

Original Article

Yhhu981, a novel compound, stimulates fatty acid oxidation via the activation of AMPK and ameliorates lipid metabolism disorder in *ob/ob* mice

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Aim: Defects in fatty acid metabolism contribute to the pathogenesis of insulin resistance and obesity. In this study, we investigated the effects of a novel compound yhhu981 on fatty acid metabolism *in vitro* and *in vivo*.

Methods: The capacity to stimulate fatty acid oxidation was assessed in C2C12 myotubes. The fatty acid synthesis was studied in HepG2 cells using isotope tracing. The phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) was examined with Western blot analysis. For *in vivo* experiments, *ob/ob* mice were orally treated with yhhu981 acutely (300 mg/kg) or chronically (150 or 300 mg·kg⁻¹·d⁻¹ for 22 d). On the last day of treatment, serum and tissue samples were collected for analysis.

Results: Yhhu981 (12.5–25 μmol/L) significantly increased fatty acid oxidation and the expression of related genes (*Sirt1*, *Pgc1α* and *Mcad*) in C2C12 myotubes, and inhibited fatty acid synthesis in HepG2 cells. Furthermore, yhhu981 dose-dependently increased the phosphorylation of AMPK and ACC in both C2C12 myotubes and HepG2 cells. Compound C, an AMPK inhibitor, blocked fatty acid oxidation in yhhu981-treated C2C12 myotubes and fatty acid synthesis decrease in yhhu981-treated HepG2 cells. Acute administration of yhhu981 decreased the respiratory exchange ratio in *ob/ob* mice, whereas chronic treatment with yhhu981 ameliorated the lipid abnormalities and ectopic lipid deposition in skeletal muscle and liver of *ob/ob* mice.

Conclusion: Yhhu981 is a potent compound that stimulates fatty acid oxidation, and exerts pleiotropic effects on lipid metabolism by activating AMPK.

Keywords: yhhu981; fatty acid oxidation; fatty acid synthesis; AMPK; compound C; sirtinol; EX527; STO609; AICAR; metformin; metabolism disorder; C2C12 myotubes; HepG2 cells; *ob/ob* mice

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Introduction

Lipid metabolism disorder is one of the leading causes of insulin resistance. Among the peripheral tissues involved in energy metabolism, skeletal muscle plays an important role in lipid homeostasis^[1]. In the presence of high concentrations of circulating non-esterified fatty acid (NEFA), lipid flux into skeletal muscle exceeds its oxidation capacity, leading to the ectopic accumulation of lipids and their metabolites, such as triglycerides, diacylglycerol and ceramide. These lipids are detrimental and ultimately impair insulin signaling pathways, contributing to skeletal muscle insulin resistance^[2]. In the hyperglycemic state, elevated glucose levels alter the metabolic partitioning of fatty acids by shifting toward their esteri-

fication and away from their oxidation^[3]. As a consequence, the direction of fatty acid metabolism switches from oxidation to synthesis, resulting in excess lipid accumulation in skeletal muscle^[4]. Therefore, the fine-tuning of fatty acid oxidation represents a particularly attractive strategy for ameliorating skeletal muscle insulin resistance.

Fatty acid oxidation is regulated by multiple factors, of which AMP-activated protein kinase (AMPK) is the most important. Acting as a major cellular energy sensor, AMPK activation induces a variety of beneficial effects on glucose and lipid metabolism in peripheral tissues, such as skeletal muscle, liver and adipose tissue^[5, 6]. Research has demonstrated that AMPK increases fatty acid oxidation by directly inhibiting acetyl-CoA carboxylase (ACC) and by stimulating malonyl CoA decarboxylase (MCD), two enzymes responsible for malonyl CoA synthesis and degradation, respectively^[7, 8]. The result of these actions is to cause a net reduction in malonyl CoA levels, a release of the malonyl CoA-mediated inhibition of CPT-1,

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and an increase in fatty acid β -oxidation by mitochondria^[7, 9]. In addition, AMPK can regulate fatty acid oxidation by activating another downstream regulator, Sirtuin 1 (SIRT1). SIRT1 is a NAD^+ -dependent histone deacetylase that regulates life-span and lipid metabolism by deacetylating lysine residues on various transcription factors^[10]. The activation of AMPK increases both the activity of SIRT1 by modulating nicotinamide phosphoribosyltransferase (NAMPT), an NAD^+ synthetic enzyme, and the expression of SIRT1 by modulating the state of FOXO1 phosphorylation^[11, 12]. Thus, the beneficial metabolic changes induced by AMPK activation have attracted intense interest in developing AMPK activators as potential therapeutics for type 2 diabetes mellitus (T2DM) and obesity.

Recently, several compounds have been reported to improve fatty acid oxidation, primarily by activating the AMPK signaling pathway. 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) is a commonly used AMPK activator. Once inside a cell, AICAR is metabolized into ZMP, an analog of AMP, leading to the activation of AMPK^[13]. The beneficial effects of metformin, the first-line anti-diabetic drug, are mediated primarily through its effects on AMPK activation^[14]. Moreover, berberine and resveratrol are two natural products that have been reported to exert beneficial effects as AMPK activators^[15, 16]. All of these compounds could stimulate AMPK activation, subsequently inhibiting ACC activity and leading to increased fatty acid oxidation. Furthermore, AICAR and resveratrol have been demonstrated to regulate fatty acid oxidation and mitochondrial function through the AMPK-SIRT1 pathway^[17, 18].

In the present study, we aimed to identify novel molecules with the capacity to regulate lipid metabolism. Thus, we screened a library of synthetic small molecules for their activity to stimulate fatty acid oxidation in C2C12 myotubes and identified the potent compound yhhu981. This compound was further characterized *in vitro* and *in vivo* for its effects on lipid metabolism.

Materials and methods

Materials

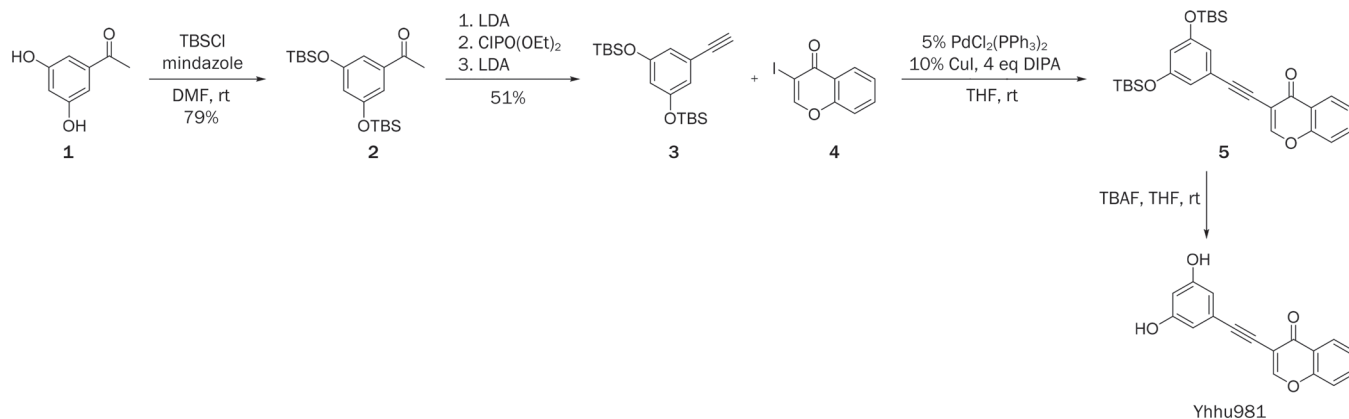
AICAR (5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside)

side) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sirtinol, EX527, STO-609, compound C and charcoal were purchased from Sigma (St Louis, MO, USA). Antibodies for phospho-AMPK(Thr172), AMPK, phospho-ACC (Ser79), ACC and GAPDH were from Cell Signaling Technology (Beverly, MA, USA). An anti-rabbit (IgG) secondary antibody conjugated to horseradish peroxidase was obtained from Bio-Rad Laboratories (Bio-Rad, CA, USA). The enhanced chemi-luminescence (ECL) plus Western Blotting Detection System was from GE Healthcare/Amersham Biosciences (Amersham, IL, USA). The vendors for all reagents and media used in cell experiments are as follows: Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM) and Fetal Bovine Serum (FBS) were from GIBCO (Carlsbad, CO, USA); TRIzol reagent was from Invitrogen (Camarillo, CA, USA); the reverse transcription kit (DRR037A) and real-time PCR detection kit (DRR041A) were from Takara Biotechnology (Dalian) Co, Ltd (Dalian, China); the primers were synthesized by Sangon Biotech Co, Ltd (Shanghai, China); the triglyceride kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); the serum free fatty acid (NEFA) test kit was from Wako Chemicals GmbH (Neuss, Germany); the serum triacylglycerol (TG) kit was from Wenzhou Dongoujinma Reagent Co, Ltd (Wenzhou, China); the ultra-sensitive mouse insulin ELISA kit was from Crystal Chem Inc (Downers Grove, IL, USA). All other reagents and chemicals were of the highest grade available commercially.

Synthetic route of yhhu981

Yhhu981 was prepared via the route shown in Scheme 1. Intermediates 2 and 3 were prepared according to a method previously reported in the literature^[19].

Compound 5. To a mixture of 3 (540 mg, 1.5 mmol), 4 (272 mg, 1 mmol), CuI (19 mg, 0.1 mmol) and $\text{PdCl}_2(\text{PPh}_3)_2$ (35 mg, 0.05 mmol) in THF (5 mL) was added DIPEA (0.7 mL, 4 mmol) under nitrogen. The resulting mixture was stirred overnight at room temperature. The solvent was removed and the residue was purified by column, eluted with hexane: ethyl acetate (4:1) to give product 5 (395 mg, 78%) as a white solid.



Scheme 1. Synthetic route of yhhu981.

Compound yhhu981. To a solution of 5 (250 mg, 0.5 mmol) in THF (5 mL) was added TBAF (260 mg, 1 mmol). The resulting mixture was stirred at room temperature for 3 h. The solvent was removed and the residue was purified by column, eluted with hexane: ethylacetate (2:1) to give product yhhu981 (118 mg, 85%) as a white solid with purity >95%.

C2C12 cell differentiation and fatty acid oxidation

The skeletal muscle cell line C2C12 myoblasts were maintained in DMEM supplemented with 10% FBS at 37°C with 95% air and 5% CO₂. Differentiation into myotubes was performed as previously described^[20]. Briefly, confluent cells were differentiated into myotubes by culturing with HG-DMEM containing 2% (*v/v*) horse serum (HS) for 5 d. Fatty acid oxidation was performed with differentiated C2C12 myotubes as previously described^[21]. Briefly, C2C12 myotubes in 12-well plates were serum starved overnight. The cells were then incubated with 0.5 mL DMEM, supplemented with [9, 10(*n*)-³H] palmitic acid (PerkinElmer, 2.5 μCi/mL media) along with yhhu981 or the indicated treatment. The medium was collected and 0.2 mL of the cell supernatant was mixed with 0.9 mL charcoal slurry (10 g charcoal powder mixed with 100 mL 0.02 mol/L Tris-HCl buffer, pH 7.5) and shaken for 30 min at room temperature. Next, the samples were centrifuged at 13000 r/min for 15 min, 0.2 mL of the supernatant was carefully withdrawn and added to 1 mL scintillation vials containing 0.8 mL of scintillation liquid and counted by liquid scintillation counting (PerkinElmer). The total cellular protein concentration was measured by the Bradford method (BioRad) and used for normalization.

HepG2 cell culture and fatty acid synthesis

The HepG2 cells were maintained in MEM supplemented with 10% FBS at 37°C with 95% air and 5% CO₂. Fatty acid synthesis was determined by measuring acetate incorporation into lipids. HepG2 cells were treated with various concentrations of yhhu981 or AICAR in serum-free MEM for 21 h, followed by co-incubation for 3 h in fresh medium containing [1-¹⁴C] acetate sodium salt (0.02 μCi/mL media) with or without indicated compound. Thereafter, cells were washed three times with ice-cold PBS and analyzed as previously described^[22].

Protein extraction and Western blot analysis

All protein extractions and Western blots were performed as previously described^[23]. After treatment with yhhu981 or the other compounds, C2C12 myotubes or HepG2 cells were harvested by scraping into ice-cold lysis buffer (20 mmol/L Tris, pH 7.8, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 1% Triton X-100, 10 mmol/L NaF, 0.5 mmol/L Na₃VO₄, 10% glycerol, 1 μg/mL leupeptin, 0.2 mmol/L PMSF, 1 μg/mL aprotinin, 1 mmol/L EDTA, 1 mmol/L DTT, 5 mmol/L pyrophosphate sodium, 1 mmol/L benzamidine) and homogenized. The homogenates were rotated for 1 h and centrifuged (15000×g for 10 min at 4°C). The supernatant was collected and the total protein concentration was measured by the Bradford method. An equal amount of protein was loaded and resolved by SDS-PAGE, transferred to polyvinylidene difluo-

ride membranes (Millipore, MA, USA), and blocked with 7.5% non-fat milk. Next, the membranes were blotted with primary antibodies against AMPK, phospho-AMPK (Thr172), ACC, phospho-ACC (Ser79), and GAPDH overnight at 4°C, followed by a 2-h incubation with the horseradish peroxidase conjugated secondary antibody. The immunoreactive proteins were detected by ECL plus Western Blotting Detection System, and the Western blot signals were quantified by densitometry (BioRad) and normalized to total protein or GAPDH.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from C2C12 myotubes using TRIzol reagent. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed via a two-step RT-PCR kit, followed by PCR using a SYBR[®] Premix Ex Taq[™] II kit and ABI Prism 7500 Sequence Detection System (Life Technologies Corporation, California, USA). The primer sequences for all genes are listed here: *Cpt1b* forward: 5'-TGGATTCTGTGCGGCCCTTATTG-3', reverse: 5'-TTTGCCTGGGATGCGTGTAGTGT-3'; *Pgc1* forward: 5'-GAAGCGGGAGTCTGAAA-3', reverse: 5'-GGTGTAAACGGTAGGTGATG-3'; *Mcad* forward: 5'-GAGAAGAAGGGTGACGAGTATGT-3', reverse: 5'-GGGTACTTTAGGATCTGGGTTAG-3'; *Sirt1* forward: 5'-AGGGAACCTTTGCCTCATCTA-3', reverse: 5'-GTGC-CACTGTCACCTGTTACTGC-3'; β-actin forward: 5'-TGCTGTCCCTGTATGCCTCTG-3', reverse: 5'-TTGATGTCACG-CACGATTTC-3'. The 7500 Fast System Software was used for data analysis. β-actin mRNA was used as an endogenous control to normalize expression levels. The data were presented as the fold change relative to the endogenous control.

Animal experiments

B6.V-*Lep^{ob}/Lep^{ob}* mice (from Jackson Laboratory, Bar Harbor, ME, USA) were bred in the Shanghai Institute of Materia Medica (SIMM), Chinese Academy of Sciences. The animals were individually housed under constant temperature and humidity on a 12-h light-dark cycle with free access to water and food. The mice were assigned to four groups (*n*=8) based on fasting blood glucose and body weight and subjected to once daily oral gavage with vehicle (0.5% carboxymethyl cellulose, CMC), yhhu981 (150 or 300 mg/kg) or metformin (250 mg/kg) for 22 d. Body weights and food intake were measured daily during the whole treatment. At the end of treatment, blood was collected for glucose, insulin, NEFA, and triacylglycerol analysis. The gastrocnemius and liver were dissected for triacylglycerol measurement. The acute effect of yhhu981 (300 mg/kg) on the respiratory exchange ratio (RER) was measured by indirect calorimetry using the Oxymax system (Columbus Instruments, Columbus, OH, USA) as described^[24]. All the animal studies were approved by the Animal Care and Use Committee, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Determination of triacylglycerol content in gastrocnemius and liver

Triacylglycerol in the gastrocnemius muscle and liver was

measured as previously described^[25]. In brief, 2 mL each of muscle and liver tissue were homogenized in a Heptan-Isopropanol-Tween mixture (3:2:0.01 by volume), vortexed and allowed to separate into two phases, and then centrifuged at 500×g for 10 min at 4 °C. An equal volume of the organic phase was evaporated by vacuum drier until dry. Triacylglycerol was then measured using a triacylglycerol kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The triacylglycerol content was normalized to tissue weight and expressed as micromole per gram of tissue.

Statistical analysis

The data were expressed as the mean±SEM. All statistical analyses were performed using Prism 5 (GraphPad Software Incorporate, CA, USA). Comparisons were performed with Student's *t*-test or one-way ANOVA when appropriate. For ANOVA, if a significant variance was found, the Newman-Keuls test was used for *post hoc* analysis. The difference was deemed significant when $P < 0.05$.

Results

Yhhu981 increased fatty acid oxidation in C2C12 myotubes

We screened a series of compounds for their ability to stimulate fatty acid oxidation in C2C12 myotubes, and yhhu981 was identified as the most potent candidate (Figure 1A). We tested the yhhu981 dose- and time-response of fatty acid oxidation in C2C12 myotubes. A significant increase in fatty acid oxidation was observed when yhhu981 was tested at 12.5 μmol/L (131%±7.76% of control, $P < 0.05$), with a maximum response reached at 25 μmol/L (159%±6.17%, $P < 0.001$, Figure 1B). The maximal response of fatty acid oxidation to yhhu981 reached at 2 h and sustained for more than 12 h (Figure 1C). At the concentrations tested, yhhu981 did not affect cell morphology or viability as assessed either by microscopic visualization or by MTT testing (data not shown). The effects of yhhu981 on fatty acid oxidation prompted us to investigate whether yhhu981 stimulates fatty acid oxidation by regulating gene expression. We employed real-time RT-PCR to profile several genes related to fatty acid oxidation. Our results showed that yhhu981 upregulated the expression of *Sirt1*, *Pgc1α* and *Mcad* in C2C12 myotubes by more than 1.5-fold. The expression of *Cpt1b* remained unchanged (Figure 1D).

Yhhu981 activated the AMPK signaling pathway in C2C12 myotubes

Thr172 phosphorylation serves as a marker for AMPK activation. We examined whether yhhu981 could activate AMPK and thereby promote fatty acid oxidation in C2C12 myotubes. Our results showed that yhhu981 stimulated AMPK Thr172 phosphorylation in a dose- and time-dependent manner. Consistent with an effect on AMPK activation, the phosphorylation of the AMPK substrate ACC was also significantly increased (Figure 2A–2D). AMPK activation is associated with improved lipid metabolism in rodents^[26–28]. To determine whether AMPK activation mediates elevated fatty acid oxida-

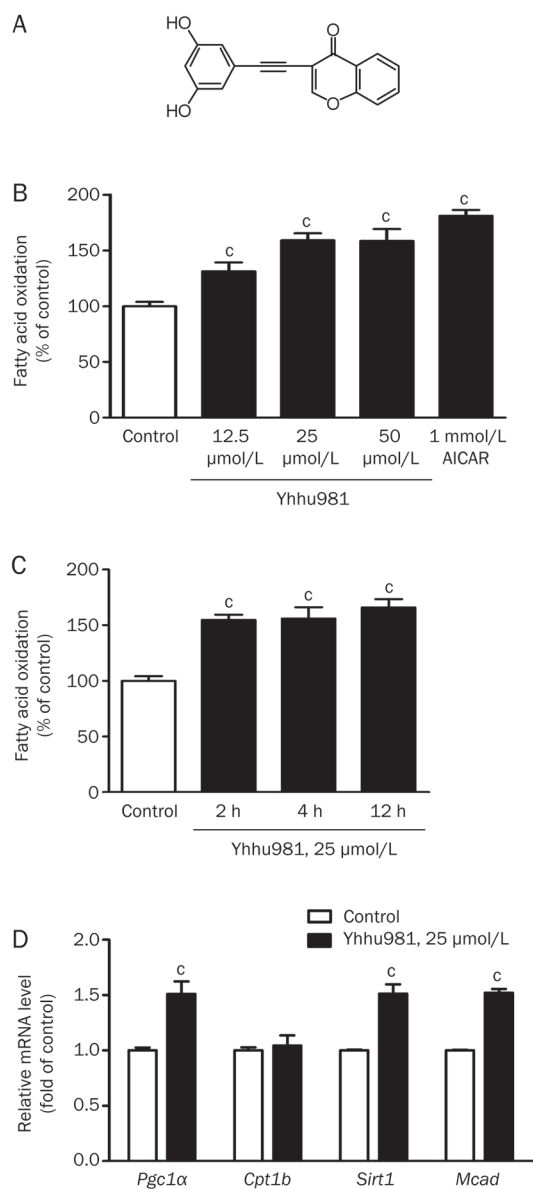


Figure 1. Yhhu981 stimulated fatty acid oxidation in C2C12 myotubes. (A) Structure of yhhu981. (B) C2C12 myotubes were incubated with yhhu981 at indicated concentrations in serum-free media for 2 h or (C) with yhhu981 (25 μmol/L) for the indicated periods. (D) C2C12 myotubes were treated with yhhu981 (25 μmol/L) for 24 h, total RNA was extracted and real-time PCR was used to measure the mRNA levels of genes involved in fatty acid oxidation. The expression of each gene in the control cells was set as 100%. The results represent the mean±SEM for more than three independent experiments in duplicates. * $P < 0.01$ compared with control.

tion by yhhu981, we pre-incubated C2C12 myotubes with the AMPK inhibitor compound C for 30 min before co-incubation with yhhu981. Compound C abolished AMPK phosphorylation and blocked the yhhu981-induced increase in fatty acid oxidation (Figure 2E). It has been shown that CaMKK is an upstream kinase of AMPK^[29]; therefore, we tested the CaMKK inhibitor STO-609 for its effects on the yhhu981-stimulated fatty acid oxidation. Pretreatment with STO-609 before incu-

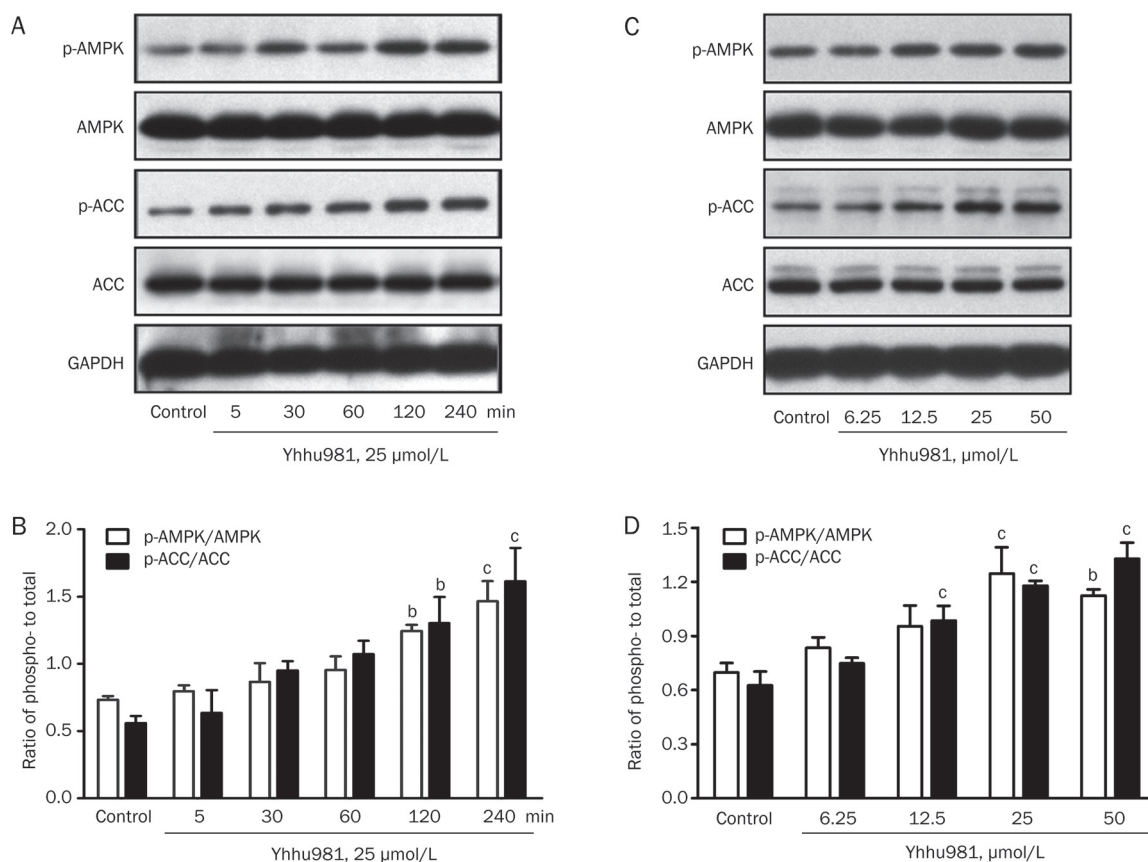


Figure 2A–2D. Yhhu981 promoted fatty acid oxidation by activating the AMPK signaling pathway in C2C12 myotubes. C2C12 myotubes were exposed to 25 μmol/L yhhu981 for the indicated time period or indicated concentrations of yhhu981 for 2 h. (A, C) Protein samples were prepared and subjected to Western blot. (B, D) Ratio of phospho- to total-AMPK, ACC was presented as a bar graph.

bation with yhhu981 resulted in an attenuation of yhhu981-stimulated AMPK phosphorylation and fatty acid oxidation (Figure 2F). Collectively, these results demonstrated that yhhu981 stimulated fatty acid oxidation in myotubes through the activation of the CaMKK-AMPK signaling pathway.

Previous reports showed that Sirt1 activation was related to its regulatory effect on fatty acid oxidation^[16, 30, 31]. The induction of Sirt1 expression by yhhu981 prompted us to test whether Sirt1 is involved in the yhhu981-stimulated fatty acid oxidation. We pre-incubated C2C12 myotubes with the Sirt1 inhibitors EX527 or sirtinol before yhhu981 treatment^[32]. No significant increase was observed in C2C12 myotubes treated with yhhu981 in the presence of sirtinol (Figure 2G). Moreover, EX527 significantly attenuated yhhu981-induced fatty acid oxidation (Figure 2H). These results suggested an important role for Sirt1 in yhhu981-stimulated fatty acid oxidation.

Effect of yhhu981 on fatty acid synthesis in HepG2 cells

Liang *et al* reported that the activation of AMPK or Sirt1 alleviates ethanol-induced alcoholic fatty liver and improves hepatocyte lipid metabolism in mice^[33–35]. We carried out a fatty acid synthesis assay in HepG2 hepatoma cells in the presence of increasing concentrations of yhhu981. Consistent with the results from C2C12 myotubes, yhhu981 dose-dependently

increased the phosphorylation of AMPK (Figure 3A) and suppressed fatty acid synthesis (Figure 3B); the AMPK inhibitor compound C reversed the suppression of fatty acid synthesis by yhhu981 (Figure 3C). These results indicated that yhhu981 repressed fatty acid synthesis through a mechanism involving the activation of AMPK.

Acute effects of yhhu981 on respiration exchange ratio in *ob/ob* mice

Based on the *in vitro* data, we examined whether yhhu981 could acutely affect whole-body metabolism in *ob/ob* mice. Mice were administered 300 mg/kg yhhu981 and monitored for the following 22 h. We observed a significant decrease in the respiratory exchange ratio (Figure 4A and 4B) without affecting food intake (Figure 4C), indicating a shift to fatty acid utilization.

Chronic yhhu981 treatment improved fatty acid metabolism in *ob/ob* mice

To test whether yhhu981 improves fatty acid metabolism *in vivo* after chronic treatment, we treated *ob/ob* mice with yhhu981 for 22 d. Yhhu981 treatment did not significantly change body weight (Figure 5A) or average food intake (Figure 5B), but significantly reduced the TG content in the

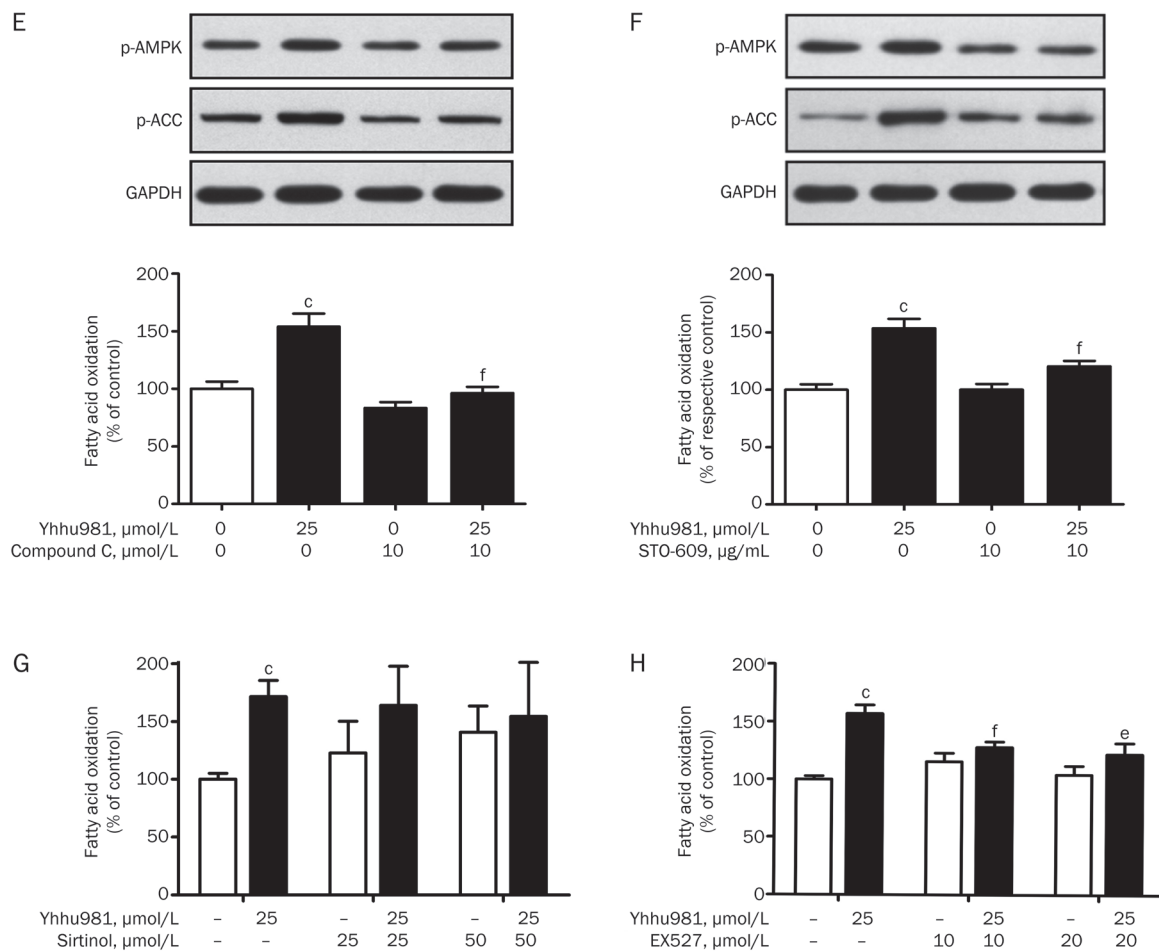


Figure 2E–2H. (E, F) C2C12 myotubes were pretreated with or without 10 μmol/L compound C or 10 μg/mL ST0609 for 30 min and then co-treated with 25 μmol/L yhu981 for another 2 h, followed by the measurement of fatty acid oxidation. The values of fatty acid oxidation in Figure 2E were normalized to the control group, and the values of fatty acid oxidation in Figure 2F were normalized to the respective control. (G, H) C2C12 myotubes were pretreated with SIRT1 inhibitors EX527 or sirtinol, then co-incubated with yhu981 for another 2 h, followed by the measurement of fatty acid oxidation. The values of fatty acid oxidation in Figure 2G and 2H were normalized to the control group. The results represent the mean ± SEM for three independent experiments. ^b $P < 0.05$, ^c $P < 0.01$ compared with related control. ^e $P < 0.05$, ^f $P < 0.01$ compared with yhu981 treatment alone.

gastrocnemius muscle (Figure 5C) and the serum NEFA levels (Figure 5E). The TG content in liver (Figure 5D), serum TG levels (Figure 5F) and fasting blood glucose (Figure 5G) all showed a trend towards lower levels, although the decrease did not reach statistical significance. However, the serum insulin level was not significantly changed (Figure 5H).

Discussion

The regulation of fatty acid metabolism represents a critical strategy for improving metabolic disorders, such as insulin resistance and type 2 diabetes mellitus. Recently, a series of reported compounds were defined as fatty acid metabolic regulators. AICAR, an AMPK activator, stimulated fatty acid oxidation by the allosteric regulation of CPT-1, as well as by activating PPARα and PGC-1^[36–40]. Resveratrol, the first reported Sirt1 activator, increased fatty acid oxidation in skeletal muscle cells and adipocytes through the activation of AMPK^[16, 41]. Thus, a compound capable of regulating fatty acid metabolism

may hold promise for the treatment of diabetes. In this study, we aimed to identify a new compound capable of potentially regulating fatty acid oxidation in C2C12 myotubes. Yhu981, a compound with a novel structure was found to stimulate fatty acid oxidation in C2C12 myotubes and to repress fatty acid synthesis in HepG2 cells. Further studies showed that yhu981 reduced circulating NEFA levels and ectopic lipid deposition in skeletal muscle and liver of *ob/ob* mice.

In an attempt to uncover the mechanisms underlying yhu981-stimulated fatty acid oxidation, we investigated the potential involvement of AMPK. AMPK plays a key role in the regulation of lipid metabolism through multiple signaling pathways, including by directly catalyzing its downstream substrates, and through the transcription of multiple genes^[42, 43]. In the present study, yhu981 was found to stimulate fatty acid oxidation and upregulate the related genes, which was also accompanied by the activation of AMPK signaling pathways in C2C12 myotubes. More importantly,

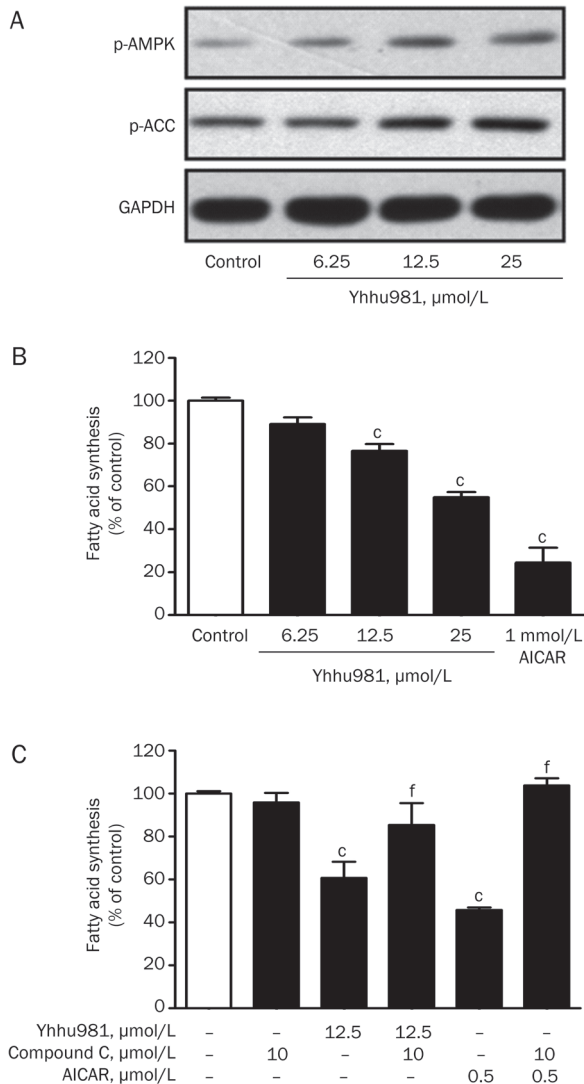


Figure 3. Yhhu981 inhibited fatty acid synthesis in HepG2 cells. (A) HepG2 cells were incubated with yhhu981 at indicated concentration in serum-free media for 24 h, protein samples were prepared and subjected to Western blot. (B) HepG2 cells were incubated with yhhu981 at the indicated concentrations in serum-free media for 24 h, followed by the measurement of fatty acid synthesis. (C) HepG2 cells were pretreated with or without 10 μmol/L compound C for 30 min and then treated with 25 μmol/L yhhu981 or 1 mmol/L AICAR for another 24 h, followed by the measurement of fatty acid synthesis. AICAR treatment was used as a positive control. The results represent the mean±SEM for more than three independent experiments in duplicates. ^c*P*<0.01 compared with control. ^f*P*<0.01 compared with yhhu981 or AICAR treatment alone.

yhhu981-stimulated fatty acid oxidation was blunted by the AMPK inhibitor compound C and the CaMKK inhibitor STO-609. Collectively, these data suggest that yhhu981 stimulates fatty acid oxidation through the activation of the CaMKK-AMPK signaling pathway. SIRT1 plays a key role in regulating fatty acid oxidation and lipid metabolism in mammals^[35, 44]. The activation of SIRT1 has been considered as a promising approach to ameliorate lipid disorders^[45]. Recently, reports

showed that AMPK acted upstream of SIRT1 and increased its activity by modulating NAMPT, an NAD⁺ synthetic enzyme^[11, 12]. However, AMPK has also been shown to regulate SIRT1 expression by modulating the state of FOXO1 phosphorylation^[46, 47]. We did not detect direct SIRT1 activation by yhhu981 in the *in vitro* assay. However, yhhu981 induced the expression of *Sirt1* in C2C12 myotubes. Furthermore, the selective SIRT1 inhibitors EX527 and sirtind reversed the stimulatory effect of yhhu981 on fatty acid oxidation. Given that the induction of *Sirt1* by yhhu981 is downstream of AMPK, the results demonstrated that yhhu981 stimulates fatty acid oxidation by activating the CaMKK-AMPK-SIRT1 signaling pathway in myotubes^[11, 48].

The liver is the major organ for buffering quick fatty acid storage and utilization. Hepatic fatty acid metabolism plays an important role in the regulation of whole-body energy homeostasis. The evidence that activation of AMPK in the liver or hepatocytes resulted in increased fatty acid oxidation and decreased lipogenesis holds considerable potential for ameliorating metabolic disorders^[49, 50]. Because yhhu981 was shown to stimulate AMPK signaling pathways in C2C12 myotubes, we further investigated its effects on hepatocytes. Consistently, yhhu981 increased the phosphorylation of AMPK and ACC in HepG2 cells, suggesting that the activation of AMPK has occurred. Moreover, yhhu981 dose-dependently inhibited fatty acid synthesis in HepG2 cells, and the AMPK inhibitor compound C reversed the inhibition caused by yhhu981. These results again demonstrated that yhhu981 suppressed fatty acid synthesis by activating AMPK in hepatocytes.

To investigate the physiological relevance of the above cellular observations, we treated *ob/ob* mice with yhhu981. Initially, we administered to mice a single dose of yhhu981, which did not significantly affect whole-body energy expenditure (data not shown); however, we did observe a reduction in the respiratory exchange ratio (RER) compared with vehicle throughout the monitored time after treatment, indicating a switch to whole-body fatty acid oxidation^[21]. This elevated lipid utilization is consistent with the AMPK signaling pathway activation^[39] that we observed in the C2C12 and HepG2 cells treated with yhhu981. We next assessed the chronic effects of 22 d of yhhu981 treatment in *ob/ob* mice and phenotyped the mice for alterations in lipid metabolism. As a genetically obese rodent model, *ob/ob* mice displayed obesity, hyperglycemia, dyslipidemia and significant ectopic lipid deposition in both the liver and skeletal muscle. Yhhu981 treatment did not alter body weight or food intake, but significantly decreased circulating NEFA levels. In addition, yhhu981 reduced TG content in both the gastrocnemius muscle and the liver, indicating the amelioration of ectopic lipid accumulation. These observations in *ob/ob* mice are consistent with the enhanced fatty acid oxidation and diminished fatty acid synthesis that was uncovered in our *in vitro* study.

In conclusion, here we identified yhhu981 as a new compound that enhances free fatty acid oxidation in C2C12 myotubes. Further studies revealed the pleiotropic effects of

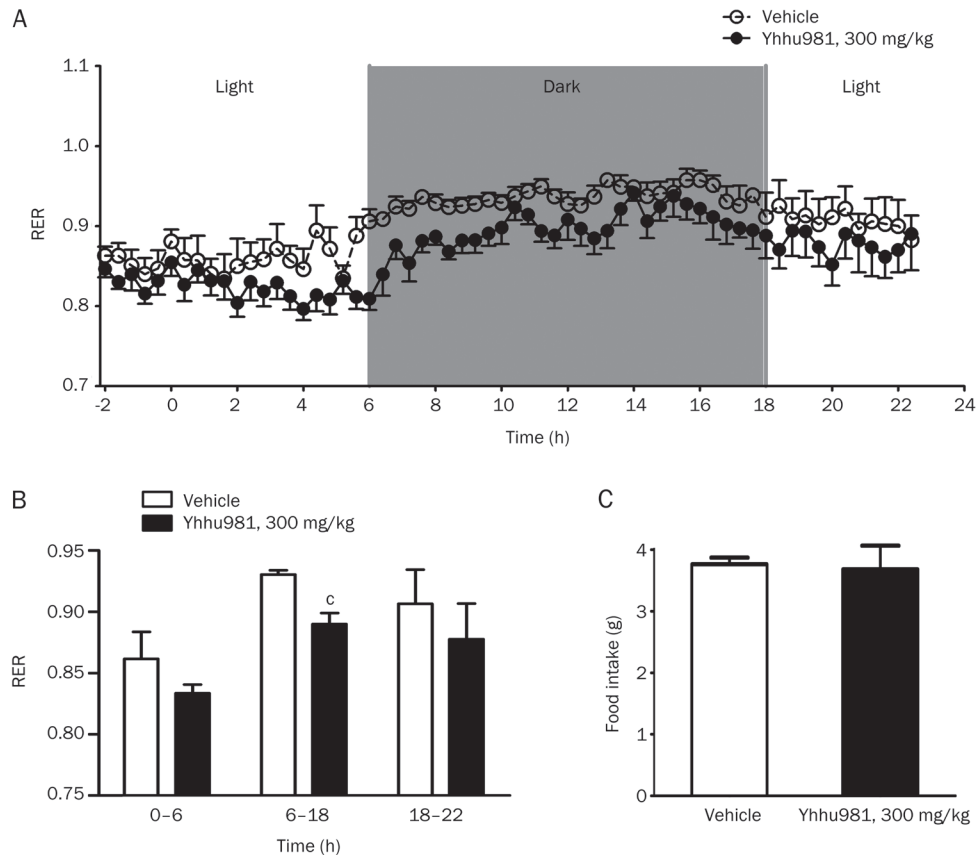


Figure 4. A single administration of yhhu981 decreased the respiration exchange ratio in *ob/ob* mice. Respiration exchange ratio (A, B) and food intake (C) were measured from -2 h (2 h before administration) to +22 h (22 h after administration) by indirect calorimetry using the Oxymax system. Data are presented as the mean±SEM. $n=6$. ^c $P<0.01$ compared with the vehicle group.

yhhu981 on lipid metabolism by activating the AMPK signaling pathway. The lipid abnormalities and ectopic fat deposition in skeletal muscle and liver of *ob/ob* mice were ameliorated by chronic treatment with yhhu981. These results highlight the potential value of yhhu981 as a leading compound for the treatment of metabolic disorders and provide support for strategies that target the activation of free fatty acid oxidation for the prevention of lipid abnormalities and ectopic fat accumulation, which were associated with the development of metabolic syndrome and type 2 diabetes.

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Author contribution

Ying LENG and You-hong HU designed research; Hong-liang ZENG, Su-ling HUANG, Fu-chun XIE, and Li-min ZENG performed research; Hong-liang ZENG, Su-ling HUANG, and Ying LENG wrote the paper.

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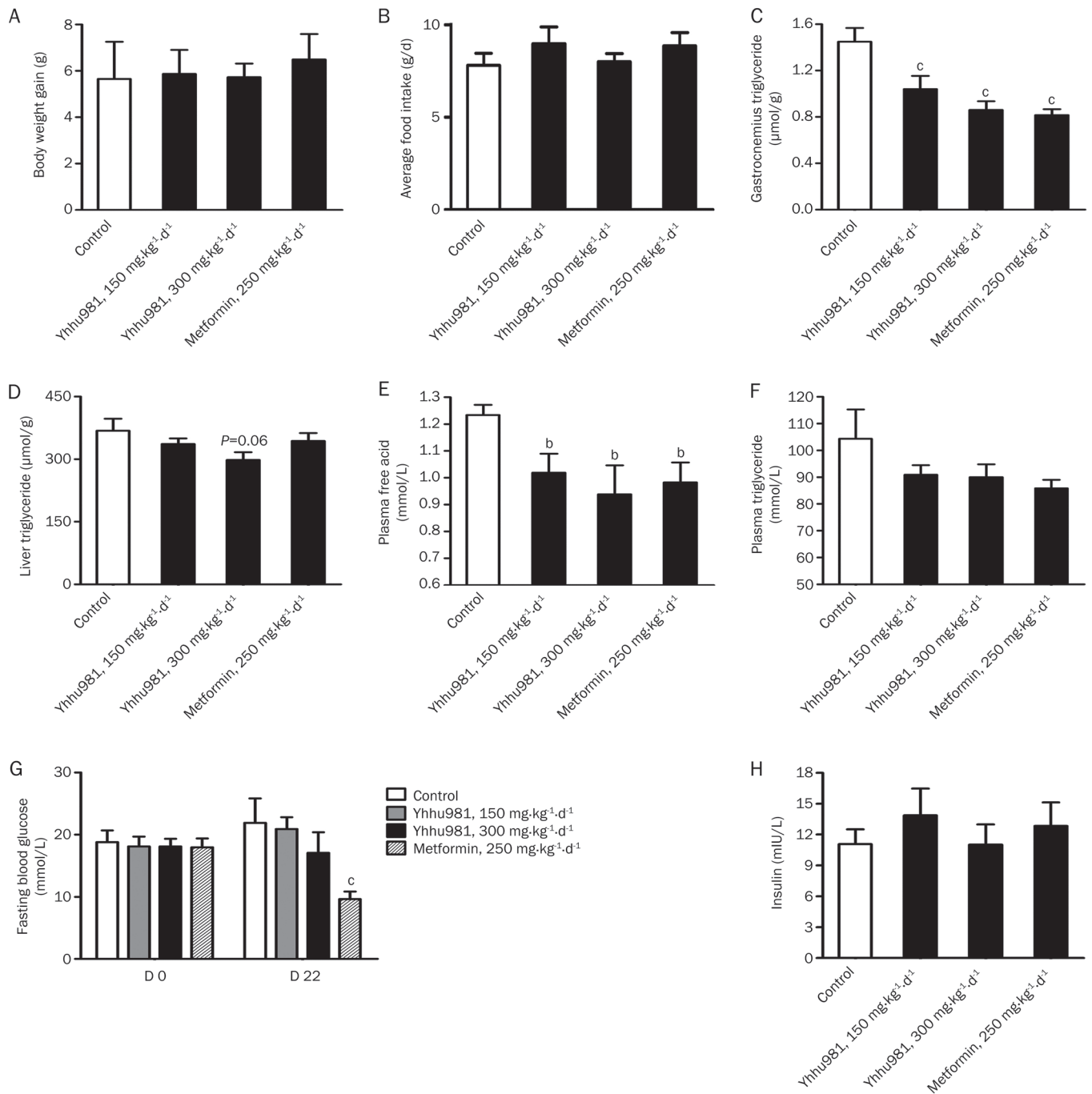


Figure 5. Yhhu981 improved lipid metabolism in *ob/ob* mice. *Ob/ob* mice were subjected to gavage treatment once daily with vehicle (0.5% CMC), 150 mg·kg⁻¹·d⁻¹ or 300 mg·kg⁻¹·d⁻¹ compound yhhu981 or 250 mg·kg⁻¹·d⁻¹ metformin for 22 d. At the end of treatment, blood, liver and gastrocnemius were collected for posterior analysis. Body weight (A) and food intake (B) were recorded regularly during the treatment period. The content of triglyceride in the gastrocnemius (C) and liver (D) were determined by alkane-isopropyl alcohol-extract methods. Plasma free fatty acid (E) and triglyceride (F) were determined as described in Materials and methods. Fasting blood glucose concentrations (G) were measured before and after 22 d treatment. Insulin levels (H) were measured at the end of the treatment. Values are expressed as the mean±SEM for *n*=6–8 mice. ^b*P*<0.05, ^c*P*<0.01 vs control group.

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