delta\Delta$ CT method was used to calculate the results.

2.5 Western blot analysis

Whole-protein lysates of liver tissue were extracted using 10 ml lysis buffer containing 9.8 ml NP40 cell lysis buffer (Invitrogen, Camarillo, CA, USA), 100 μ l protease inhibitor cocktail (Sigma–Aldrich), 100 μ l 50 mM β -Glycerophosphate (Invitrogen) and 33.3 μ l 0.3M phenyl-methylsulfonyl fluoride (Sigma–Aldrich). Bio-Rad DCTM Assay was used to measure protein concentration. We loaded 30 μ g of proteins onto an 8% to 12% sodium dodecyl sulfate–polyacrylamide gel, transblotted onto polyvinylidene difluoride membrane (Bio-Rad), blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20, and then incubated with the primary antibodies, including anti-PPAR γ (1:1000, Abcam, ab209350) and anti- β -actin (1:2000, Merck, MAB1501). After incubating overnight, the membrane was then rinsed and incubated with anti-rabbit IgG, HRP-linked Antibody (1:2000, Cell Signaling, #7074s) or anti-mouse IgG, HRP-linked Antibody (1:5000, Cell Signaling, #7076s). The band complexes were recorded using Amersham Gel Imager (GE Healthcare, Life Science, USA) and quantified by a Fiji Image processing package. To ensure consistency, Western blot experiments were performed in duplicate. The results were presented as fold changes through divided by β -Actin and compared to the control.

2.6 Chromatin immunoprecipitation (ChIP)-qPCR and ChIP-Seq

ChIP experiments for H3K4me and K3K9me were performed using EpiQuik™ Tissue

Methyl-Histone H3-K4 ChIP Kit, EpiQuik™ Tissue Methyl-Histone H3-K9 ChIP Kit, and an EpiQuik™ Tissue ChIP Kit (Epigentek, USA) according to the manufacturer's instructions. In brief, 40 mg of liver tissue was homogenized after it was cross-linked by 1% formaldehyde using Precellys®24 homogenizer (Bertin Technologies, France). The chromatin DNA was then sheared to an average fragment length of 200–1,000 bp using a Branson 450 Digital Sonifier (Branson, USA). The sheared DNA was diluted with ChIP dilution buffer at a 1:1 ratio, transferred to a strip well for binding with chip-grade anti-dimethyl-H3-K4, anti-trimethyl-H3-K9, or anti-normal mouse IgG (negative control) and incubated at room temperature for 60 minutes. Cross-Linked DNA was reversed using both a DNA release buffer containing Proteinase K and a reverse buffer in a 65 °C dry bath for one and half hours. Purified DNA was extracted using a spin column. The preliminary quantitation of the ChIP DNA was determined with NanoDrop 2000 (Thermo Fisher, USA). qPCR was performed with SYBR Green Master Mix (Qiagen, USA). The primers used for ChIP-qPCR were in accordance with the references (34). ChIP data (including input DNA) were normalized to negative control IgG and expressed as a percentage of input and fold enrichment. In parallel, ChIP DNA for H3K4me2 and H3K9me3 were further analyzed for deep sequencing. Paired-end sequencing libraries were sequenced using an Illumina Hiseq2500 50SE sequencer. BWA software was applied to align sequence reads or assembly contigs against the rat reference genome (rn6). Profiles of histone methylation on gene loci were visualized using Integrative Genomics Viewer (IGV) 2.5.0 software.

2.7 Statistical analysis

SPSS software (version 21.0, IBM, NY, USA) was used to analyze all data. The outliers were identified and removed using Boxplot. The Kolmogorov–Smirnov test was used to

examine the distribution of data from all experiments. Data were analyzed by a Student t-test. A nonparametric Mann–Whitney *U*-test was performed for data without normal distribution. Statistical significance was accepted when $p < 0.05$. The results were calculated as the mean $\pm$ SEM.

3. Results

3.1 *The effects of Olanzapine in metabolic disturbance*

After 9-weeks' treatment, olanzapine led to significant body weight gains compared to the control ($p < 0.05$; Fig. 1A). Consistently, WAT mass was significantly increased in Olanzapine-treated rats ($p < 0.05$), while no change in BAT mass was observed ($p > 0.05$; Fig.1B). However, Olanzapine significantly increased liver weight (Fig 1A), as well as total ORO staining in lipids (both $p < 0.01$) and lipid droplet size in livers (both $p < 0.05$, Fig.1D and 1E). Meanwhile, the concentrations of plasma TG ($p < 0.001$), TC ($p < 0.01$), and NEFA ($p < 0.05$) in the olanzapine group were significantly higher than in the control (Fig.1C).

3.2 *Dynamic modulation of PPAR γ expression in the liver of Olanzapine treated rats*

The mRNA expression and protein levels of PPAR γ in the liver were measured at two time points after the final drug treatment. Two hours after the final drug treatment, both protein and mRNA levels of PPAR γ were significantly upregulated in the Olanzapine group (both $p < 0.05$) compared to the control (Fig. 2A, 2C, 2E). However, after overnight drug-washout, PPAR γ protein levels in Olanzapine-treated rats were significantly lower than in the control ($p < 0.05$, Fig. 2B and 2D), while no significant difference was observed in mRNA expression from the control ($p > 0.05$, Fig. 2F).

3.4 The effect of Olanzapine on hepatic H3K4me2 and H3K9me3 histone modifications- ChIP experiments

To probe into the effect of olanzapine on histone modification in the liver, we investigated the effect of histone methylation on gene loci encoding transcriptional factors by performing ChIP experiments for H3K4me2 and H3K9me3 in liver samples of rats sacrificed two hours after the final treatment. The ChIP-seq experiment showed that an increased H3K4me2 binding was observed on a list of gene loci, especially in the transcriptional start sites (TSS), in rats treated with Olanzapine, while no H3K4me2 binding was observed in these gene loci of the control group (Fig. 3 and Fig. 5). As shown in Fig. 3A and 3B, Olanzapine induced a spike of H3K4me2 binding at the promotor region of the *Pparg* and *Srebp1* gene loci. Olanzapine also increased H3K4me2 binding in other genes that had been confirmed to modulate the cascade of adipogenesis and lipogenesis, including *Cebps* (*C/ebpa*, *C/ebpb* and *C/ebpd*; Fig. 3 C-E), *Klfs* (*Klf15*, Fig. 3 F and *Klf9*, data unrepresented), and *Stat5* (*Stat5a* and *Stat5b*; Fig. 3 G-H). It is interesting that opposite results have been observed in H3K9me3 histone methylations in these genes. Although there was some H3K9me3 binding in *Pparg* gene locus of the control rats, Olanzapine inhibited H3K9me3 binding in this gene locus. There was no clear H3K9me3 binding in other gene loci (Fig. 3). These results were confirmed by the ChIP-qPCR for primers of *Pparg1* and *Pparg2*; H3K9me3 on the promoter of *Pparg2* was significantly lower in Olanzapine rats than in controls ($p < 0.01$; Fig. 4B). No significant differences of H3K4me2 bindings on *Pparg2* were observed (Fig. 4A), while for PPAR γ 1, we did not observe significant changes in Olanzapine treated rats.

In addition, as shown in Fig. 5A, Olanzapine increased H3K4me2 binding at the whole

gene loci of the muscarinic M3 receptor *Chrm3* including the TSS region compared to the control rat. Although there was strong H3K9me3 binding in the *Chrm3* gene locus of the control rats, Olanzapine inhibited H3K9me3 binding in this gene locus (Fig. 5 A). On the contrast, Olanzapine had not any effects on both H3K4me2 and H3K9me3 bindings in the dopamine D2 receptor *Drd2* gene locus (Fig. 5B).

3.5 Modulation of histone methyltransferases and demethylases

In order to investigate the role of site-specific histone methyltransferases and demethylases in the current change of histone methylations, we measured mRNA levels of methyltransferases (EHMT2 for H3K9me; KMT2C for H3K4me) and demethylases (KDM1A for H3K9me and H3K4me; KDM4B and PHF2 for H3K9me). Two hours after the drug treatment, inhibitors of H3K9me such as *Kdm1a*, *Kdm4b* and *Phf2* had significantly higher expression in the Olanzapine group than the control (*Kdm1a* and *Phf2*, $p<0.01$; *Kdm4b*, $p<0.05$; Fig. 6A-C). There was also a significantly increased expression of methyltransferase *Ehmt2* at 2 hours of drugs treatment ($p<0.05$; Fig. 6D), however, no significantly change was observed in *Kmt2c* ($p>0.05$, Fig. 6E).

4. Discussion

In this research, we observed that chronic treatment of olanzapine caused weight gain and abnormal lipid metabolism including white fat accumulation in the liver of female rats. Upregulation of hepatic PPAR γ was observed two hours after final drug treatment, but was not shown after 15 hours' overnight drug-washout, which suggested a direct modulation of olanzapine on this master transcriptional factor in adipogenesis. Further ChIP-Seq analysis showed an enhanced H3K4me peak on the gene loci of *Pparg*, *Srebp1*, *Cebps* (*C/ebpa*,

276 *C/ebpb* and *C/ebpd*), *Klfs* (*Klf9* and *Klf15*), *Stat5* (*Stat5a* and *Stat5b*) and *Chrm3*, especially
277 on the transcriptional specific site (TSS) in olanzapine treated rats. It has been reported
278 previously that a heightened H3K4me mark on the TSS of genes promoted the activation
279 of PPAR γ , and some members of the C/EBPs and KLFs, which promoted the cascade of
280 adipogenesis induced by olanzapine treatment (9, 22). H3K9me binding on the *Pparg2*
281 promoter region was reduced by Olanzapine, which is supported by significant mRNA
282 upregulation of H3K9me-specific histone demethylases, *KDM4B*, *PHF2* and *KDM1A*.
283 These results suggested that epigenetic histone modulation of hepatic PPAR γ and related
284 lipogenesis and adipogenesis pathways play a key role in olanzapine-induced metabolic
285 disorders.

286
287 Our understanding of the mechanism of adipogenesis is largely based on the *in vitro* murine
288 cell lines and *in vivo* mice studies, and very little is known in humans (9). Further, the
289 PPAR γ modulations in metabolic syndromes observed from mouse models were not fully
290 consistent with data from humans. For example, both dominant negative P465L
291 heterozygous for the *Pparg* gene can cause abnormal adipose tissue distribution and high
292 blood pressure in both mice and humans, while insulin resistance was observed only in
293 humans (35, 36). On the other hand, the PPAR γ mutant rat has been found to be a better
294 model than mice in their physiological characteristics, which are more relevant to humans
295 in studying PPAR γ regulation of adipogenesis (37). Thus, the rat model used in this study
296 would be suitable to investigate the hepatic PPAR γ regulation of adipogenesis induced by
297 olanzapine medication. In this data, hepatic PPAR γ were increased in both transcriptional
298 and protein levels after 2 hours' of olanzapine treatment but not after 15 hours' wash-out,

which strongly suggested that the PPAR γ modulation for adipogenesis was directly promoted by olanzapine.

The role of histone methylation in biological processes is different, depending on the lysine side of modification, including adipose differentiation. Generally, H3K4me correlates with gene activation, while H3K9me restricts gene expression (38). Our H3K4me2 ChIP-seq analysis is consistent with previous reports on histone methylation in the PPAR γ pathways during adipogenesis using embryonic stem cells (ESCs) (39, 40). Our results showed that, compared to the control rats without clear H3K4me2 bindings, olanzapine treatment led to peak enrichment of H3K4me2 on the TSS of gene loci encoding *Ppar γ* , *Srebp1*, *Stat5a* and *Statb*, and 2 *Klfs* (*Klf9* and *Klf15*). In addition, H3K4me2 was enriched on the whole loci of *C/ebpa*, *C/ebp β* and *C/ebp δ* . On the other hand, previous genome-wide profiling by ChIP-Seq revealed that H3K9me3, but not H3K4me2, is enriched on the whole loci (including TSS) of *Ppar γ* genes in preadipocytes (41). Consistently, our ChIP-Seq data showed that H3K9me2 was enriched on the whole loci of *Ppar γ* in the control rats, while this enrichment was largely reduced by olanzapine treatment. This was further confirmed by our ChIP-qPCR focused on the promoter area of the *Ppar γ 2* isoform that a significantly lower H3k9me3 specific binding was caused by olanzapine treatment. Therefore, these results indicated these adipogenesis and lipogenesis related TFs may be upregulated by the increased methylation of the positive histone marks H3K4me, and decreased methylation of the negative histone marks H3K9me.

Several histone site-specific methyltransferases and demethylases have been identified

which regulate adipogenesis (40). In this study, we investigated mRNA levels of *KMT2C* (*MLL3*) and *EHMT2* (G9a) that can specially promote methylation on H3K4me and H3K9me, respectively. *KMT2C* (*MLL3*) could be recruited directly to the PPAR γ promoter and increase H3K4me promoting adipogenesis, while a *KMT2C* (*MLL3*) knockout reduces H3K4me₃ levels at PPAR γ target genes leading to a 30-40% WAT reduction and triglyceride accumulation (42). However, unexpectedly, although olanzapine slightly increased the expression of *MLL3* mRNA, it was not significant in this study, suggesting that olanzapine-enhanced H3K4me₃ was not mainly due to the contribution of *KMT2C*. More recently, it has been reported that *KMT2D* (*MLL4*) is a major mammalian H3K4 methyltransferase for enhanced activation of PPAR γ and C/EBP β (43). Therefore, further study should investigate whether olanzapine promoted H3K4me through *KMT2D* (*MLL4*). *EHMT2* (G9a) is a major H3K9-specific methyltransferase, and acts as an inhibitor in PPAR γ expression and adipogenesis (41, 44). It was unexpectedly to observe an increased expression of *EHMT2* mRNA after 2 hours of the final olanzapine treatment. However, a previous study on 3T3-L1 cells had demonstrated that G9a was responsible for the epigenetic mechanism for the delayed expression of PPAR γ through transactivation of C/EBP β (45). It was interesting that we observed a reduced protein expression of PPAR γ after 15 hours drug wash-out. Therefore the late decrease of PPAR γ in this study may be modulated by *EHMT2* (G9a).

PHF2 and KDM4B (*JMJD2B*) are the H3K9-specific histone demethylases that have been identified as modulating the lipid metabolism process (46, 47). PHF2 potentiates adipogenesis by facilitating H3K9me histone demethylation at the C/EBP α promoter, while

a *Phf2* overexpression may promote hepatic fat accumulation (46, 48). However, conditional knockout of *Kdm4b* in the liver of mice has been reported to play no role in the obesity phenotype (47). It is worthy of note that KDM1A (LSD1) is a histone demethylase for both H3K4me2 and H3K9me3 (38). It has been reported that knockdown *KDM1A* resulted in increased H3K9me3 binding at the *C/EBPα* promoter and inhibited adipogenesis, but increased energy expenditure through H3K4me2 regulation, while increased *KDM1A* gene expression by a high fat diet had the opposite effects, leading to fat accumulation (49). In this study, we observed an increased mRNA expression of *Phf2*, *Kdm4b* and *Kdm1a* (*Lsd1*) in the liver of rats after 2 hours' treatment with olanzapine. Therefore, in consideration of the increased PPAR γ expression and the inhibited profile of H3K9me3 binding on the *Pparg2* promoter region, but not the *C/EBPα* promoter, under the olanzapine treatment, these results suggested that olanzapine recruited PHF2, KDM4B and KDM1A (LSD1) to erase histone methylation on the H3K9me mark, and subsequently reduced H3K9me3 levels on the *Pparg* promoter region leading to an inhibition of its expression.

Finally, accumulating evidence confirms that the process of activation of transcriptional factors and chromatin remodeling events is dynamic and most happen within hours after initiation of adipocyte differentiation (50). The possible explanation was that the structure of the ligand binding domain for key TFs is dynamic, such as the PPAR γ ligand (51, 52). Furthermore, the time of interaction between trans-acting factors and TFs corresponding DNA cis-acting elements is also a dynamic change. For example, mRNA peaks in *KLF4* and *KLF5* were at around 2 h and 6 h, respectively (53-56), while

KLF9 and *KLF15* were at around day 6–8 in 3T3-L1 adipogenesis (54, 55). In the current study, we observed that both TFs and histone modifications were modulated for provoking PPAR γ and related pathways after two hour' of olanzapine treatment when the blood drug concentration peaked (57), while this provocation retreated when the drug was washed out. These indicated that epigenetic regulation through histone modifications in hepatic PPAR γ and related pathways was activated by the olanzapine medication.

The vagal nerve innervates and regulates liver metabolisms *via* muscarinic M3 receptors in hepatocytes, since the M3 receptor is the only subtype of muscarinic receptors expressed in the rodent liver/hepatocytes (27, 58). Our previous studies have revealed that the hepatic M3 receptor was significantly upregulated by chronic olanzapine treatment and contributed to olanzapine-induced dyslipidemia in rats (7). In this study, we observed an increased H3K4me2 but decreased H3K9me3 on the *Chrm3* gene loci. Therefore, the upregulated hepatic M3 receptors by chronic olanzapine treatment could be modulated through increased H3K4me2 methylation and decreased H3K9me3 methylation on the *Chrm3*. It is interesting that olanzapine did not have any effects on methylation of both H3K4me2 and H3K9me3 on the *Drd2* gene locus in this study, which is consistent with a previous report that antipsychotic drug sulpiride (also a dopaminergic D2 receptor antagonist) did not directly act at the D2 receptors of the rat liver in regulating functions of hepatic Cytochrome P450 enzymes (59). These findings suggest that, although olanzapine has high binding affinities to D2, 5-HT2A/2C, and M3 receptors, it may modulate hepatic functions via directly acting at M3 but not D2 and 5-HT2A/2C receptors. A recent study found that activation of M3 receptors reduced lipid synthesis in hepatocytes via activation of AMPK α

(60), while olanzapine, as a M3 antagonist, has been found to increase lipogenesis via deactivation of AMPK α (61, 62). Since it has been established that AMPK plays a key role in the modulation of histone H3 methylation and demethylation (63), olanzapine may provoked histone regulation in the PPAR γ pathway via the hepatic M3 receptor-AMPK pathway (Fig. 7). On the hand, as a cationic amphiphile, olanzapine can penetrate cellular membranes and, therefore, interferes with lipid organization and disrupts lysosomal function (3, 64). Olanzapine has been reported to directly inhibit cholesterol biosynthesis and disrupt cholesterol intracellular trafficking that leads an up-regulation of SREBPs (3, 65), while it is unknown whether these direct lipid interferences have any effects on epigenetic modulations. It is also worthy to note that olanzapine could direct interact with nuclear receptors, such as the liver X receptor. Although a previous *in vitro* study found that olanzapine did not directly activate the liver X receptor in CHO-7 cells (a Chinese hamster ovarian cell line) (65), our recent study showed that olanzapine increased the expression of the liver X receptor in HepG2 cells (a human hepatoma cell line) (17). Using HepG2 cells, recently, the liver X receptor has been reported to upregulate histone H3K9 demethylase JMJD2B leading an increased expression of PPAR γ 2 (66). In fact, JMJD2B (KDM4B) has been found to be significantly increased in the liver of rats treated with olanzapine in this study. Therefore, independently from acting *via* the M3 receptor-AMPK pathway, olanzapine may penetrate cellular membranes and regulate histone modifications *via* acting at the liver X receptor and possibly other nuclear receptors in the hepatocytes (Fig. 7). More studies are important to further confirm these regulation pathways.

Taken together, this study is the first to demonstrate the time-dependent epigenetic

regulation of key transcriptional factors in the process of hepatic lipogenesis and adipogenesis induced by olanzapine treatment. The results clearly suggested that histone modifications (Fig. 7) could be a molecular mechanism underlying olanzapine induced metabolic disorders, particularly lipid metabolic dysfunctions. It provided us with a new insight to find effective strategies for prevention and treatment of adverse metabolic events induced by olanzapine and other antipsychotic drugs.

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Author contributions

YS and CD designed the experiments. YS, XL, and JL performed the experiments. YS and CD analysed the data. YS prepared the initial draft of the manuscript. CD revised the manuscript. All authors commented on and approved the final draft.

Conflict of interest

None of the authors has a conflict of interest.

Appendix A Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phrs.2020.104703>.

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656 **Chemical compounds**

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658 Olanzapine (PubChem CID: 4585)

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662 **Figure 1. The effects of olanzapine on (A) body gain and liver weight, (B) weight of**
663 **white adipose (WAT) and brown adipose tissue (BAT), (C) plasma triglyceride (TG),**
664 **total cholesterol (TC) and non-esterified fatty acid (NEFA) (D) Oil-Red-O (ORO)**
665 **staining of liver sections. Insert images were magnified eight times to show the lipid**
666 **staining morphology. (E) Data from ORO staining of liver sections were presented. The**
667 **sample size is 12 per group. * $p < 0.05$, ** $p < 0.01$, vs. control.**

670 **Figure 2 Expression of PPAR γ at 2 hours or 15 hours after final Olanzapine treatment.**
671 **(A-B)** The representative images of Western blot for PPAR γ (57-KDa) and β -actin (43-
672 KDa; as loading controls). **(C-D)** relative protein levels of PPAR γ . **(E-F)** mRNA expression
673 of PPAR γ . Abbreviations: 2H, 2 hours after final drug treatment; 15H, 15 hours drug wash-
674 out. The sample size is 6 per group. * $p < 0.05$ vs. control.

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678 **Figure 3. ChIP-Seq profiles - snapshots of H3K4me2 and H3K9me3 binding on (A)**
679 ***Pparg*, (B) *Srebp1*, (C) *Cebpa*, (D) *Cebpb*, (E) *Cebpd*, (F) *Klf15*, (G) *Stat5a*, and (H)**
680 ***Stat5b* gene loci.** ChIP-Seq on H3K4me2 and H3K9me3 were performed in the liver
681 samples of rats at 2 hours after final drugs treatment. Enrichment of peaks was visualized
682 using IGV-2.5.0 software.

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687 **Figure 4. ChIP-qPCR analyses of histone methylations on PPAR γ 2 promoter at 2**
688 **hours after final drugs treatment.** Histone modifications were measured by using ChIP-
689 qPCR with antibodies against **(A)** H3k4me2 (active mark), **(B)**, H3K9me3 (inactive mark)
690 in the liver of rats treated with olanzapine or control (n=6/group). * $p < 0.05$, vs. control.

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694 **Figure 5. ChIP-Seq profiles - snapshots of H3K4me2 and H3K9me3 binding on (A)**
695 **muscarinic M3 receptor *Chrm3*, and (B) dopamine D2 receptor *Drd2* gene loci. ChIP-**
696 **Seq on H3K4me2 and H3K9me3 were performed in the liver samples of rats at 2 hours**
697 **after final drugs treatment. Enrichment of peaks was visualized using IGV-2.5.0 software.**

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702 **Figure 6. Effects of olanzapine treatment on histone methyltransferases (HMTs) and**
703 **histone demethylase (HDMs).** mRNA levels of specific HMTs and HDMs contributing to
704 H3K4me and H3K9me at 2 hours (A-E) or 15 hours (F-J) after the final drug treatment
705 (n=6/group). KMT2C (MLL3) is an HMT for H3K4me; KDM1A (LSD) is an HDM that
706 erases methylation of both H3K4 and H3K9; KDM4B (JMJD2B) and Plant homeodomain
707 finger 2 (PHF2) are HDMs that erase methylation of H3K9. EHMT2 (G9a) is a HMT that
708 catalyses methylation of histone H3K9. Abbreviations: 2H, 2 hours after final drug
709 treatment; 15H, 15 hours drug wash-out. * $p < 0.05$, ** $p < 0.01$: vs. control.

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713 **Figure 7. A schematic diagram of histone modifications for epigenetic regulation of**
714 **hepatic PPAR γ and related pathways leading to increased lipogenesis and**
715 **adipogenesis contributing to olanzapine-induced metabolic disorders. (The dashed**
716 **arrows indicate the proposed regulation pathways based on previous findings from our and**
717 **other laboratories (7, 17, 60, 61,63, 66))**

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