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Betahistine co-treatment ameliorates dyslipidemia induced by chronic olanzapine treatment in rats through modulation of hepatic AMPK α -SREBP-1 and PPAR α -dependent pathways

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Betahistine co-treatment ameliorates dyslipidemia induced by chronic olanzapine treatment in rats through modulation of hepatic AMPK α -SREBP-1 and PPAR α -dependent pathways

Abstract

Second-generation antipsychotics including olanzapine are associated with weight gain, dyslipidemia and other metabolic disorders. Both animal and clinical studies have shown that co-treatment with betahistine (a histamine H1 receptor agonist/H3 receptor antagonist) is effective in controlling olanzapine-induced weight gain. In the present study, we investigate whether co-treatment with betahistine is able to prevent dyslipidemia induced by chronic olanzapine treatment and the underlying mechanisms. Female rats were orally administered with olanzapine (1 mg/kg, t.i.d.) for 3.5 consecutive weeks and then a 2.5-week drug withdrawal. Then, rats were divided into 4 groups for 5 weeks treatment: (1) vehicle, (2) olanzapine-only (1 mg/kg, t.i.d.), (3) betahistine-only (9.6 mg/kg, t.i.d.), and (4) olanzapine and betahistine (O + B) co-treatment. After completing treatment, hepatic mRNA expression was measured by qRT-PCR, while the protein levels were detected by western blot. In our study, olanzapine-only treatment significantly increased triglyceride accumulation and non-esterified fatty acids (NEFA), and upregulated mRNA expression of sterol regulatory element binding protein 1 (SREBP-1) and its target genes, while these alterations were ameliorated by O + B co-treatment. Hepatic AMP-activated protein kinase α (AMPK α) was activated in the O + B co-treatment group, with a significant reduction in nuclear SREBP-1 protein expression but an increased expression of peroxisome proliferator-activated receptor- α (PPAR α) and its-responsive molecule (CPT1A), compared with olanzapine-only treatment. In addition, olanzapine significantly increased hepatic histamine H1 receptors, while O + B co-treatment significantly reversed them to normal levels. This study provided the first evidence that betahistine could act on hepatic H1 receptors via modulation of AMPK α -SREBP-1 and PPAR α -dependent pathways to ameliorate olanzapine-induced dyslipidemia in rats.

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Title: Betahistine co-treatment ameliorates dyslipidemia induced by chronic olanzapine treatment in rats through modulation of hepatic AMPK α -SREBP-1 and PPAR α -dependent pathways

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19 **Abstract**

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21 dyslipidemia and other metabolic disorders. Both animal and clinical studies have shown that co-
22 treatment with betahistine (a histamine H1 receptor agonist/H3 receptor antagonist) is effective
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26 (1 mg/kg, t.i.d.) for 3.5 consecutive weeks and then a 2.5-week drug withdrawal. Then, rats were
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28 betahistine-only (9.6 mg/kg, t.i.d.), and (4) olanzapine and betahistine (O+B) co-treatment. After
29 completing treatment, hepatic mRNA expression was measured by qRT-PCR, while the protein
30 levels were detected by western blot. In our study, olanzapine-only treatment significantly
31 increased triglyceride accumulation and non-esterified fatty acids (NEFA), and upregulated
32 mRNA expression of sterol regulatory element binding protein 1 (SREBP-1) and its target genes,
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34 kinase α (AMPK α) was activated in the O+B co-treatment group, with a significant reduction in
35 nuclear SREBP-1 protein expression but an increased expression of peroxisome proliferator-
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37 only treatment. In addition, olanzapine significantly increased hepatic histamine H1 receptors,
38 while O+B co-treatment significantly reversed them to normal levels. This study provided the
39 first evidence that betahistine could act on hepatic H1 receptors via modulation of AMPK α -
40 SREBP-1 and PPAR α -dependent pathways to ameliorate olanzapine-induced dyslipidemia in
41 rats.

42 **Keywords:** olanzapine; dyslipidemia; betahistine; sterol regulatory element binding protein
43 1; AMP-activated protein kinase α ; peroxisome proliferator-activated receptor α

44 **Abbreviations**

- 45 ACC1: acetyl-CoA carboxylase 1
- 46 AMPK α : AMP-activated protein kinase α
- 47 CPT1A: carnitine palmitoyltransferase 1A
- 48 FASN: fatty acid synthetase
- 49 H1R: histamine H1 receptors
- 50 H3R: histamine H3 receptors
- 51 HDL-C: high density lipoprotein cholesterol
- 52 HMGCoA: 3-hydroxy-3-methyl-glutaryl-CoA
- 53 LDL-C: low density lipoprotein cholesterol
- 54 m-SREBP-1: the mature form of SREBP-1
- 55 NEFA: Non-esterified fatty acid
- 56 ORO: Oil-Red-O
- 57 PPAR α : peroxisome proliferator-activated receptor α
- 58 p-SREBP-1: the precursor form SREBP-1
- 59 SCD1: stearoyl-CoA desaturase-1
- 60 SGAs: second-generation antipsychotic drugs
- 61 SREBPs: sterol regulatory element-binding proteins
- 62 TC: total cholesterol
- 63 TG: triglyceride

64 1. Introduction

65 Second-generation antipsychotics (SGAs) including olanzapine and clozapine are commonly
66 used in clinics for controlling schizophrenia symptoms and other psychotic disorders [1-3].
67 Over the last few decades, weight gain and other metabolic disorders caused by SGAs have
68 attracted increasing attention [4, 5]. Dyslipidemia is one of the troublesome side-effects associated
69 with chronic antipsychotic medication, which may further cause cardiovascular disease, stroke,
70 and premature death [6, 7].

71

72 Although dyslipidemia could be the consequence of obesity, recent studies demonstrate that
73 SGAs have direct effects on the liver and/or adipose tissue in inducing dyslipidemia without
74 weight gain [8-10]. First, the lipogenic transcription factor sterol regulatory element binding
75 protein 1 (SREBP-1) and target genes are thought to be one of the possible pathways involved in
76 SGA-induced hepatic lipid dysregulation. We have recently shown that mRNA expression
77 of SREBP-1 and target genes in the liver and fat tissue was strongly affected by a single oral
78 olanzapine administration [11]. In addition, SGAs (especially clozapine) exposure in the cultured
79 cells induces a time-dependent proteolytic cleavage of SREBP-1, in which mRNA expression of
80 the cluster of lipid biosynthesis-related genes is directly up-regulated, without a preceding
81 elevation of SREBP gene expression [12]. These data suggest that SGA-mediated upregulation of
82 the SREBP-1-controlled transcription system and activation of the SREBP-1 cleavage process in
83 the peripheral tissues may play an important role in the elevation of lipid accumulation. Second,
84 peroxisome proliferator-activated receptor- α (PPAR α) is a critical regulator of fatty acid
85 oxidation, in which activation of PPAR α promotes the expression of genes required for fatty acid
86 and lipoprotein metabolism in mitochondria, such as carnitine palmitoyltransferase 1 (CPT1)

[13]. Recently, it has been reported that olanzapine downregulated transcriptional activity of PPAR α to augment hepatic lipid accumulation in an AMPK-dependent manner in rat primary hepatocytes [14]. Given the pivotal role of SREBP-1 and PPAR α in mediating hepatic dysregulation of lipid metabolism caused by SGAs, it is important to identify and characterize factors as targets for reversing lipid dysfunction.

AMPK also plays an important role in regulating hepatic lipid metabolism [15]. In short-term regulation, AMPK phosphorylates and inactivates acetyl CoA carboxylase (ACC) and 3-hydroxy-3-methyl-glutaryl-CoA (HMGCoA) reductase, thus inhibiting both cholesterol and fatty acid biosynthesis [16]. In addition, phosphorylation by AMPK could also affect PPAR α activity including ligand affinity and DNA binding [17]. Olanzapine-induced hepatic dysregulation of lipid metabolism has been reported to be resulted from reduced PPAR α activity by inhibiting AMPK signalling in hepatocyte [14]. In the long-term, AMPK has been reported to regulate hepatic lipogenic gene expression by inhibiting transcription factors including SREBP to reduce the transcription rate [16]. However, Schmidt *et al.* found that olanzapine-induced disturbances in hepatic lipid metabolism were mediated through activation of AMPK and mammalian target of rapamycin pathways in female mice [18]. Therefore, further study is necessary to assess the potential contribution of chronic SGAs on lipid metabolism from hepatic AMPK pathway.

Although it is not known whether the hepatic histamine receptors are involved in SGA-induced dyslipidemia, histamine is a classic inflammatory mediator in peripheral tissues and also functions as a neurotransmitter in the brain [19]. The histaminergic receptors in the hypothalamus appear to be an important mediator of feeding behaviour and body weight

regulation [20], which may further regulate obesity and dyslipidemia [21, 22]. The high binding affinity of SGAs to H1R and its modulating role in the hypothalamic AMPK pathway have been evidenced as one key mechanism for a marked increase in food-intake/weight gain [21-24].

Betahistine, a histamine H1R agonist/H3R antagonist, has been successfully used to prevent/reduce olanzapine-induced weight gain in both rats and schizophrenia patients [21, 25, 26]. Recent reports demonstrate that co-treatment of olanzapine with betahistine significantly reduces weight gain and feeding efficiency [25, 27]. In a clinical trial, when co-administered with olanzapine (10 mg/day) for 6 weeks, the mean weight gain in schizophrenia patients with betahistine/olanzapine co-treatment (3.1 ± 0.9 kg) was lower than those with olanzapine-only treatment (5.0 ± 1.2 kg) [28]. Another short-term clinical trial in first episode schizophrenia patients showed that patients co-treated with olanzapine, betahistine and reboxetine (a selective norepinephrine reuptake inhibitor) had significantly less weight gain than those treated with olanzapine only treatment [26]. It is interesting that betahistine has been found to reduce lipid accumulation in the liver of rats caused by chronic olanzapine treatment [25]. Therefore, it is important to further investigate the long-term effects of co-treatment with betahistine on lipogenesis and lipolysis in the liver and the underlying mechanisms.

Here, we reported that up-regulated mRNA of SREBP-controlling genes and increased expression of nuclear SREBP-1 as well as decreased CPT1A protein levels was an underlying mechanism for dysregulation of hepatic lipid metabolism induced by chronic olanzapine treatment. An important consideration is long-term prevention and therapy of SGA-induced dyslipidemia, since schizophrenia patients face life-long and repeated treatment with SGAs.

Using an animal model with repeated and chronic olanzapine treatment, we further showed that chronic co-treatment with betahistine stimulated AMPK α phosphorylation to reduce the cleavage of p-SREBP-1 and to enhance CPT1A-dependent fatty acid oxidation, possibly through acting on hepatic histamine H1R. These data provided the first evidence to indicate that betahistine could act at H1R via the AMPK α -SREBP-1 and PPAR α -dependent pathways to ameliorate olanzapine-induced dyslipidemia in rats.

2. Materials and methods

2.1 Animal housing and treatment

Forty-eight female Sprague-Dawley (SD) rats (200-220 g) were obtained from the Animal Resources Centre (Perth, WA, Australia) and housed in individual cages and allowed ad libitum access to water and a standard laboratory chow diet under environmentally controlled conditions (22°C, with light cycle from 07:00-19:00 and dark cycle from 19:00-07:00) throughout the experimental period. After one week of environmental familiarization, rats were trained to self-administer a sweet cookie dough pellet (0.3 g, including 30.9% cornstarch, 30.9% sucrose, 6.3% gelatine, 15.5% casein, 6.4% fibre, 8.4% minerals and 1.6% vitamins) without drugs for one week [27, 29]. A chronic and repeated olanzapine treatment rat model was used as described previously [25]. In brief (Fig.1), for the first 3.5 weeks, 48 rats were divided into two groups: half of them (n=24) were treated with olanzapine (1 mg/kg, Eli Lilly, Indianapolis, USA, t.i.d.), and the other half were treated with vehicle. From week 3.5 to week 6, no rats received any treatment during this period (e.g. olanzapine was withdrawn). From week 6 to week 11, the two groups were divided into 4 subgroups (n=12) for further treatment of 5 weeks: subgroup I was control (received a sweet cookie dough pellet without drug; C); subgroup II was olanzapine-only (1

mg/kg, t.i.d.; O), subgroup III was betahistine-only (9.6 mg/kg, t.i.d., Manus Aktteva, India; B);while subgroup IV was co-treatment of olanzapine and betahistine (O+B). The dosage translation between species based on body surface area followed an FDA guideline [30, 31]. 1 mg/kg olanzapine and 9.6 mg/kg betahistine is equivalent to ~10 mg olanzapine and ~93mg betahistine in humans at 60 kg body weight, respectively. In addition, betahistine has 3~4 hours of plasma half-life in humans with one day of urine excretion [32]. The dose (9.6 mg/kg rat body weight, t.i.d.) used in this study should be equivalent to the human dose (48 mg) used in clinical trials [26, 28]. Furthermore, betahistine treatment at the dosage range (8-24mg) can significantly reduce food intake for 24 hours in rats[33]. Body weight, food intake and water intake were measured once per week.All experimental procedures have beenapproved by the Animal Ethics Committee, University ofWollongong, Australia (AE11/10); and complied with theAustralian Code of Practice for the Care and Use of Animals forScientific Purposes (7th edition, 2004).

After completing treatment, all rats were sacrificed by carbon dioxide asphyxiation. The liver was weighed, and then collected and frozen in liquid nitrogen immediately followed by storage in a -80 °C freezer until further analysis. Cardiac blood was collected in EDTA-coated tubes, and then centrifuged (3000 rpm, 10 min, 4 °C) to separate the plasma which was stored at -80 °C freezer until assay.

2.2 Plasma and liver lipid analyses

Plasma was assayed for total cholesterol, triglycerides, low-density lipoproteins-cholesterol (LDL-C) as well as high-density lipoproteins-cholesterol (HDL-C) by using the Thermo Scientific Kit on a Konelab 30i biochemistry analyzer (Thermo Fisher Scientific Oy,Vantaa,

Finland). Non-esterified fatty acid (NEFA) was measured by using NEFA C kit (Wako Chemicals, Richmond, VA).

Liver lipids were extracted in Folch solution (chloroform/methanol, 2:1, v/v) [34]. Liver samples (0.1 g) were homogenized in 2.0 ml Folch solution. Extracts were then washed once with 0.2 volume of 0.9% NaCl solution and twice with methanol/water (1:1, v/v). The organic phase was separated and brought up to 2.0 ml with chloroform. Fifty microliters of each sample were mixed with 10 μ l of 50% Triton X-114 in chloroform (v/v). Samples were air dried and then subjected to colorimetric enzymatic assays for total cholesterol or triglycerides (Thermo Scientific)[35].

2.3 Oil Red O staining and liver histology

For the visualization of hepatic lipid content, cryostat sections were cut at 12 μ m, fixed with 10% formalin for 5 minutes and lipid droplets deposition was detected by Oil-Red-O (ORO) staining (Sigma-Aldrich 01516, St Louis, MO, USA). Sections were rinsed with 60% isopropanol and stained for 15 min with filtered ORO solution (0.5% in isopropanol followed by 60% dilution in distilled water). After two rinses with 60% isopropanol and distilled water, slides were counterstained with hematoxylin (Sigma-Aldrich GHS232, St Louis, MO, USA) for 15 seconds, rinsed with water and mounted. For quantitative analyses of ORO staining, images were randomly sampled using a Leica DMRB microscope (Leica Systems, Toronto, Ontario, Canada) across 2 adjacent sections on the same slide (N=6 individual livers) in each treatment group. The area of positive staining for ORO was calculated as a percentage of total section area, and an average lipid droplet size was calculated by utilizing morphometry software ImageJ (version 1.46) [36].

202

203 **2.4 RNA extraction, cDNA synthesis, and gene expression analysis**

204 Total RNA from liver tissue was prepared using a PureLink® RNA Mini Kit (Life
205 technologies™, USA). First strand cDNA was synthesized from 1.4 µg of DNase I(On-Spin
206 Column DNase, USA)-treated total RNA with random hexamer primers using SuperScript®
207 VILO™ cDNA synthesis Kit (Invitrogen™, USA) by incubation at 42 °C for 60 min. *Acc1*
208 (Rn00573474_ml), *Fasn* (Rn01463550_ml), *Scd1*(Rn00821391_gl), *Ppara*
209 (Rn00566193_ml), *Cpt1a* (Rn00580702_ml) and *Srebp-1* (Rn01495769_ml) mRNA
210 expression were determined by qRT-PCR in duplicate using TaqMan® Gene Expression
211 Assays (Applied Biosystems, Foster City, USA) on LightCycler® 480 (Roche, Penzberg,
212 Germany). The cycling parameters were 95 °C 10 min followed by 40 cycles (95 °C 15 s, 60 °C
213 1 min). All gene expression levels were normalized relative to two endogenous control genes, β -
214 *Actin* (Actb)(Rn00667869_ml) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)
215 (Rn01775763_gl). The $2^{-\Delta\Delta CT}$ method was used to calculate the results.

216

217 **2.5 Preparation of nuclear and cytoplasmic extracts**

218 Nuclear and cytoplasmic extracts were prepared from rat livers using the NE-PER™ nuclear and
219 cytoplasmic extraction reagent kit (Pierce Biotechnology, USA), according to the manufacturer's
220 instructions. Protein concentration was detected by SpectraMax Plus 384 absorbance microplate
221 reader (Molecular Devices, USA) using the Bio-Rad DC™ Assay. Transcription factors including
222 SREBP-1 (1:1000, Santa Cruz, sc-364), and PPARα (1:1000, Santa Cruz, sc-9000) were detected
223 with western blot analysis.

224

225 **2.6 Western blot analysis**

226 Whole-protein lysates of liver tissue were extracted using 10% Nonidet P-40 lysis buffer
227 l(Invitrogen, Camarillo, CA, USA) supplemented with 1% protease inhibitor cocktail (Sigma-
228 Aldrich), 0.5 mM β -Glycerophosphate (Invitrogen) and 1.0 mM phenylmethylsulfonyl fluoride
229 (Sigma-Aldrich). Protein concentrations were measured with a protein assay reagent using the
230 Bio-Rad DC™ Assay. Aliquots containing 10 μ g of proteins were loaded onto an 8% to 12%
231 sodium dodecyl sulfate–polyacrylamide gel, transblotted onto polyvinylidene difluoride (PVDF)
232 membrane (Bio-Rad), blocked with 5% BSA in Tris-buffered saline with 0.1% Tween-20, and
233 then incubated with the primary antibodies including anti-AMPK(1:2000, CellSignally, #2532),
234 anti-phospho-AMPK (1:2000, Cell Signally, #2535), anti-histamineH1R (1:1000, Santa Cruz, #
235 SC-20633), anti-histamineH3R (1:1000, Santa Cruz, # SC-390140),anti-ACC1 (1:1000, Cell
236 Signaling, #3662), anti-phospho-ACC1 (1:1000, Merck Millipore, 07-303), ATGL (1:2000,
237 Abcam, ab109251), anti-CPT1A (1:1000, Abcam, ab176320), and anti- β -actin (1:2000, Santa
238 Cruz, sc-47778). The membrane was then incubated with horseradish peroxidase–conjugated
239 goat anti-rabbit (1:5000, Millipore) or goat anti-mouse IgG (1:5000, Millipore). The bound
240 complexes were detected with Amersham Hyperfilm ECL (GE Healthcare, Life Science, USA)
241 and quantified by a GS-800 image densitometry (Bio-Rad). The ratio to β -Actin was calculated
242 and presented as fold changes, setting the values of control rats as one.

243

244 **2.7 Statistical analysis**

245 All data were analysed using SPSS software (version 21.0, IBM, NY, USA). The Kolmogorov-
246 Smirnov test was used to examine the distribution of data from all experiments. Data were

analysed by Two-way analysis of variance (ANOVA) (OLANZAPINE \times BETAHISTINE), followed by multiple comparisons using *post-hoc* Dunnett t-tests. A nonparametric Mann-Whitney U test was performed for data without normal distribution. Pearson's correlation test was used to analyse the relationships among the measurements. Statistical significance was accepted when $p < 0.05$. The results were calculated as the mean \pm SEM.

3. Results

Body weight, adipose mass and energy intake data have been reported previously [25]. Briefly, olanzapine-only rats displayed a significantly higher body weight gain, white adipose mass (including inguinal, periovarian, mesentery and perirenal fat), food intake and feeding efficiency, whereas the O+B co-treatment reversed weight gain, white fat mass, and feeding efficiency compared with the olanzapine-only treatment group.

3.1 Effects on lipid concentration in the liver and blood

As shown in Table 1, plasma TG levels were significantly increased (1.06 ± 0.11 (C) vs. 1.26 ± 0.13 (O) mmol/l, $p < 0.05$), as were plasma NEFA levels (0.28 ± 0.03 (C) vs. 0.35 ± 0.03 (O) mEq/l, $p < 0.05$) in olanzapine-only treatment. Although betahistine-only treatment had no effect on plasma TG and NEFA levels compared to the control, O+B co-treatment significantly reversed olanzapine-induced plasma TG ($p < 0.05$) and circulating NEFA ($p < 0.01$) levels to near the control levels. A slight correlation was observed between NEFA levels and TG levels in the plasma ($r = 0.298$, $p = 0.077$). However, plasma HDL-C, LDL-C, as well as total cholesterol showed no significant changes in all treatment groups.

After chronic treatment with olanzapine, a 14% increase in the liver weight of the control group was detected (13.76 ± 0.44 (O) vs. 12.09 ± 0.26 (C) g, $p < 0.05$). Oppositely, O+B co-treatment led to a significant reduction in liver weight (12.78 ± 0.39 (O+B) vs. 13.76 ± 0.44 (O)g; $p < 0.05$). Similar to what we observed in plasma, olanzapine-only treatment induced a significant accumulation of lipids in the hepatic crude extracts (11.2 ± 0.21 mg/g, $p < 0.01$; Table 1) compared with the control (7.91 ± 0.85 mg/g). Moreover, a positive correlation was observed between plasma and hepatic TG levels ($r = 0.359$, $p = 0.040$). Although hepatic cholesterol levels were increased by olanzapine treatment (0.41 ± 0.13 mmol/g), it was not significant compared with the control rats ($p = 0.14$).

In addition, olanzapine-only treatment rats significantly increased positive ORO staining ($133.65 \pm 6.09\%$ of the control value, $p < 0.001$) for neutral lipids and increased lipid droplet size ($152.39 \pm 28.27\%$ of the control value, $p < 0.01$) compared with the control (Fig.2). It should be noted that there was a significantly lower positive ORO staining in the O+B co-treatment group than the olanzapine-only group ($\sim 31.57\%$ reduction, $p < 0.01$). Although the betahistine-only group had a lower lipid accumulation than the control, the difference was not significant ($86.97 \pm 4.10\%$ of control value, $p = 0.170$). The images counterstained with hematoxylin showed that there were no notable histological changes in treatment groups compared with the control (Fig. 2A-2D).

3.2 Effects of olanzapine and/or betahistine treatment on the SREBP-1 pathway

To investigate one of the most important pathways controlling lipid biosynthesis, we quantified mRNA expression of genes involved in the SREBP-1 pathway, and examined the protein level of the mature form of SREBP-1 (m-SREBP-1) in the liver. At the transcription levels, olanzapine-

only treatment led to a significant transcriptional response with upregulation of *Srebp-1* and its target lipid-related genes (*Acc1*, *Fasn*, *Scd1*) ($p < 0.01$; Fig. 3A-3D). In particular, *Scd-1* had 5.18 ± 0.56 -fold increase in the olanzapine-only treated group ($p < 0.01$; Fig. 3D). Compared to olanzapine-only treatment, co-treatment with betahistine exhibited a significant decrease in mRNA expression of *Acc1* (2.36 ± 0.23 -fold (O) vs. 1.41 ± 0.01 -fold(O+B), $p < 0.05$; Fig. 3B), *Fasn* (2.05 ± 0.33 -fold(O) vs. 1.49 ± 0.12 -fold(O+B), $p < 0.01$; Fig. 3C) and *Scd1* (5.31 ± 0.56 -fold(O) vs. 1.27 ± 0.23 -fold(O+B), $p < 0.01$; Fig. 3D). Although it was not significant, in O+B co-treatment rats, mRNA expression of *Srebp-1* was $\sim 30\%$ less compared to the olanzapine-only group ($p = 0.175$). At the protein levels, olanzapine significantly increased the abundance of the mature form of SREBP-1 (m-SREBP-1; $p < 0.05$) as well as the ratio of m-SREBP-1 over precursor form SREBP-1 (p-SREBP-1) protein levels (0.32 (O) vs. 0.22 (C), $p < 0.05$; Fig. 3F), whereas the p-SREBP-1 did not show any significant changes (Fig. 3G). It was important that betahistine-only and O+B co-treatment led to a significant decrease in m-SREBP-1 protein levels compared to olanzapine-only treatment (both $p < 0.05$, Fig. 3E), and the ratio of m-SREBP-1 over p-SREBP-1 in the O+B co-treatment group returned normal levels (Fig. 3F). Furthermore, we also observed a positive linear relationship between plasma TG concentration and m-SREBP-1 protein levels ($r = 0.459$, $p = 0.012$)

3.3 Effects of olanzapine and/or betahistine treatment on the PPAR α -dependent pathway

To determine whether betahistine was able to enhance fatty acid β -oxidation to reverse dyslipidemia induced by olanzapine, we examined mRNA and protein levels of PPAR α (a nuclear receptor) and CPT1A (the rate-limiting enzyme of mitochondrial β -oxidation). As shown in Fig. 4A and 4D, both betahistine-only and O+B co-treatments significantly up-regulated mRNA

expression levelsof PPAR α ($p<0.01$),as well as its protein levels,compared to the control ($p<0.05$). Furthermore, a negative linear relationship between hepatic triglyceride content and PPAR α protein levels was observed ($r=-0.316$, $p=0.039$). Surprisingly, olanzapine-only treatment did not cause a significant change in *Pparam*RNA levels but led to a markedincreaseat PPAR α protein levels ($p<0.01$; Fig. 4A and 4D).On the other hand, both in the betahistine-only and O+B co-treatment group, mRNA expression of *Cpt1a* was significantly higher than in the control rats (both $p<0.05$; Fig. 4B).Importantly, olanzapine-only significantly decreased the protein levels of CPT1A ($p<0.05$; Fig. 4E), while no significant change in transcription levels was observed. As another PPAR α target gene, ATGL protein expression was not affected by olanzapine and/or betahistine treatment (Fig. 4F).

3.4 Effects of olanzapine and/or betahistine treatment on AMPK α phosphorylation

To determine whether AMPK α is involved in olanzapine and/or betahistine-induced dyslipidemia, the protein levels of AMPK α , phosphorylated AMPK α (pAMPK α , Thr172), and phosphorylated ACC1 (pACC1, Ser79), a downstream target of AMPK α , were measured. As shown in Fig. 5A-5C, although the AMPK α levels were not significantly altered in all groups, the protein levels of pAMPK α and the ratio of pAMPK α /AMPK α demonstrated that betahistine efficiently stimulated the phosphorylation of AMPK α at T172 in the liver ($p<0.05$).However, olanzapine-only treatment had no effect on pAMPK α levels. Moreover,O+B co-treatment also caused a ~25%increase in hepaticlevels of pAMPK α compared with the olanzapine-only treatment ($p<0.05$; Fig. 5B). Consistent with the observed changes in pAMPK α expression, phosphorylation in the liver of acetyl-CoA carboxylase 1 (ACC1) (a downstream molecule of AMPK)wassignificantly elevated at ~54% and ~47% of control value by betahistine-only

treatment and O+B co-treatment, respectively (both $p < 0.05$; Fig. 5F). By contrast, co-treatment with betahistine significantly reduced ACC1 protein levels to ~63% of the control value ($p < 0.05$), while olanzapine-only treatment led to a ~73% increase compared with the control ($p < 0.01$; Fig. 5E). ACC1 protein levels were positively correlated with m-SREBP-1 protein levels ($r = 0.603$, $p = 0.004$) which was negatively correlated with AMPK phosphorylation in the liver ($r = -0.387$, $p = 0.009$). Additionally, a strong positive correlation of protein levels between pAMPK α and CPT1A was observed ($r = 0.849$, $p < 0.001$).

3.5 Effects of olanzapine and/or betahistine treatment on histamine H1 receptor

To test the hypothesis that the effects of olanzapine and/or betahistine on lipid metabolism might be via action on hepatic histamine receptors to modulate AMPK, we determined the expression of H1R and H3R. As shown in Fig. 5F, the protein levels of H1R were significantly increased (~67%) by olanzapine-only treatment ($p < 0.05$), and positively correlated with m-SREBP-1 protein levels ($r = 0.343$, $p = 0.05$). It was interesting that O+B co-treatment significantly reduced the H1R levels to ~76% ($p < 0.01$; Fig. 5F). We further found a strong negative correlation between H1R levels and circulating NEFA concentration ($r = -0.502$, $p = 0.005$) as well as hepatic pAMPK levels ($r = -0.772$, $p < 0.001$). However, H3R was not affected by any of these treatments (all $p > 0.05$; Fig. 5G).

4. Discussion

The present study showed that chronic treatment with olanzapine led to a significant increase in body weight gain and elevated the circulating NEFA, TG and hepatic lipid accumulation without altering cholesterol synthesis in rats. Interestingly, co-treatment with betahistine

improved olanzapine-induced dyslipidemia. Our data further revealed that olanzapine-only treatment enhanced the cleavage of 125-kDa p-SREBP-1 and increased transcriptional expression of the crucial genes involved in lipogenesis, while it reduced CPT1A protein levels involved in fatty acid β -oxidation. By contrast, as expected, co-treatment with betahistine reduced lipid accumulation, possibly through the following process: 1) inhibited olanzapine-induced transcriptional activation of lipogenesis genes including *Srebp-1*, *Acc1* and *Scd1*; 2) increased AMPK α phosphorylation to inhibit the cleavage of p-SREBP-1 and to suppress the protein levels of ACC1; and 3) stimulated PPAR α and CPT1A expression to modulate fatty acid β -oxidation. Furthermore, H1R was elevated by olanzapine-only, while it was reversed by O+B co-treatment. The results suggest that amelioration of olanzapine-induced dyslipidemia after betahistine co-treatment may be attributable not only to alteration of the transcriptional responses of lipid-metabolic genes but also, at least in part, to activation of the AMPK signalling pathway via action at hepatic H1 receptor.

Previous studies showed that olanzapine treatment enhanced mRNA expression of SREBP-1 and downstream target genes involved in lipogenesis [9, 12, 37]. Our findings further confirmed that under chronic olanzapine treatment, the induction of increased lipid synthesis was associated with transcriptional up-regulation of *Srebp-1* and its-controlling genes such as *Acc1*, *Fasn*, and *Scd1* in the liver. However, it was noteworthy that olanzapine-only treatment led to an up-regulation of *Srebp-1* mRNA expression but not a significant increase of p-SREBP-1 protein expression in this study. Olanzapine augmented the abundance of the transcriptionally active (68 kDa) form of SREBP-1 in the nucleus without a significant effect on the 125-kDa precursor form of SREBP-1 in the cytoplasm, resulting in an increase in the 68/125-kDa protein ratio.

385 Interestingly, it is observed in previous studies that post-translational activation of SREBP-1 was
386 enhanced to regulate lipid metabolism by chronic olanzapine treatment[38-40]. Overall, these
387 findings suggested that olanzapine might have a stronger effect on modulating the post-
388 translational process of p-SREBP-1 than just elevating its transcription. In the case of co-
389 treatment, O+B had significantly down-regulated olanzapine-induced mRNA expression of
390 lipogenesis-related genes, although betahistine-only treatment did not affect these genes. We
391 further investigated whether the concentration of circulating NEFA was positively correlated
392 with m-SREBP-1 expression in our experiment. These findings raise the possibility that O+B co-
393 treatment may modulate the post-translational process of SREBP-1 to ameliorate olanzapine-
394 induced lipid accumulation in liver.

395
396 Of note, olanzapine induced the greatest increase in *Scd1* mRNA expression involved in SREBP-
397 controlling lipogenic genes (a greater than 5-fold up-regulation). Although SCD1 is just a
398 desaturase that catalyse the synthesis of monounsaturated fatty acids, previous studies have
399 shown that the elevated SCD1 activity is implicated in a wide range of disorders, including
400 obesity[41, 42] and hepatic insulin resistance[43]. Moreover, evidence for up-regulation of *Scd1*
401 mRNA expression had been obtained in cultured human and rat cell lines following exposure to
402 SG treatment[44, 45], as well as in blood cells of patients treated with
403 olanzapine[46]. Therefore, increased lipid accumulation caused by olanzapine was particularly
404 likely due to the promotion of *Scd1* mRNA expression. By contrast, co-treatment with
405 betahistine reversed olanzapine-induced up-regulation of *Scd1* mRNA expression. Interestingly,
406 metformin (a classic approach to treat the metabolic syndrome) also suppressed SCD1 gene
407 expression through activation of AMPK[47]. Taken together, SCD1 may act as an important

modulator controlling the development of obesity and dyslipidemia induced by olanzapine and may be targeted by betahistine co-treatment.

As an activator of mitochondrial and peroxisomal fatty acid β -oxidation, both betahistine-only and O+B co-treatment obviously improved the transcriptional response as well as protein levels of PPAR α compared with the control, with a negative correlation between hepatic triglyceride content and PPAR α protein levels. Moreover, co-treatment with betahistine also significantly induced CPT1A (a PPAR α -target molecule) expression, contributing to an enhancement of β -oxidation. Thus, co-treatment with betahistine might enhance lipolysis to reduce accumulation of hepatic TG via activation of PPAR α . Although there was a significantly up-regulated expression of PPAR α and CPT1A by betahistine-only treatment, hepatic lipid droplets had only a slight (and non-significant; $p=0.17$) decrease compared with the control in lipid accumulation. It is worthy to note that there are over 800 genes, including *Srebp-1* and *Hsl* (Hormone-sensitive lipase), involved in the lipid droplets accumulation [48, 49]. Therefore, betahistine-induced increase in the expression of PPAR α and CPT1A may only partially affect the assembly of lipid droplets. In the olanzapine-only group, we investigated a significant decrease in CPT1A protein levels, suggesting that olanzapine-induced dyslipidemia could also be relevant for antilipolytic effects related to fatty acid β -oxidation. However, unexpectedly, there was no significant difference of PPAR α mRNA levels between the olanzapine-only and control groups, but a marked increase in its protein expression in the olanzapine-only group. It should be noted that clozapine treatment caused the hepatic transcriptional response of *Ppar α* with an initial up-regulation at 1~3 h and a subsequent down-regulation at 6 h after drug exposure [50], while in HepG2 cells rather than primary hepatocytes of clozapine-fed mice, olanzapine decreased transcriptional activity of

PPAR α in an AMPK-dependent manner [14]. Therefore, it is possible that the regulation of PPAR α expression is not only a direct stimulation of SGAs but also a feedback-response to significantly increased fatty acid levels as well as multiple changes of hormone[51] during chronic olanzapine treatment.

In this study, elevated H1R levels were observed in the liver, while O+B co-treatment significantly down-regulated H1R expression and induced an increase in the pAMPK α as well as pACC1 (downstream molecule of AMPK). It was worth noting that an inverse correlation was found previously between AMPK and SREBP-1 activities in hepatocytes and in mice livers[52, 53]. Consistent with previous reports, there was a negative correlation between hepatic m-SREBP-1 protein levels and pAMPK α levels in this study. With an increase in pAMPK levels, betahistine-only treatment led to a significant inhibition of the transcription levels of *Acc1*, while O+B co-treatment down-regulated olanzapine-induced mRNA expression of SREBP-controlled lipogenic genes. Although the physiological significance of the phosphorylation of AMPK α by betahistine is far from understood, these findings support the idea that the effects of betahistine on fatty acid synthesis are at least partially due to AMPK α -mediated regulation of SREBP-1 activity via hepatic H1R (Fig. 6). As a potent histamine H1 antagonist, olanzapine-only treatment showed an up-regulation of *Srebp-1* and its-controlling genes, as well as an increase of the post-translational process of SREBP-1, while co-treatment with betahistine (as a H1 agonist) downregulated olanzapine-induced mRNA expression of lipogenesis-related genes. Therefore, it is possible that betahistine, via H1 receptors, could activate phospholipase C (PLC), with a subsequent increase in cytosolic Ca²⁺ concentration and activation of AMPK through CAMKK β (Ca²⁺ calmodulin-dependent protein kinase kinase β) [54]. Lauret et al. have found that

the transcriptional inductions (*Fasn*, *Scd1*, *HmgCoAr*, *Ldlr* mRNA) promoted by clozapine could be completely reversed when BAPTA-AM (Selective Ca^{2+} chelator) was co-incubated with clozapine in the Immortalized Human Hepatocyte cell model, showing that calcium chelation may prevent SREBP-1, SREBP-2 gene expression induced by clozapine [44]. Therefore, it is important in further studies to investigate the effects of betahistin/olanzapine co-treatment on PLC expression and Ca^{2+} concentration in the liver. In addition to the effects on the H1R levels, histidine decarboxylase (HDC) could be affected by olanzapine and/or betahistine treatment, which may further cause changes in histamine synthesis. Therefore, it is important in further study to analyse the alteration of the HDC protein levels and histamine content. Furthermore, the “pleiotropic effects” of betahistin might be involved in improving lipogenesis. Since betahistine is also a H3R antagonist, it might modulate SREBP-1 expression through H3 receptors. However, this study did not find any effects on H3 receptors by either olanzapine or O+B co-treatment, which may suggest a key role of histamine H1 receptors on the regulation of betahistine. In fact, it is also worth to note that the antibody used in this study targets at an epitope mapping between amino acids 301-405 near the C-terminus of H3R[55]. Due to a large variety of H3R isoforms in the protein sequences existed between different tissues[56, 57], the limitation of the antibody used in this study may not allow us to detect the possible changes in other H3R isoforms. Therefore, we could not completely exclude the role of histamine H3R in betahistin’s effects, in which betahistine may act on H3R to activate other effector pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [58, 59]. Taken together, other specific antibodies could be used in further studies to investigate whether betahistine has any effect on other isoforms of H3R in the liver.

On the other hand, it should also be noted that pAMPK α levels positively correlated with CPT1A protein levels. *Cpt1a* is a PPAR α -responsive gene, and that induction of PPAR α in human hepatocytes increases CPT1A mRNA levels [60]. Therefore, betahistine-induced activation of AMPK may 1) decrease the activation of p-SREBP-1; 2) lead to the phosphorylation at Ser-79 and inhibition of ACC1 [61]. This inhibition may result in a drop in the level of malonyl-CoA which itself is an inhibitor of CPT1A [16]. With a drop in the inhibition of CPT1A, β -oxidation of fatty acid within the mitochondria will be increased [62]; or 3) increase PPAR α levels to promote CPT1A expression, enhancing fatty acid oxidation.

The female rats were used in this study, because the SGA-induced weight gain model has been consistently established and validated in female rats in our and other laboratories [27, 63-65], while it could not be consistently modelled in male rodents [66]. Clinically, it is also a common observation that female patients have a much higher risk than males for SGA-induced weight gain and other metabolic side-effects [67-70]. Previous studies have shown that betahistine could reduce olanzapine-induced weight gain in both male and female schizophrenia patients [26], as well as in female rats [27]. Betahistine is also effective to inhibit food intake in male rats [33]. Therefore, it is most likely that betahistine co-treatment is also effective to ameliorate olanzapine-induced dyslipidemia in male rats, although future study in male rat model is necessary.

Conclusion

This study provided further evidence that chronic olanzapine induces accumulation of hepatic lipid. More importantly, co-treatment with betahistine appears to be effective in ameliorating

olanzapine-induced dyslipidemia through modulation of the AMPK α -SREBP-1 and PPAR α -dependent pathways (Fig. 6). A great deal of further work needs to be carried out to delineate not only the precise molecular mechanisms by which betahistine activates AMPK α to regulate lipolysis, but also the signalling pathways that link hepatic H1R with AMPK α . Furthermore, clinical trials have shown that short-term betahistine co-treatment produces a clinically significant weight-attenuating effect in schizophrenia patients treated with olanzapine [26, 28]. Therefore, further studies are important to identify whether betahistine is also effective to control dyslipidemia caused by olanzapine and other SGAs in patients.

Contributors

Chao Deng and Xuemei Liu managed the literature searches designed the experiment. Jiamei Lian performed the animal treatment. Xuemei Liu and Jiamei Lian performed lipid assays and Q-PCR. Xuemei Liu and Chang-Hua Hu performed Western blot. Xuemei Liu conducted statistical analysis and prepared the initial draft of the manuscript. All authors contributed to and have approved the final manuscript.

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717 **Chemical compounds**

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719 Betahistine (PubChemCID: 2366)

720 Olanzapine (PubChem CID: 4585)

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Figure Legends

Figure 1 Outline of the experimental design.

Figure 2 O+B co-treatment reduced liver fat accumulation induced by olanzapine. Representative images of randomly selected sections of liver stained for ORO in rats treated with drugs. A-D: Representative images counterstained with hematoxylin; E-H: (ORO)-stained histological sections of livers of rats treated with Vehicle (E), Olanzapine-only (F), Betahistine-only (G), and O+B co-treatment (H). Intense red color indicates the presence of neutral lipids, mainly triacylglycerols. Scale bars, 100 μ m. Inset images were magnified eight times in order to highlight the lipid-staining morphology. (I) Lipid level of liver. The data from ORO staining are shown as mean \pm SEM. O+B: olanzapine and betahistine co-treatment; ORO: Oil-Red-O. ***, $p < 0.001$; **, $p < 0.01$ vs. control group. #, $p < 0.05$; ##, $p < 0.01$ vs. olanzapine-only group.

Figure 3 Olanzapine stimulated the hepatic lipogenesis related SREBP-1 pathway. (A) *Srebp-1*, (B) *Acc1*, (C) *Fasn*, and (D) *Scd1* mRNA expression shown relative to untreated controls; (E) The mature form of hepatic SREBP-1 protein levels shown relative to untreated controls; (F) the ratio of m-SREBP-1 over p-SREBP-1 protein levels; (G) Representative western-blotting images. m-SREBP-1 and p-SREBP-1 denote the mature, nuclear (~68 kDa) and the precursor (~125 kDa) forms of SREBP-1. Data represent mean \pm SEM (n=6).

per group). O+B: olanzapine and betahistine co-treatment. *, $p < 0.05$; **, $p < 0.01$ vs. control group. #, $p < 0.05$; ##, $p < 0.01$ vs. olanzapine-only group.

Figure 4 Effects of chronic olanzapine and/or betahistine treatment on the PPAR α -dependent pathway. (A) *Ppara* and (B) *Cpt1a* mRNA expression shown relative to untreated controls; (C) Representative western-blotting images; Protein levels of (D) PPAR α , (E) CPT1A, and (F) ATGL shown relative to untreated controls. Data represent mean \pm SEM (n=6 per group). *, $p < 0.05$; **, $p < 0.01$ vs. control group. #, $p < 0.05$; ##, $p < 0.01$ vs. olanzapine-only group.

Figure 5 Impact of olanzapine and/or betahistine treatment on the AMPK signalling pathway in the liver. Protein levels of (A) AMPK α , (B) pAMPK α , (C) the ratio of pAMPK α /AMPK α protein levels, (D) ACC1, (E) pACC1 (F) H1R and (G) H3R shown relative to untreated controls; (H) Representative western-blotting images. Data represent mean \pm SEM (n=6 per group). *, $p < 0.05$; **, $p < 0.01$ vs. control group. #, $p < 0.05$; ##, $p < 0.01$ vs. olanzapine-only group.

Figure 6 A schematic diagram of the proposed mechanisms for olanzapine-induced dyslipidemia and preventing effects of betahistine co-treatment. The solid arrow displays two proposed pathways causing hepatic metabolic disorder of lipid induced by chronic olanzapine treatment: I olanzapine may directly up-regulate the expression of *Srebp-1* and its target genes involved in lipogenesis, and down-regulate the expression of *Cpt1a* involved in fatty

acid β -oxidation; and II olanzapine may elevate the cleavage of p-SREBP-1 through acting at H1R. The dotted arrow displays possible pathways underlying betahistine-mediated improvement of hepatic dyslipidemia: betahistine-induced activation of AMPK α may ① decrease the activation of p-SREBP-1; ② lead to the phosphorylation of ACC1, with a drop in the inhibition of CPT1A, and therefore increasing β -oxidation of fatty acid; or ③ increase PPAR α levels to promote CPT1A expression, enhancing fatty acid oxidation. Betahistine co-treatment appears to be effective in ameliorating olanzapine-induced dyslipidemia through modulation of the AMPK α -SREBP-1 and PPAR α -dependent pathways.

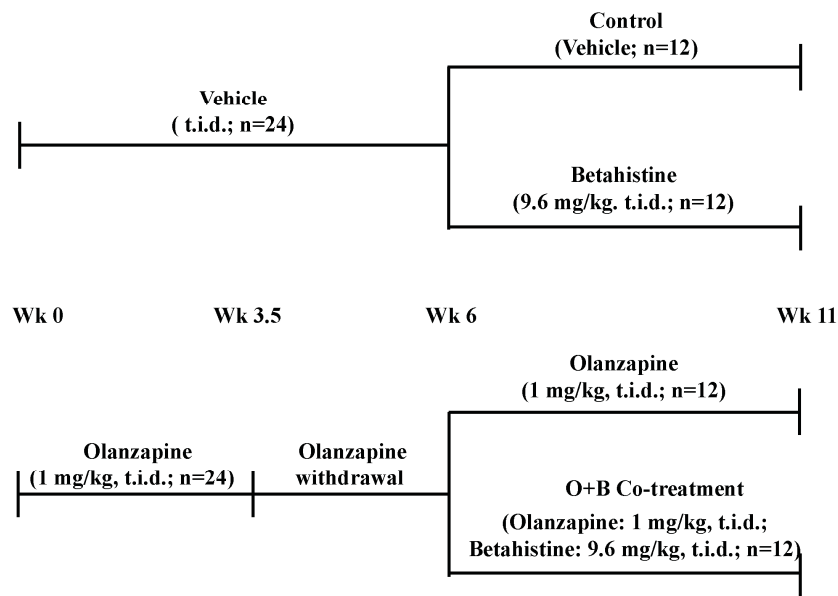
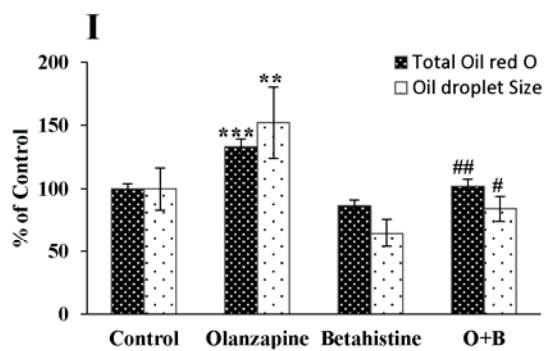
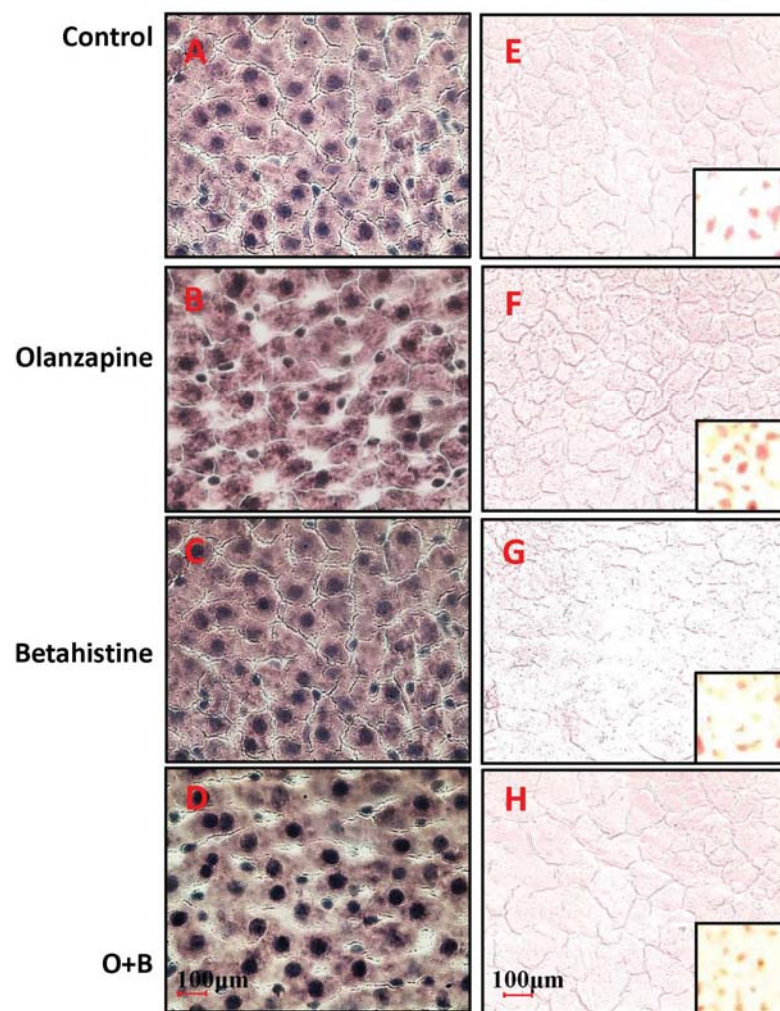


Fig. 1



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Fig. 2

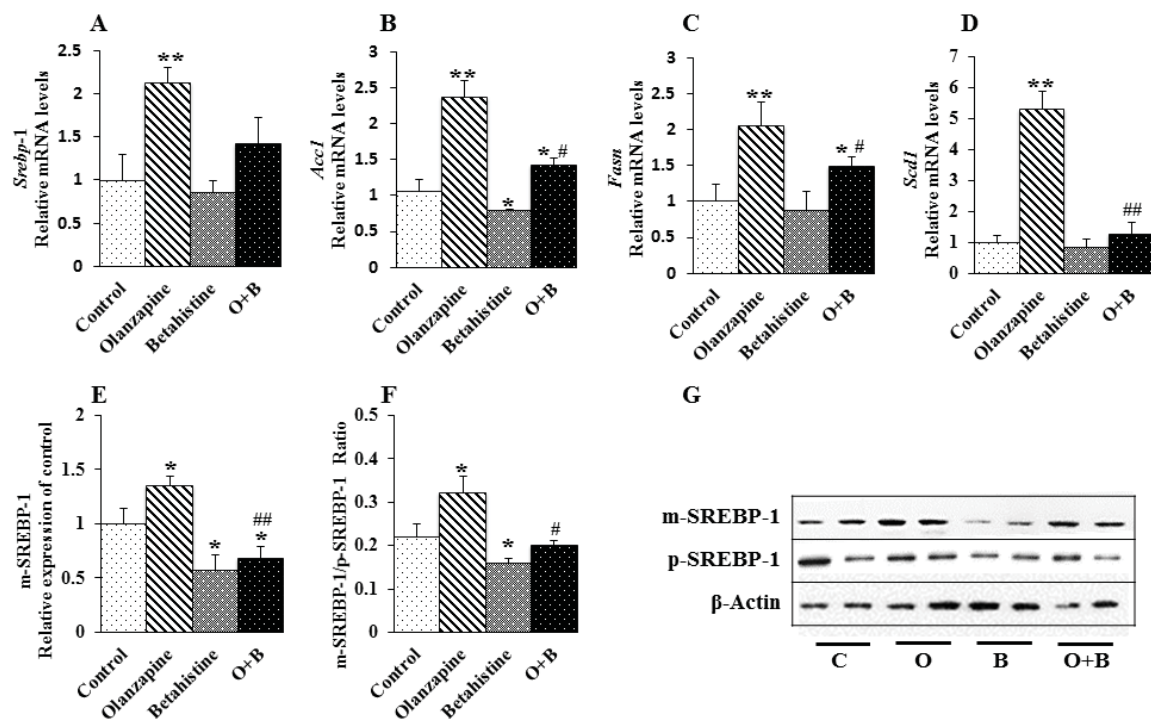


Fig. 3

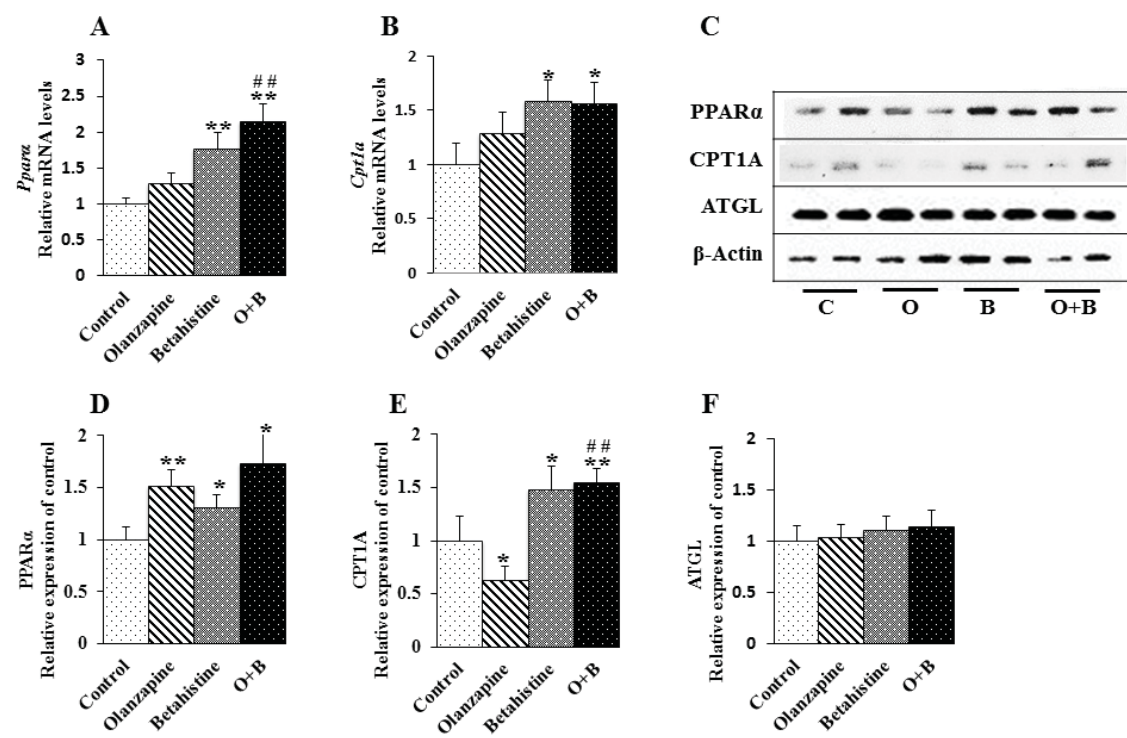


Fig. 4

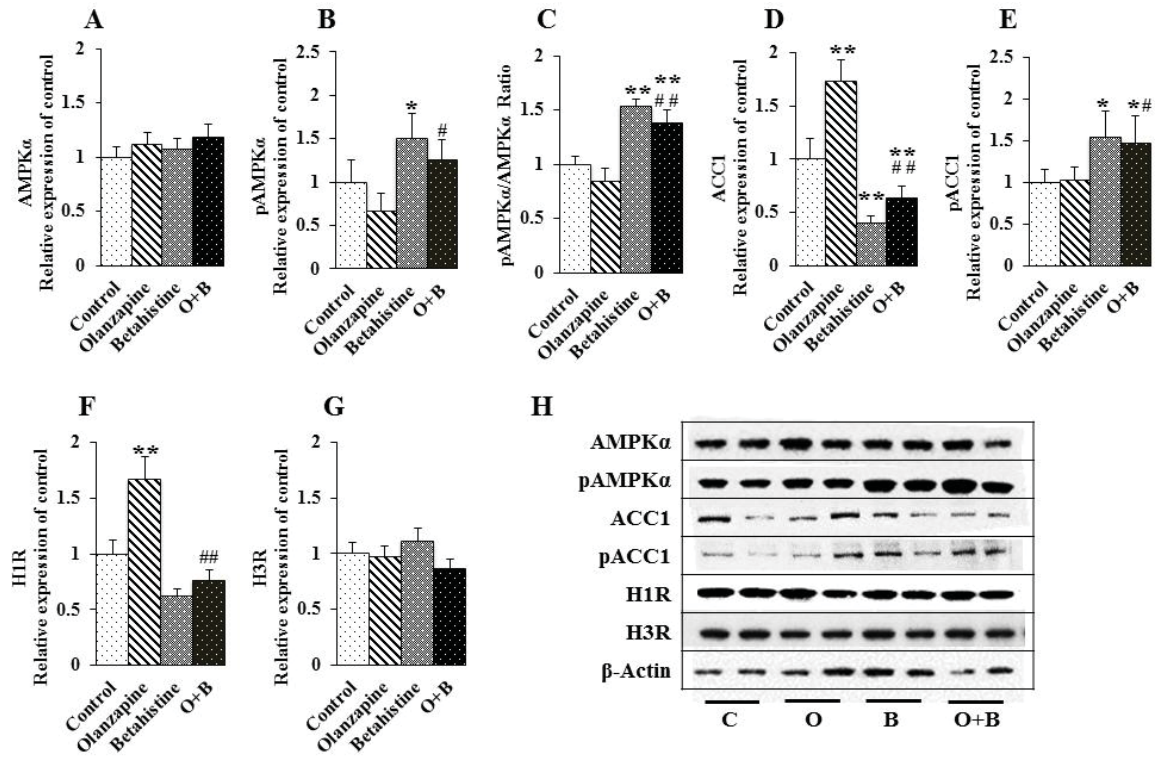
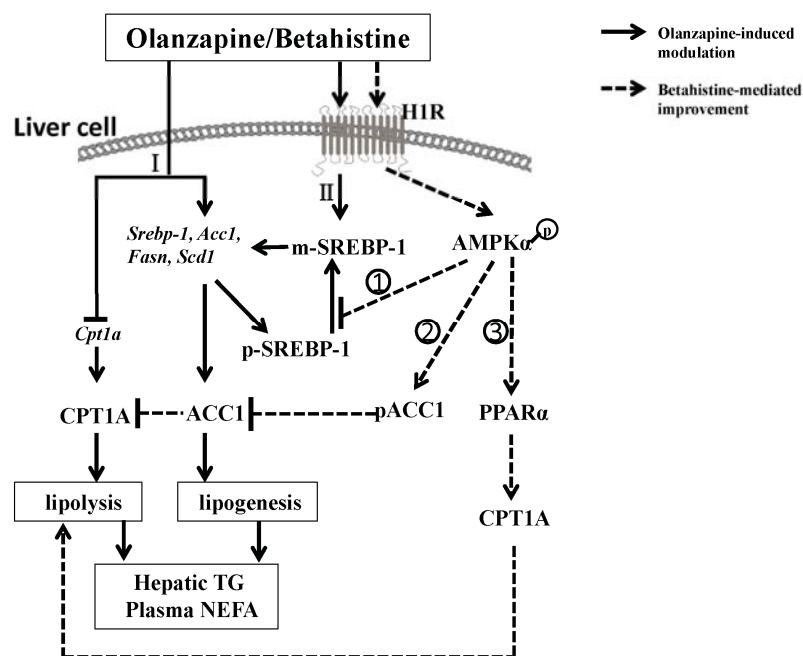


Fig. 5



Page 38



Graphical abstract

Table 1 Lipid response to the olanzapine and/or betahistine treatment in the plasma and the liver

	Marker	Control (n=12)	Olanzapine-only (n=12)	Betahistine-only (n=12)	O+B (n=12)
Plasma	Cholesterol (total), mmol/l	1.87±0.08	2.14±0.12	1.98±0.06	1.94±0.15
	Triglycerides, mmol/l	1.06±0.11	1.26±0.13*	0.85±0.11	1.05±0.08[#]
	HDL, mmol/l	1.00±0.04	0.95±0.09	1.13±0.06	1.02±0.04
	LDL, mmol/l	0.20±0.02	0.23±0.03	0.22±0.01	0.18±0.01
	NEFA, mEq/l	0.28±0.03	0.35±0.03*	0.22±0.03	0.24±0.02^{##}
Liver	Cholesterol (total), mmol/g	0.32±0.05	0.41±0.13	0.28±0.03	0.33±0.03
	Triglycerides, mg/g	7.91±0.85	11.20±1.01**	7.58±0.827	9.27±0.918[#]

LDL, low-density lipoprotein; HDL, high-density lipoprotein; NEFA, non-esterified fatty acids.

Hepatic lipids were extracted from control, olanzapine-treated, betahistine-treated, and O+B co-treatment rats and measured against known standards. Data are representative of independent measurements for each parameter (n=12). *, $p < 0.05$; **, $p < 0.01$ vs. control group. #, $p < 0.05$; ##, $p < 0.01$ vs. olanzapine-only group.

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