Atypical antipsychotic drugs perturb AMPK-dependent regulation of hepatic lipid metabolism

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Running title: Antipsychotics inhibit AMPK activity in hepatocytes.

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Abstract

Dysregulation of lipid metabolism is a key feature of metabolic disorder related with side effects of antipsychotic drugs. Here, we investigated the molecular mechanism by which second-generation atypical antipsychotic drugs (AAPDs) affect hepatic lipid metabolism in liver. AAPDs augmented hepatic lipid accumulation by activating expression of sterol regulatory element-binding protein (SREBP) transcription factors, with subsequent induction of downstream target genes involved in lipid and cholesterol synthesis in hepatocytes. We confirmed the direct involvement of SREBPs on AAPDs-induced expression of lipogenic and cholesterogenic genes by utilization of adenovirus for dominant negative SREBP (Ad-SREBP-DN). Interestingly, AAPDs significantly decreased phosphorylation of AMPK and expression of fatty acid oxidation genes. Treatment of constitutive active AMPK restored AAPDs-mediated dysregulation of genes involved in both lipid synthesis and fatty acid oxidation. Moreover, AAPDs decreased transcriptional activity of PPAR, a critical transcriptional regulator for controlling hepatic fatty acid oxidation, via AMPK-dependent manner. Close investigations revealed that mutations at the known p38 MAPK phosphorylation sites (S6/12/21A), but not mutations at the putative AMPKα phosphorylation sites (S167/373/453A), block AAPDs-dependent reduction of PPARα transcriptional activity, suggesting that p38 MAPK might be also involved in the regulatory pathway as a downstream effector of AAPDs-AMPK. Taken together, these data suggest that AAPDs-stimulated hepatic dysregulation of lipid metabolism could result from the inhibition of AMPK activity, and pharmaceutical means to potentiate AMPK activity would contribute to restore hepatic lipid homeostasis occurred during AAPDs treatment.

Key words: AAPDs, AMPK, hepatic lipid metabolism, SREBPs, PPARα
Introduction

Atypical antipsychotic drugs (AAPDs) have been used in the pharmacological treatment of schizophrenia (1). Although the second-generation AAPDs are much potent than the classical antipsychotics, treatment of AAPDs could cause troublesome side effects; obesity, insulin resistance, dyslipidemia, abnormal glucose tolerance, and diabetes (5, 11, 23, 36). Patients taking stable dose of clinically assigned OLZ or CLZ have significantly higher fat mass, plasma insulin, glucose, triglyceride and cholesterol levels (4, 5, 28). However, the exact molecular mechanism by which AAPDs lead to metabolic disorder remains unknown.

Dysregulation of lipid metabolism is a critical instigator for the metabolic disorder. Recently, it has been reported that antipsychotic drugs such as OLZ and CLZ activate maturation of sterol regulatory element binding proteins (SREBPs) in cultured liver cells, glioma cells, and adipocytes (6, 33, 41). The SREBPs are major transcriptional regulators of cellular lipid and cholesterol synthesis (12, 14). Therefore, it is plausible to suspect that SREBP-mediated stimulation of lipogenesis might be crucial for antipsychotic drugs-mediated dyslipidemia.

AMP-activated protein kinase (AMPK) plays a critical role in the regulation of hepatic energy metabolism (7, 31, 38). Activation of AMPK in the liver leads to the inhibition of lipogenesis as well as the stimulation of fatty acid oxidation. Inhibition of Acetyl CoA Carboxylase (ACC) by AMPK reduces malonyl-CoA content, resulting in a subsequent decrease in lipid synthesis and an increase in mitochondrial fatty acid oxidation. In addition, AMPK has been shown to directly inhibit expression and/or activity of SREBP1c (25, 31, 44, 48), and enhance activity of peroxisome proliferator-activated receptor α (PPARα), a critical transcriptional factor for fatty acid oxidation (2, 45). Failure of a proper regulation of such pathways by AMPK may contribute to the development of non-alcoholic fatty liver disease (NAFLD). Indeed, treatment of anti-diabetic drug metformin reduces hepatic steatosis via activation of AMPK, suggesting a critical role of AMPK in the regulation of hepatic lipid homeostasis (24, 47).

Here, we report that increased expression of SREBPs is the key mechanism for AAPDs-induced hepatic lipid synthesis. Furthermore, we show that AAPDs repressed AMPK activity to reduce PPARα-dependent fatty acid oxidation, and to enhance SREBP-dependent lipid synthesis. These data support that AMPK
activation would contribute to restore hepatic dysregulation of lipid metabolism occurred during AAPDs treatment.
Materials and Methods

Drug Preparation

For the cell culture studies, olanzapine (OLZ) (LY170053, 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno [2,3-b][1,5]benzodiazepine; Zyprexa-Velotab, Eli Lilly and Co.) and clozapine (CLZ) (Sigma) were dissolved in DMSO at the final concentration of 50mM. Cells were treated with 25 to 50 μM OLZ or CLZ for 24 h to 72 h. The concentration of drugs and the duration of the treatment were described in the figure legends in detail.

Animal Experiments

Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories, and were housed in a specific pathogen-free facility at the Sungkyunkwan University School of Medicine (12:12-h light-dark cycle). Clozapine was chronically self-administered in a small quantity of cookie dough with 0.1 mg/g dough to mice for 5 weeks. Control mice received an equal quantity of drug-free dough. All procedures were approved by the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee (IACUC).

Isolation and culture of rat primary hepatocytes

Primary hepatocytes were prepared from 200–300 g Sprague-Dawley rats by collagenase perfusion method as described (43). Cells were plated in medium 199 (Sigma) supplemented by 10% FBS, 10 units/ml penicillin, 10 mg/ml streptomycin, and 10 nM dexamethasone. After attachment, cells were infected with various adenoviruses and treated with drugs as indicated in the figure legends.

Plasmids and Recombinant adenoviruses

PPAR\(\alpha\) mutants (S6/12/21A, S167/373/453A and both) were generated using site-directed mutagenesis (44). Expression plasmid for SREBP Y320A (SREBP-DN, amino acid 1-403) (18) was purchased from Addgene (Addgene plasmid 8885). Adenoviruses expressing GFP only, 6X SRE-1 luc, SREBP Y320A (SREBP-DN, amino acid 1-403), and AMPK\(\alpha\) T172D (AMPK\(\alpha\)-CA, amino acid 1-312) were generated as described previously (20).
Oil-red O staining
Rat primary hepatocytes were washed twice with PBS. Mono-layer cells were fixed on dishes with 3.7 % formaldehyde in PBS for 30 min. Cells were stained with 40% isopropanol containing 0.5% Oil-red O for 1 h, followed by rinsing in 60% isopropanol and dH2O to remove unbound dye. To quantify lipid accumulation, Oil-red O was extracted with 100 % isopropanol and the optical density (O.D) of the solution was detected at 500 nm.

Total RNA preparation and Quantitative PCR analysis
Total RNA from primary hepatocytes was extracted using RNA extraction kit (Intron). Complementary DNA (cDNA) generated by Reverse transcriptase (GenDEPOT) was analyzed by Q-PCR using a SYBR green PCR kit and a TP 800 Thermal Cycler Dice model (Takara) as described (21). Primers were designed using Oligoperaft Design (Invitrogen). The primers used for PCR are as follows;
L32, forward 5'-TCTGGTGAAGCCCAAGATCG-3' and reverse 5'-CTCTGGGTTTCCGCCAGTTT-3',
SREBP-1c, forward 5'-GTACCTGCGGGACAGCTTAG-3' and reverse 5'-TCAGGTCTAGTTGGAAAACCA-3',
SREBP-2, forward 5'-GCCTTCTGGAGACCATGGA-3' and reverse 5'-ACAAGTTGCTCTGAAAACAAAATGA-3',
ACCo, forward 5'-GCGGGAGGAGTTCCTAATTC-3' and reverse 5'-TGTCAGCACGTGCTT TTC-3',
MCAD, forward 5'-GCTAGTGGAGCAGGAAGGAG-3' and reverse 5'-CCTTCGCAATAGGCCAAGAG-3',
HMG CoA Reductase, forward 5'-CTTGTGGAATGCCTTGATT-3' and reverse 5'-AGCCGAAGCAGCACATGAT-3',
FAS, forward 5'-CGACCTCGCAGGGATACC-3' and reverse 5'-TTCACGAATGGGTAGCACCAG-3',
LDLR, forward 5'-AGGCTGTGGGCTCCATAGG-3' and reverse 5'-TGCGGTCCAGGGTGATCT-3',
HMG CoA Synthase, forward 5'-GCCGTGGAGGTTCCTAATTC-3' and reverse 5'-GCATATAGCTCGAGGCTT-3',
SPOT14, forward 5'-CCCAGCTTCTGAGGGATGT-3' and reverse 5'-GCCGTGCTCCTACCTACA-3',
CPT-1α, forward 5'-GTCTGAGCCATGGAGGTTGT-3' and reverse 5'-
GGCTTGTCTCAAGTGCTTCC-3',
PPARα, forward 5'-AGAAGTTGCAGGAGGGATT-3' and reverse 5'-
TCGGACTCGGTCTTCTTGAT-3',
PGC-1α, forward 5'-GCCTATGAGCACGAAAGGCT-3' and reverse 5'-
GCGCTCTTCAATTGCTTTCT-3'.
All data was normalized to ribosomal L32 expression.

Western blot analysis
Western blot analyses on 10-60ug of protein extracts from cells and liver
tissue were performed as described (35). Antibodies for phospho-Ser79 ACC,
ACC, phospho-Thr172 AMPKα, AMPKα, phospho-Thr389 p70 S6 Kinase, p70
S6 Kinase, and phospho-Thr37/46 4E-BP1 were obtained from Cell signaling
technology. HSP90α/β was used as a loading control and its antibody was
purchased from Santa Cruz.

Cell culture and transient transfection assays
Human hepatoma HepG2 cells were maintained with Ham's F12 medium
(cellgro) supplemented with 10 % FBS, 10 units/ml penicillin, and 10 mg/ml
streptomycin as described (35). For transfection, we used TransIT-LT1 reagent
(Mirus) with 300 ng (3X PPRE :5'-TGACCTXTGACCT-3')/ 150 ng (LXRE :5'-
AGGTCAAXXXXAGGTCA-3') luciferase construct, 50 ng β-galactosidase
plasmid and 2.5 ng PPARα, 100 ng AMPKα2 T172D/ 1-5 ng pcHA, pcMX, LXR,
RXR expression vectors. Mutant PPARα constructs were kindly provided by Dr.
Jae-Bum Kim. Cells were treated with either DMSO or 25-50 μM OLZ for 24 h.
Luciferase activity was normalized to β-galactosidase activity.

De novo Lipogenesis
De novo lipogenesis (DNL) activities were determined by the incorporation of
[14C]acetate into the lipids (16). Rat primary hepatocytes were plated in
medium 199 and cultured overnight at 37°C. After the incubation period, the
hepatocytes were treated with or without OLZ for 24h in serum-free or serum-
containing media. Cells were labeled with 2μCi [2-14C] sodium acetate
(American radiolabeled chemicals, St. Louis, MO) for 1 h before being
harvested. Lipids were extracted from the cells with methanol and chloroform
according to the Folch method (8). Radioactivities in the lipid fraction were counted with a liquid scintillation counter.

**Measurement of metabolites**

Blood glucose for basal conditions was monitored from tail-vein blood using an automatic glucose monitor (OneTouch; LifeScan, Inc.). Plasma and liver triglyceride levels, and 3-hydroxybutyric acid (BOH) were measured by colorimetric assay kits (TG; Wako and BOH; BioAssay System). Total liver lipids were extracted with chloroform-methanol (2:1. v/v) mixture according to Folch method (8).

**Statistical analysis**

Results are shown as mean ± SD. Differences between two groups were assessed using two-tailed unpaired Student’s t test, and comparison among more than two groups were carried out using one-way analysis of variance (ANOVA) with the Tukey’s multiple comparison test. A P value less than 0.05 was considered statistically significant and indicated in the figure.
Results

Atypical antipsychotics (AAPDs) promote lipid synthesis through regulation of SREBP transcription in primary hepatocytes.

Recently, it was shown that the AAPDs-dependent induction of hepatic lipogenic and cholesterogenic gene expression is mediated by SREBP proteins in cultured hepatocytes, suggesting that AAPDs might directly promote lipid synthesis in peripheral tissues (33). In line with previous reports, olanzapine (OLZ) treatment promoted lipid accumulation and *de novo* lipogenesis in primary hepatocytes (Fig. 1A ad 1B). We were also able to observe an increase in expression of key genes in the fatty acid synthesis (SREBP1c, ACC, fatty acid synthase (FAS), and SPOT14) and cholesterol metabolism (SREBP2, low density lipoprotein receptor (LDLR), HMG CoA Synthase, and HMG CoA Reductase) by either OLZ or clozapine (CLZ) in primary hepatocytes, suggesting that AAPDs are able to modulate hepatic lipid synthesis via a transcriptional mechanism (Fig. 1C). To directly assess the role of SREBPs on AAPDs-induced expression of hepatic lipid synthetic genes, we generated adenovirus for dominant negative SREBP (SREBP-DN) and infected it into rat primary hepatocytes (Fig. 2A). AAPDs-induced expression of lipogenic (ACCα and FAS) and cholesterogenic (LDLR and HMG CoA Reductase) genes was suppressed by SREBP-DN (Fig. 2A), suggesting that the effect of OLZ might be directly mediated by SREBPs. To further investigate the OLZ-dependent transcriptional regulation of SREBP target genes, we generated adenovirus for a luciferase reporter containing a sterol regulatory element (SRE-1), and tested it in rat primary hepatocytes (Fig. 2B). Indeed, AAPDs-dependent induction of SRE-1 promoter activity was decreased by 3.5 fold in the presence of Ad-SREBP-DN (Fig. 2B), showing that AAPDs regulate hepatic lipid synthesis by controlling transcriptional activity of SREBP.

AAPDs inhibit expression of fatty acid oxidation genes in primary hepatocytes.

Hepatic lipid metabolism is tightly regulated by a balance between energy-requiring lipid synthetic pathway (anabolism) and energy-releasing β-oxidation pathway (catabolism). Thus, we attempted to investigate whether AAPDs-dependent accumulation of hepatic lipid can also result from the decreased fatty acid oxidation. Indeed, genes involved in the fatty acid oxidation such as PGC-
carnitine palmitoyltransferase1 (CPT1α), and medium chain acyl CoA dehydrogenase (MCAD) were all down-regulated by either OLZ or CLZ treatment in primary hepatocytes (Fig. 3A). To test a potential role of SREBPs on AAPDs-dependent regulation of fatty acid oxidation genes, primary hepatocytes were infected with Ad-SREBP-DN in the presence of OLZ. However, unlike the case for lipogenic or cholesterogenic genes (Fig. 2A), infection of Ad-SREBP-DN did not restore the OLZ-dependent changes in expression of fatty acid oxidation genes, suggesting that SREBPs are not directly involved in AAPDs-dependent regulation of fatty acid oxidation (Fig. 3B).

AAPDs target AMPK to perturb hepatic lipid homeostasis

Activation of AMP-activated protein kinase (AMPK) in liver leads to both stimulation of fatty acid oxidation and inhibition of lipid and cholesterol synthesis, suggesting that AAPDs may perturb hepatic lipid metabolism by regulating AMPK activity (34, 47). Indeed, AAPDs treatment reduced AMPK activity as evidenced by reduced phosphorylation of AMPK at Thr 172 and ACC at Ser 79 (Fig. 4A). Furthermore, phosphorylation of RPS6-p70-protein kinase (S6K1) and eIF4E-binding protein 1 (4E-BP1), downstream targets of mammalian target of rapamycin (mTOR), was also induced, indicating that AAPDs also inhibit AMPK-dependent down-regulation of mTOR activity (Fig. 4B). To directly test whether AAPDs-mediated suppression of AMPK activity is the main cause for the dysregulation of lipid homeostasis, we utilized adenovirus expressing constitutively active AMPK (Ad-AMPK-CA). Infection of Ad-AMPK-CA did not affect basal expression levels of lipogenic and cholesterogenic genes, perhaps due to the minimal expression of such genes in the absence of insulin or OLZ (data not shown). However, we were able to observe that OLZ or insulin-dependent induction of SREBPs and their target gene expression were significantly reduced in the presence of AMPK-CA. (Fig. 4C). Similarly, infection of Ad AMPK-CA relieved basal or OLZ-mediated repression of fatty acid oxidation genes in primary hepatocytes (Fig. 4D). These data confirmed that AAPDs target AMPK to disturb hepatic lipid homeostasis.

AMPK restores AAPDs-mediated inhibition on transcriptional activity of PPARα

PPARα is a critical transcriptional regulator for controlling hepatic fatty acid oxidation (15, 46). AMPK has been shown to potentiate the activity of PPARα,
thus influencing expression of its downstream target genes (2, 42). To further understand the mechanism by which AAPDs-dependent repression of AMPK on expression of fatty acid oxidation genes, we tested whether AAPDs can directly reduce PPARα transcriptional activity. As expected, OLZ decreased ability of PPARα to transcriptionally stimulate PPRE promoter activity, whereas AMPK-CA expression restored it close to the normal level (Fig. 5A), suggesting that PPARα is a potential proximal target of AAPDs. To investigate the molecular mechanism by which AAPDs and AMPK regulate transcriptional activity of PPARα, we generated various PPARα expression constructs that carry mutations on putative AMPK phosphorylation sites (S167/373/453A), verified p38 MAPK phosphorylation sites (S6/12/21A), or both sites (double mutants). p38 MAPK was previously shown to be a downstream of AMPK, and could affect transcriptional activity of PPARα (42). As shown in figure 5B, OLZ could still reduce the transcriptional activity of S167/373/437A mutant. However, OLZ was unable to repress the transcriptional activity of either S6/12/21A mutant or double mutant, suggesting that AAPDs-dependent down-regulation of PPARα activity is mediated via AMPK-p38 MAPK pathway.

AAPDs regulate hepatic lipid metabolism in vivo.

Finally, in order to confirm the effects of AAPDs on hepatic lipid metabolism in vivo, we fed wild type C57BL/6 mice with either control diet or CLZ-containing diet. Five-week feeding of CLZ significantly increased serum triacylglycerol (TG) levels as well as hepatic TG contents (Fig. 6A). Interestingly, body weight was not altered by CLZ consumption, indicating that five-week period may not be enough to provoke changes in body weight (Fig. 6B, left). Alternatively, a balance between physical activity and food consumption possibly hinders the detection of subtle changes in total body mass. Blood glucose levels and serum beta hydroxybutyrate tend to be lower in the CLZ-fed group than in the control, although it did not reach the statistical significance (Fig. 6B, middle and right). As in the case for in vitro studies, CLZ-feeding greatly induced expression of both lipogenic genes (SREBP1c, ACCα, and FAS) and cholesterogenic genes (SREBP2, LDLR, and HMG CoA Red) (Fig. 6C). On the other hand, no significant changes were shown on the expression levels of fatty acid oxidation genes (PGC-1α, and MCAD), which was in line with subtle changes in serum ketone bodies upon CLZ feeding. CLZ-feeding also affected phosphorylation of hepatic AMPK slightly (Fig. 6D). These data suggest that while CLZ-feeding is
able to induce hepatic lipogenic program \textit{in vivo}, potential secondary effects from other tissues may hinder the detection of subtle changes in lipid metabolism in liver. Collectively, we propose that AAPDs promote hepatic lipid accumulation in part via a direct action on peripheral AMPK signalings and SREBP-mediated transcriptional cascades \textit{in vivo}.
The present study reports a novel role of AMPK in AAPDs-induced lipid accumulation in hepatocytes. It has recently reported that OLZ treatment could cause metabolic disorders such as dyslipidemia (5, 23). AAPDs also stimulate lipogenesis in cultured adipocytes, glioma cells, and hepatocytes, hinting that SREBP-mediated stimulation of lipogenesis in peripheral tissues could be a major mechanism for antipsychotic drugs–mediated metabolic side effects (6, 32, 41). Our data show that AAPDs increase expression of SREBP-1c and its downstream target genes such as ACCα and FAS, and expression of SREBP-2 and its downstream cholesterogenic genes such as HMG CoA Reductase, HMG CoA Synthase, and LDLR. These data suggest that both SREBP-1c and 2 are involved in AAPDs-induced dysregulation of lipid synthesis in liver.

We confirmed the direct involvement of SREBPs on AAPDs-dependent induction of lipogenic and cholesterogenic gene expression by showing that SREBP-DN blocks AAPDs-mediated effects in hepatocytes. However, the exact mechanism by which AAPDs activate expression of both SREBPs was not elucidated in the current study. It is possible that AAPDs-mediated induction of SREBP processing might be critical in activating its own transcription. Alternatively, AAPDs could activate other known transcription factors that activate SREBP expression.

Hepatic lipid homeostasis is tightly maintained by a balance between energy-requiring lipid synthetic pathway and energy-releasing fatty acid oxidation pathway. AMPK is shown to reduce lipid synthesis by suppressing SREBP activity and induce fatty acid oxidation in liver (9, 24, 47, 48). Therefore, the activation of AMPK may improve hyperlipidemia and the progression of the non-alcoholic fatty liver (NAFLD) (34). We found that AAPDs were able to inhibit AMPK signalings in liver, as evidenced by the reduced phosphorylation levels of AMPKα and ACC, as well as enhanced phosphorylation of S6K1 and 4E-BP1, direct substrates for mTOR complex1 (Fig. 4). Interestingly, recent reports suggest that mTOR signaling is responsible for the transcriptional activation of SREBP-1c and hepatic lipogenesis (22). Since AMPK was known to inhibit mTOR complex activity by direct phosphorylation of raptor (13), it is possible to postulate that AAPDs-dependent activation of hepatic lipogenesis might stem from the activation of mTOR complex1 due to the reduced AMPK activity. Further study is necessary to assess the potential contribution of AAPDs on
lipid metabolism from this pathway. As mentioned previously, AMPK exerts the effects on target gene expression by modification of transcriptional machinery. It is reported that AMPK potentiates the transcriptional activity of PPARα on downstream target genes (42). Our data show that AAPDs decreased transcriptional activity of PPARα, however, unlike the case for SREBPs, it did not affect the expression of PPARα. Rather, AAPDs-mediated reduction of PPARα transcriptional activity was recovered by AMPK-CA expression (Fig. 5), suggesting that AAPDs reduced PPARα activity by regulating AMPK signaling.

Unlike the inhibitory effects of AAPDs on hepatic AMPK activity, recent reports suggest that hypothalamic AMPK activity was rather enhanced upon acute CLZ or OLZ infusion (19, 26). The differences might stem from the duration of drug treatment; while we performed longer term treatment of AAPDs (24-72 h for hepatocytes, and 5 weeks for feeding studies), they focused more on the acute effect of AAPDs on hypothalamic AMPK activity. Alternatively, AAPDs may utilize tissue-specific mechanisms to affect AMPK activity. While AAPDs activate AMPK through histamine H1 receptor in hypothalamus, it is unlikely that hepatic AMPK is also influenced by the same mechanism due to the lack of expression of histamine H1 receptor in liver. Interestingly, leptin is shown to influence AMPK activity differentially in a tissue-specific manner. While leptin increases AMPK activity in the peripheral tissues such as the skeletal muscle, it reduces AMPK activity in the arcuate nucleus and paraventricular nucleus in the hypothalamus (27, 37), indicating that central and peripheral AMPK activities can be differentially regulated by the same stimulus. Further study is necessary to identify the potential receptor(s) for AAPDs in peripheral tissues.

The cannabinoid receptors (CB receptors) are a class of cell membrane receptors that belong to the G-protein coupled receptor (GPCR) superfamily. There are currently two known subtypes, termed CB1 and CB2. CB1 receptors (CB1R) are expressed at high levels in the brain but are also present at much lower levels in peripheral tissues (10, 40). The activation of hepatic CB1R was shown to stimulate expression of SREBP-1c and its downstream target genes, and promote de novo lipid synthesis (29). In addition, endocannabinoids and CB1R could inhibit AMPK activity and repress fatty acid oxidation in liver (17, 30). Therefore, it is possible that AAPDs might serve as a CB1R agonist and activate hepatic CB1R. In line with this idea, Weston and colleagues demonstrated that OLZ treatment significantly decreased cannabinoid receptor
binding in the dorsal vagal complex (39). These data suggest that AAPDs-induced hepatic lipid synthesis might be regulated by the modulation of CB1R. Investigations are underway to test whether hepatic CB1R are directive targets of AAPDs.

In summary, our data describe that a novel relationship between AAPDs and AMPK on AAPDs-mediated dysregulation of lipid metabolism in liver (Fig. 7). Thus, one could speculate that co-treatment of patients with metformin, a known pharmacological drug to activate AMPK signaling, may be useful to prevent hepatic dysregulation of lipid metabolism that seem to occur during AAPDs treatment. To understand AAPDs-induced signaling pathway, it is necessary to further delineate AAPDs targets both in central and peripheral tissues in in vivo models. These studies will help to further extend our understanding on how AAPDs perturb hepatic lipid metabolism to promote dyslipidemia-related syndrome, and provide clues for relieving these side effects of atypical antipsychotic drugs.
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References


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Legends

Figure 1. AAPDs stimulate lipogenesis in primary hepatocytes.
A) Oil red O staining and optical density (O.D.) value showing hepatic lipid accumulation. Rat primary hepatocytes were treated with DMSO or 50 μM olanzapine for 72 h. B) OLZ-induced de novo lipogenesis in rat primary hepatocytes. C-D) Q-PCR analysis showing effects of OLZ on expression of lipogenic (SREBP-1c, FAS, ACCα, and SPOT14) and cholesterogenic (SREBP2, LDLR, HMG CoA reductase, and HMG CoA synthase) genes in rat primary hepatocytes. Cells were treated with DMSO, 25 μM OLZ (C) or 50 μM CLZ (D) for 24 h. Data in A - D represent mean ± S.D.

Figure 2. AAPDs increase expression of lipid synthetic genes via SREBPs.
A) Q-PCR analysis showing effects of GFP or SREBP-DN adenovirus on OLZ-dependent increase of lipogenic (ACCα and FAS) and cholesterogenic (LDLR and HMG CoA reductase) gene expression. Rat primary hepatocytes were infected with Ad-GFP or Ad-SREBP-DN, and then treated with DMSO, 50 μM OLZ, or 100 nM insulin for 24 h. B) Promoter assays showing effect of SREBP-DN on OLZ-induced Ad-SRE-1 luciferase activity in rat primary hepatocytes. Data represent mean ± S.D.

Figure 3. AAPDs decrease expression of fatty acid oxidation genes.
A) Q-PCR analysis showing effects of AAPDs on expression of fatty acid-oxidation genes (PGC1α, PPARα, CPT1α, and MCAD). Cells were treated with DMSO, 25 μM OLZ or 50 μM CLZ for 24 h. B) Q-PCR analysis showing effects of Ad-GFP or Ad-SREBP-DN on OLZ or insulin-reduced expression of fatty acid-oxidation genes (PPARα, CPT1α, and MCAD). Data represent mean ± S.D.

Figure 4. AAPDs decrease AMPK signaling to perturb lipid metabolism in primary hepatocytes.
A) Western blot analysis showing effects of AAPDs on total and phospho-serine/threonine levels for AMPKα (top) and ACC (bottom). Rat primary hepatocytes were treated with DMSO, 25 - 50 μM OLZ or 50 μM CLZ for 24 h. Representative data from five independent experiments was shown. B) Western blot analysis showing effects of OLZ on mTOR activation by examining phospho-S6K1 and phospho-4E-BP1. Rat primary hepatocytes were treated
with DMSO or 50 μM OLZ for 24 h. Representative data from three independent experiments was shown. C -D) Rat primary hepatocytes were infected with Ad-GFP or Ad-AMPK CA, and then treated with DMSO or 50 μM OLZ for 24 h. Q-PCR analysis showing effects of Ad-AMPK CA on OLZ-induced expression of lipid synthetic genes (C) and fatty acid-oxidation genes (PPARα, CPT1α, and MCAD) (D). Data in C and D represent mean ± S.D.

**Figure 5. AMPK restores AAPDs-mediated inhibition on transcriptional activity of PPARα.**

A) Transient transfection assay showing effects of Ad-AMPK CA on OLZ-reduced transcriptional activity of PPARα. HepG2 cells were treated with DMSO or 50 μM OLZ for 24 h after co-transfection of 3X PPRE-TK luciferase construct with pcf, PPARα WT, or AMPK-CA. Representative data from six independent experiments (n = 3 for each condition) was shown. B) Transient transfection assay showing effects of mutation of putative AMPK phosphorylation sites (S167/373/453A), known p38 MAPK phosphorylation sites (S6/12/21A), or both sites (double mutants) on PPARα. HepG2 cells were treated with DMSO or 50 μM OLZ for 24 h after co-transfection of 3X PPRE-TK luciferase construct with pcf, wild type or mutant forms of PPARα. Representative data from three independent experiments (n = 3 for each condition) was shown. Data represent mean ± S.D.

**Figure 6. AAPDs regulate lipid metabolism in vivo.**

8-week-old male C57BL/6 mice were provided with dough alone or dough containing 0.1mg/g of CLZ for 5 weeks. A) Effects of CLZ on plasma and liver triglyceride levels in mice (n=5). B) Effects of CLZ on body weight, blood glucose and serum 3-hydroxybutyric acid (BOH) level in mice (n=5). C) Q-PCR analysis showing effects of CLZ on expression of lipid synthetic and fatty acid oxidation genes in mice (n=4). D) Western blot analysis showing effects of CLZ on total and phospho-Ser/Thr protein levels of AMPKα in mice. Representative data from three independent experiments (n = 4 for each condition) was shown. Data in A – C represent mean ± SD.

**Figure 7. A proposed model of AAPDs-dependent regulation of AMPK affecting lipid synthesis and fatty acid oxidation in liver.**
AAPDs increase mRNA levels of SREBP\(\alpha\)s to enhance lipid synthesis, whereas they decrease PPAR\(\alpha\)-mediated fatty acid oxidation program by reducing AMPK signaling. SREBP-induced lipid synthesis might be further enhanced due to the reduction in AMPK activity.
Figure 1. AAPDs stimulate lipogenesis in rat primary hepatocytes.
Figure 2. AAPDs increase expression of lipid synthetic genes via SREBP.
Figure 3. AAPDs decrease expression of fatty acid oxidation genes in SREBP-independent manner.
Figure 4. AAPDs decrease AMPK signaling to perturb lipid homeostasis in rat primary hepatocytes

A

![Image of Western blots showing p-AMPK, AMPK, and HSP90 levels with OLZ at 0, 25, and 50 uM and CLZ at 0 and 50 uM.]

B

![Image of Western blots showing p-AMPK, AMPK, and HSP90 levels with OLZ at 0, 50 uM and CLZ at 0 and 50 uM.]

C

![Graphs showing mRNA levels of SREBP1c, ACCα, SREBP2, LDLR, FAS, HMG-CoA Syn with GFP and AMPK-CA.]

D

![Graphs showing mRNA levels of PPARα, CPT1α, MCAD with DMSO and OLZ.]

* p<0.05, ** p<0.001
Figure 5. AMPK restores AAPDs-mediated inhibition on transcriptional activity of PPARα.

A

![Graph showing luciferase activity](image)

B

![Bar graph showing fold induction](image)
Figure 6. AAPDs regulate lipid metabolism in vivo.

A

![Bar graph showing serum and liver TG levels with CON and CLZ groups, with p-values indicated.]

B

![Bar graph showing body weight, blood glucose, and serum BOH levels with CON and CLZ groups.]

C

![Bar graph showing mRNA levels of SREBP1c, ACCα, LDLR, HMG CoA Red, PGC1α, and SREBP2 with CON and CLZ groups, with p-values indicated.]

D

![Western blot images of p-AMPK, AMPK, HSP90 with CON and CLZ conditions.]
Figure 7. A proposed model of AAPDs-dependent regulation of AMPK affecting lipid synthesis and fatty acid oxidation in liver.