Title page

Title: Anhydroicaritin improves diet-induced obesity and hyperlipidemia and alleviates insulin resistance by suppressing SREBPs activation

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Abbreviations: MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; SREBPs, sterol
regulatory element-binding proteins; SCAP, SREBP cleavage-activating protein; Insigs,
insulin-induced genes; COP II, coated protein II; S1P, site-1 protease -1; S2P, site-2 protease;
25-HC, 25-hydroxycholesterol; TC, total cholesterol; TG, triglyceride; LDL-C, low-density
lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; WD, western-type diet;
WAT, white adipose tissue; BAT, brown adipose tissue; LKB1, liver kinase B1; P70S6k, P70
S6 kinase; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin;
FASN, fatty acid synthase; ACC, Acetyl-CoA Carboxylase, ACC1, Acetyl-CoA Carboxylase 1;
SCD, Stearoyl-CoA dehydrogenase; SCD 2, Stearoyl-CoA dehydrogenase 2; HMGCR, 3
hydroxy 3 methyl coenzyme A reductase; HMGCS, 3 hydroxy 3 methyl coenzyme A synthase;
LDLR, low-density lipoprotein receptor; LPL, lipoproteinlipase; GK, glycerol kinase;
UCP-1, uncoupling protein 1; UCP-2, uncoupling protein 2; PGC1 α , PPAR γ Co Activator 1 α .
TNF α , Tumor necrosis factor α ; LPL, lipoproteinlipase; Glut4, glucose transporter 4; PAQR3,
progesterin and adipoQ receptor family member 3; PBS, Phosphate buffer solution; PFA,
paraformaldehyde; NMR, nuclear magnetic resonance; CMC-Na, Sodium carboxymethyl

cellulose; DMSO, dimethyl sulfoxide; BFA, brefeldin A; IRS-2, insulin receptor substrate 2; G-6-PD, glucose-6-phosphate dehydrogenase; Dio 2, Type II iodothyronine deiodinase.

Abstract

SREBPs play important roles in the regulation of lipid metabolism, and are closely related to the occurrence and development of many metabolic diseases. Small molecular inhibitors of SREBPs are important tools in developing efficient treatment of metabolic diseases. However, there are no listing drug targeting SREBPs. Therefore, there is an urgent need to develop highly specific small molecules that inhibit SREBPs. In this study, using a hepatocytes-based high-throughput screening, we identified anhydroicaritin (AHI) as a novel inhibitor of SREBPs. HepG2, HL-7702, and human primary hepatocytes were used to verify the effects of AHI. We explored the mechanism by which AHI blocks the binding of SCAP/SREBPs complex with Sec23a/24D via regulating LKB1/AMPK/mTOR pathway. AHI reduced liver cell lipid level by preventing *de novo* lipogenesis. In diet induced obese mice, AHI ameliorated obesity, insulin resistance, fatty accumulation in liver and hyperlipemia. In conclusion, AHI improves diet-induced obesity and alleviates insulin resistance by suppressing SREBPs maturation which is dependent on LKB1/AMPK/mTOR pathway. Thus, AHI can serve as a leading compound for pharmacological control of metabolic diseases.

Key words: Anhydroicaritin; SREBPs; obesity; hyperlipidemia; insulin resistance

Chemical compounds studied in this article

Anhydroicaritin (PubChem CID: 5318980); 3,5-dihydroxy-4'-methoxy-6",6"-dimethyl-4",5"-dihydropyrano(2",3":7,8)-flavone (PubChem CID: 14583584); icaritin (PubChem CID: 14427423); isoanhydroicaritin (PubChem CID: 5322079); Icariin (PubChem CID: 5318997); icarisid I (PubChem CID: 5745470); icarisid II (PubChem CID: 5488822); epimedin A (PubChem CID: 24721194); epimedin B (PubChem CID: 5748393); epimedin C (PubChem CID: 5748394); 25-hydroxycholesterol (PubChem CID: 65094); Lovastatin (PubChem CID: 53232); Rapamycin (PubChem CID: 5284616); Compound C (PubChem CID: 11524144); MHY1485 (PubChem CID: 2834965)

1. Introduction

Metabolic syndrome (MetS), including obesity, insulin resistance, dyslipidemia, hyperglycemia and hypertension, has become one of the most common health problems worldwide. In the USA, 66% of the population is overweight or obese and the recent prevalence of the MetS is 38.5% [1, 2]. Over the next 20 years, according to the forecast, the worldwide prevalence of type 2 diabetes mellitus (T2DM) will increase by 75% [3].

Sterol regulatory element-binding proteins (SREBPs) play important roles in regulating lipid homeostasis [4]. SREBPs control the expression of genes involved in biosynthesis and uptake of fatty acids, triglycerides, cholesterol and phospholipids. The central roles of SREBPs in lipid metabolism link them closely to MetS [5, 6]. Therefore, inhibition of SREBPs by small molecule may be an effective strategy to treat obesity, T2DM, fatty liver and atherosclerosis [6-8]. SREBPs are tightly associated with SREBPs cleavage-activating protein (SCAP) bonding the membranes of the ER by interacting with insulin-induced genes (Insigs) and

synthesized as inactive precursors. Under certain conditions (i.e., low sterol and insulin), the coated protein II (COP II) vesicles transport SREBPs from ER to Golgi by regulating the interaction between SCAP with Insigs and Sec23/Sec24. In Golgi apparatus, site-1 protease (S1P) and site-2 protease (S2P) sequentially cleave SREBPs to form the mature SREBPs, which are then released from Golgi and translocated to the nucleus. SREBPs bind on SRE or E-box sequences of its target genes related to fatty acid and cholesterol biosynthesis and activate the expression of these genes [9-15].

In recent years, a large number of natural compounds were reported to modulate lipid metabolism and improve insulin resistance and non-alcoholic fatty acid liver disease (NAFLD), such as quercetin[16], resveratrol[17], oleuropein[18], and betaine[19]. Anhydroicaritin (AHI), a prenylated flavonoid naturally occurring in several *Epimedium* species (Berberidaceae family), is commonly recognized as one of the effective compounds of *Epimedium* Herba, a famous traditional Chinese herbal medicine. AHI exhibits a variety of biological activities, such as suppression of steroid-associated osteonecrosis and osteoplastic activity, activation of cancer cell apoptosis and inhibition of cancer cell growth, protection against beta amyloid-induced neurotoxicity and promotion of neuronal and cardiac cellular differentiation [20-26]. However, the effects of AHI on MetS (particularly obesity, hyperlipidemia and insulin resistance) and the underlying mechanisms have not yet been reported.

Here, using a high-throughput screening, we demonstrated that AHI inhibits the processing of SREBPs by suppressing the interaction of SCAP/SREBPs complex with Sec23/Sec24 through regulating LKB1/AMPK/mTOR pathway. Moreover, AHI blocks the maturation of SREBPs

and decreases the expression of the target genes involved in the cholesterol and fatty acid biosynthesis.

2. Materials and methods

2.1 Materials

Anhydroicaritin (AHI), 3,5-dihydroxy-4'-methoxy-6",6"-dimethyl-4",5"-dihydro- pyrano (2",3":7,8)-flavone (AICT), icaritin (ICT), isoanhydroicaritin (IAHI), icariin (ICA), icarisid I (ICA I), icarisid II (ICA II), epimedin A (EPM A), epimedin B (EPM B) and epimedin C (EPM C) were purchased from Shanghai U-sea Bio-tech co., Ltd. (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT), doxorubicin, cholesterol, 25-hydroxycholesterol (25-HC), Nile-red, and Filipin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin, Compound C and MHY1485 were purchased from MedChem Express (Shanghai, China). Luciferase assay reagent and reporter lysis buffer were from Promega (Madison, Wisconsin, USA). Acetic acid sodium salt $1\text{-}^{14}\text{C}$ was purchased from Perkin Elmer (Waltham, MA, USA). Lovastatin and Compactin were purchased from Aladdin (Los Angeles, Southern California, USA). Mevalonate was synthesized by hydrolysis of mevalonolactone purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), DMEM and F12K medium were purchased from GIBCO (Grand Island, New York, USA). Hepatocyte growth supplement (HGS, Cat. No. 5252), hepatocyte medium (HM, Cat. No. 5201) and human hepatocyte (HH, Cat. No. 5200) were purchased from ScienCell Research Laboratories (San Diego, California, USA). Protein A/G PLUS-Agarose immunoprecipitation reagent (Cat. No.sc-2003) was purchased from Santa Cruz (Dallas, Texas, USA). Lipoprotein-deficient serum (LPDS) was purchased from Kalen Biomedical

(Montgomery Village, MD, USA). The active and phosphorylated AMPK ($\alpha 1\beta 2\gamma 1$) were purchased from Carna Biosciences (Kobe, Japan). The protein phosphatase 2A (PP2A) was purchased from Upstate Biotechnology (Lake Placid, New York, USA). The PP2A inhibitor okadaic acid (OA) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The LANCE Ultra kinase assay kit for AMPK containing peptide substrate Cy5-SAMS and Eu-labeled antiphospho-SAMS antibody (Eu-pSAMS-Ab) and the 384-well plates were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). *siRNA* siLKB1 (GGAUGUGUUAUACAACGAATT) and siAMPK (GAGGAGAGCUAUUUGAUUATT) were purchased from GenePharma (Shanghai, China). Scrambled siRNA was used as control. *Plasmids* sh-TSC1 was kindly provided by Prof. Aurelio Teleman at German Cancer Research Center. The pcDNA3.1-2×FLAG-SREBP-1c and pcDNA3.1-2×FLAG-SREBP-2 were gifts from Timothy Osborne (Addgene plasmid # 26802, and # 26807). The Flag-Sec23 α , RFP-Sec24A, RFP-Sec24B, RFP-Sec24C and RFP-Sec24D expression plasmids were gifts from Prof. Xiao-Wei Chen (Peking University). The Myc-SCAP, Flag-Insig-1 and GFP-PAQR3 expression plasmids were kindly provided by Prof. Yan Chen (Chinese Academy of Sciences). *Culture Medium* Medium A: DMEM containing 100 units/mL penicillin and 100 μ g/mL streptomycin sulfate; Medium B: medium A supplemented with 10% FBS; Medium C: a 1:1 mixture of Ham's F-12 medium and DMEM containing 100 units/ml penicillin and 100 μ g/ml streptomycin sulfate; Medium D: medium C supplemented with 5% LPDS, 10 μ M compactin and 50 μ M sodium mevalonate; Medium E: Hepatocyte Medium consists of 500 mL basal medium, 25 mL of FBS, 5 mL of HGS, 100 units/mL penicillin and 100 μ g/mL streptomycin sulfate.

2.2 Antibodies

Anti-SREBP-1 (cat. No. sc-8984), anti-Myc (cat. No. sc-40), anti-SCAP (cat. No. sc-9675), anti-Sec23 (cat. No. sc-12107) and anti-golgin245 (cat. No. sc-102565) antibodies were from Santa Cruz (Dallas, USA); anti-RFP tag (cat. No. A00682) antibody was from GenScript (Nanjing, China); anti-Insig1 (cat. No. ab70784), anti-Golgin-97 (cat. No. ab169287) and anti-SREBP-2 (cat. No. ab30682) antibodies were purchased from Abcam (Cambridge, UK); anti-p-ACC (Ser79) (cat. No. 11818), anti-AMPK α (Ser485) (cat. No. 2537), anti-p-mTOR (Ser2448) (cat. No. 2971), anti-p-SREBP-1c (Ser372) (cat. No. 9874) and anti-Flag (cat. No. 14793) antibodies were purchased from Cell Signaling Technology (Beverly, USA); anti-AMPK α 1 (cat. No. A1229), anti-p-AMPK α (Thr172) (cat. No. AP0116), anti-ACC (cat. No. A0110), anti-GFP-tag (cat. No. AE011), anti-Histone H3 (cat. No. A2348), anti-mTOR (cat. No. A5520), anti-p-P70S6K (Thr389) (cat. No. AP0564), anti-P70S6K (cat. No. A2190), anti-LKB1 (cat. No. A2122) and anti-TSC1 (cat. No. A0720) antibodies were purchased from ABclonal Technology (Wuhan, China); anti-Calnexin (cat. No. AC018) and anti-Actin (cat. No. AF0003) antibodies were purchased from Beyotime Biotechnology (Shanghai, China).

2.3 Cell culture

HepG2, HEK293T and HeLa cell lines were obtained from the American Type Culture Collection (ATCC). HL-7702 cells were purchased from Keygen Biotech (Nanjing China). All cell lines were grown in medium B under 37 °C, 5% CO₂. HH cells were cultured in 10 cm² flask coated with poly-L-lysine in medium E.

2.4 Generation of HL-7702/SRE-Luc reporter cell lines

Three tandem SREBP binding sites 5'-AAAATCACCCCACTGCAAATC

CTCCCCCTGC-3' were inserted into pGL4.26 by primer annealing to generate SREBP reporter plasmids, pSRE-Luc. HL-7702 cells were transfected with pSRE-Luc. After 24 h, cells were switched to culture medium with 200 µg/mL hygromycin B (Roche, Germany). The medium was refreshed every three days for about two weeks until colonies formed. Individual colonies were visually identified and isolated with cloning cylinders.

2.5 Viability assay

Cell viability was detected by MTT assay. Briefly, HepG2, HL-7702 and HH cells were seeded at the density of 2×10^4 cells/well in 96-well plate. After about 24 h, cells were treated with AHI as indicated. After 24 h, MTT (5 mg/mL) was added and incubated for 4 h. The cytotoxicity of AHI was determined by microplate reader (Multiskan FC).

2.6 Measurement of de novo fatty acid and cholesterol synthesis.

De novo fatty acid and cholesterol were measured as previously described [27].

2.7 Immunoprecipitation and western blot analysis

Immunoprecipitation with 2 µg of monoclonal indicated antibody. After the addition of 20 µL Protein A/G plus agarose beads, incubations were continued for an additional 2 h at 4 °C. The beads were then collected by centrifugation and washed three times with PBS. WB were measured as previously described [28].

2.8 Immunofluorescence staining and fluorescence microscopy

In brief, cells were fixed with 4% paraformaldehyde (PFA) for 15 min, and then permeabilized with 0.1% Triton X-100 in PBS for 15min. after blocking in 3% BSA for 1h, the cells were incubated with the primary and corresponding fluorophore-conjugated secondary antibodies. Confocal images were captured with an LSM 710 confocal microscope

(Zeiss, Germany). The merged pictures were generated by LSM 7 IMAGE browser. Fluorescence images were captured with EVOS FL Auto microscope (Life Technologies, UAS).

2.9 Quantitative RT-PCR

Total RNA was extracted from HepG2, HH or mice tissues using TRIzol reagent (Life Technologies, USA) according to the manufacturer's instructions. RNA concentrations were equalized and converted to cDNA using a kit (Hiscript II reverse transcriptase, Vazyme, Nanjing, China). Gene expression was measured by quantitative PCR (Roche, Basel, Switzerland) using SYBR-green (Roche, Basel, Switzerland). Expression was normalized to GAPDH. The sequence and GeneBank ID of primers used in the experiments are listed in Table 1.

2.10 Cell fractionation

The method for the preparation of cell fractionation was referred to a previous report [29]. The discontinuous sucrose gradients were prepared as 1.2 mL of 50% sucrose, 4.8 mL of 30% sucrose, 4.4 mL of 15% sucrose and 2.4 mL of 10% sucrose.

2.11 Filipin staining and nile-red staining

HepG2 cells were seeded in 24-well plates at the density of 5×10^4 cells/well and maintained in 5% CO₂ at 37 °C for 24 h. And then cells were incubated in medium C containing 5% LPDS, 50 µM sodium mevalonate and the indicated concentrations of AHI. After treatment for 16 h, cells were fixed with 4% PFA in PBS for 30 min, then washed with PBS for three times. 50 µg/mL fillipin or 0.5 µg/mL nile-red were used to stain the treated cells for 30 min at 24 °C. The Filipin-stained cells and nile-red stained cells were analyzed with EVOS FL Auto

microscope (Life Technologies, USA).

2.12 Cholesterol and TG measurement.

Cholesterol and TG were measured as previously described [28].

2.13 AMPK enzyme assay

As described in previous report [30].

2.14 Animal experiment

The laboratory animal facility in the animal center has been accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments and animal care in this study were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and approved by the Science and Technology Department of Jiangsu Province (SYXK (SU) 2016-0011).

The C57BJ/6L mice (SPF grade, eight weeks old, 20-22 g) were purchased from Yangzhou University (Yangzhou, China). The animals were kept under a consistent temperature (24 °C) with 12 h light/dark cycle and fed standard food pellets with access to sterile water *ad libitum*. WD was normal chow supplemented with 1.25% cholesterol, 20% fat and 0.5% cholic acid (w/w). Mice were randomly divided into 4 groups (n = 6-12/group). Vehicle, AHI (30 or 60 mg/kg) or Lovastatin (30 mg/kg) were administered to the mice by oral gavage once daily.

2.15 Fecal cholesterol and TG measurements

After receiving different treatments for 6 weeks, mice were kept in the metabolic chambers for 24 h. Feces were collected, lyophilized and ground up. About 250 mg mashed feces were extracted with 4 mL of methanol: chloroform (1:2, v/v) twice. 100μL of the supernatants were pooled and evaporated to dryness. TG was measured with determination kit. Then 4 mL

mixture of 5 N KOH: ethanol (10: 90, v/v) were added and heated at 70 °C for 1 h. After cooling to room temperature, 2 mL ultrapure water was added and saturated with sodium chloride, followed by solvent extraction with 3 mL hexane twice. The extracts were dried and re-dissolved with 50 µL hexane containing 50 µL 5- α -cholestane (1 mg/mL). Then GC-MS (gas chromatography-mass spectrometry) analyses were performed with an Agilent 7890B gas chromatograph interfaced to an Agilent 5977A mass-selective detector equipped with HP-5ms Ultra Inert (30m $\times$ 250m $\times$ 0.25m) column (Agilent Technologies, USA). The temperatures of the injector, interface, and ion source were 280 °C, 280 °C, and 230 °C, respectively. Helium was used as the carrier gas at a flow rate of 0.7 mL/min in constant flow mode.

2.16 Glucose tolerance and insulin tolerance tests

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed on mice fasted overnight with free access to water. Mice were intragastrically (i.g.) gavaged with 2 g/kg glucose (Sigma) or intraperitoneally (i.p.) injected with 0.75 U/kg insulin (Sigma). Glucose levels were measured from tail blood at 15, 30, 60, or 120 min after the glucose gavage or insulin injection. All animals were sacrificed 3 days after glucose tolerance or insulin tolerance tests, and blood and liver were harvested. Area under the curve (AUC) was calculated to quantify the GTT and ITT results.

2.17 The weight of fat analysis by NMR

To determine the fat content of animals, after six-week treatment, the mice were scanned with the minispec TD-NMR designed for experimental animals (Bruker, Germany). The fat content was calculated according to the measurement between the solid and liquid parts of the sample.

2.18 Serum and liver lipid determination

Serum total cholesterol (TC) and triglyceride (TG) levels were measured according to the manufacturer's instructions (Kehua, China), and low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels were determined by the kits (Njjcbio, China). Liver tissues were homogenized and centrifuged. Supernatants were collected, and total cholesterol and triglyceride were determined.

2.19 Histological analysis of liver and adipose

Livers, epididymal WAT and interscapular BAT were fixed in 4% PFA at 4 °C overnight and embedded in paraffin wax. Paraffin sections (5 µm) were cut and mounted on glass slides for H&E staining. Cryosections of livers were stained by oil red O and counterstained with hematoxylin to visualize the lipid droplets.

2.20 Statistical analysis

All data are expressed as the means standard error (SE). Comparisons between groups were made using one-way ANOVA (Tukey's multiple comparisons test). Differences with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Identification of AHI as a new inhibitor of SREBPs

To screen the specific small molecule inhibitor of SREBPs, human liver HL-7702 cells expressing a luciferase reporter driven by a SRE-containing promoter was established (Fig. 2A). From a small molecule pool containing about 400 natural products, AHI was found to significantly decrease the SRE-luciferase activity (Fig. 1A). To further confirm the above results, we examined the expression of SREBP-1/2 in the cells treated with AHI by western blot. Both mature SREBP-1/2 level were reduced by AHI in a dose-dependent manner in

HepG2 and HL-7702 cells (Fig. 1B). We investigated the viability of HL-7702 and HepG2 cells treated with AHI using the MTT assay. AHI showed negligible cytotoxicity in both cell lines, even at the higher concentration (Fig. 1C). Next, four kinds of flavonoids and six kinds of glycosides in *Epimedium* (Fig. 2B and E) with similar skeleton to AHI, were chosen to determine their SREBPs inhibitory effects. As shown in Fig. 2C and F, the luciferase activity was significantly decreased by IDHI, ICT and ICA, but not by AICT or other glycosides of AHI. Consistent with this, western blot results showed the expression of mature SREBPs were significantly reduced only by ICT and ICA (Fig. 2D and G).

3.2 AHI downregulates SREBPs activity and decreases cellular lipid level

SREBPs are considered master regulators of lipid homeostasis by controlling the expression of target genes which are required for cholesterologenesis and lipogenesis [6]. First, we sought to check whether AHI effects SREBPs nuclear translocation, which is positively associated with its transcription activity. The subcellular localization of SREBPs was determined by immunofluorescence staining and it was shown that nuclear localization of SREBP-1 and SREBP-2 was obviously blocked by the treatment of 25-HC and AHI (Fig. 1D). To further confirm this observation, we treated HepG2 cells with DMSO or AHI, and isolated the nuclear and cytoplasmic compartments. It was found that the nuclear SREBPs were largely decreased by AHI (Fig. 1E). To check whether AHI decreases SREBPs' activity, we detected the mRNA level of SREBP-1 and SREBP-2 target genes. The expression of the SREBP-1c target genes, such as SREBP-1c, FASN, ACC-1, SCD-1, as well as the SREBP-2 target genes, such as HMGCR, HMGCS, and LDLR were significantly decreased by AHI treatment (Fig 1F-G). Taken together, these data indicated that AHI specifically inhibits SREBPs activity.

309 Considering the reduction of SREBPs activity by AHI, we examined the changes of neutral
310 lipids and cholesterol in HepG2 cells treated with AHI. The neutral lipids and cholesterol
311 were unsurprisingly reduced by AHI treatment (Fig. 1H and I). More importantly, AHI largely
312 decreased *de novo* synthesis of both fatty acid and cholesterol in a dose-dependent manner
313 (Fig. 1J-K). Thus, our results demonstrated that AHI downregulates the target genes required
314 for lipogenesis and cholesterologenesis and inhibits fatty acid and cholesterol biosynthesis.

315 *3.3 AHI inhibits SREBPs activity and their target genes in human primary hepatocytes.*

316 Human primary hepatocytes (HH) were often used to evaluate drug efficacy and
317 hepatotoxicity. We firstly examined the toxicity of AHI in HH. The growth profiles of HH in
318 the presence of 20 μ M AHI was similar to that of DMSO control. Even at 40 μ M, there was
319 no obvious cytotoxicity (Fig. 3A). Then, we detected the expression of SREBP-1/2 and their
320 target genes, respectively. Consistent with the results in HepG2 cells, AHI significantly
321 reduced the mature SREBP-1/2 (Fig. 3B) and their target genes in HH (Fig. 3C-E).

322 *3.4 AHI suppresses binding of COP II proteins to SCAP/SREBPs complex, thereby controlling* 323 *SCAP sorting in ER*

324 To investigate how AHI suppresses the activity of SREBPs, we checked the
325 post-transcriptional regulation of SREBPs. As shown in Fig. 4A and B, AHI largely reduced
326 the endogenous mature SREBP-1/2, but did not influence the over-expressed mature
327 SREBP-1/2. These results indicated that AHI affects the maturation of SREBPs. It is not
328 surprising that AHI did not increase the degradation rate of both endogenous and
329 over-expressed n-SREBPs (Fig. 4C-I). Meanwhile, when cells were treated with Brefeldin A,
330 S1P and S2P were translocated back from Golgi to ER. The mature form of SREBPs

reappeared, indicating that AHI does not block the proteolysis of SREBPs (Fig. 4J).

When HepG2 cells were cultured in medium D, SREBPs and SCAP formed complex and translocated from ER to Golgi (Fig 5A). When HepG2 cells were treated with AHI or 25-HC, SCAP no longer co-localized with Golgi marker golgin 245, indicating SCAP could not be translocated to Golgi (Fig. 5A). Further, using sucrose gradient sedimentation, we detected the localization of SREBPs and SCAP. It was found that in low sterol condition, the majority of SCAP and SREBPs was shifted from ER to the Golgi fractions (Fig. 5B), while both AHI and 25-HC retained SCAP and SREBPs in the ER fractions (Fig. 5B). These data pinpointed that AHI suppresses the ER-to-Golgi translocation of the SCAP/SREBPs complex.

Furthermore, we checked whether AHI retains SCAP/SREBPs complex in ER through triggering the binding of Insigs to SCAP, similar to 25-HC. After both Insig-1 and SCAP were over-expressed in HEK293T cells, Myc-SCAP was used to pull down Flag-Insig-1. As shown in Fig. 5C, unlike 25-HC, AHI did not promote the interaction between SCAP and Insig-1. COP II proteins Sec23/Sec24 are responsible for the trafficking of SCAP/SREBPs complex from ER to Golgi. Through co-immunoprecipitation, we found that SCAP brought down both Sec23 α and Sec24D in LPDS medium condition (Fig. 5D). Encouragingly, after treated with AHI or 25-HC, SCAP no longer pulled down either Sec23 α or Sec24D (Fig. 5D). Moreover, we found that the interaction between SCAP and PAQR3 (the anchor protein of SCAP in the Golgi) was blocked by AHI (Fig. 5E). It was worthwhile to note that the over-expression of SCAP, Sec23 α and Sec24D together, but not alone, in HepG2 cells upturned the loss of mature SREBP-1/2 induced by AHI, but not by 25-HC (Fig. 5F). These data suggested that AHI inhibits the ER-to-Golgi translocation of the SCAP/SREBPs complex by blocking the

binding of Sec 23 α /Sec24D with SCAP.

3.5 AHI inhibits SREBPs processing through regulating AMPK/mTOR signaling pathway which is dependent on LKB1

SREBPs processing and lipogenesis are regulated by mTOR activity. Therefore, we examined the activity of mTOR and its downstream kinase p70s6k. As shown in Fig.6, AHI decreased the phosphorylation level of mTOR and p70s6k in time-dependent and dose-dependent manners. Moreover, MHY1485, which is a potent mTOR activator (Fig. 7A and 8A), rescued SREBPs abundance and activity (Fig. 7A and Fig. 8B-D). Similarly, the inhibition of mature SREBP-1/2 could also be reversed by shTSC1 (Fig. 7B). These data indicated that mTOR mediates the inhibition of SREBPs processing by AHI. The activity of mTOR is regulated by AMPK, which serves as a key cellular energy sensor. AHI increased the phosphorylation of both AMPK and its substrates ACC (Fig. 6). When the activity of AMPK was inhibited by Compound C (Fig. 9A) or siRNA (Fig. 7D), the reduction of SREBPs activity (Fig 9C), mature SREBP-1/2 (Fig. 7C and D), and their target genes (Fig. 9D and E) were reversed. AMPK also interacts and directly phosphorylates SREBP-1c and SREBP-2, but not SREBP-1a [31]. However, with the increased dose and the extended time of AHI treatment, the phosphorylation of SREBP-1c did not alter (Fig. 6). These results demonstrated that AHI inhibits SREBP-1/2 processing through AMPK/mTOR pathway, rather than a direct effect on SREBPs, because AHI could not directly inhibit the activity of AMPK using an *in vitro* AMPK kinase assay (Fig. 9B). We reasoned AHI could affect AMPK by LKB1 [32]. Firstly, AHI increased the phosphorylation of AMPK at Thr172 (Fig. 6), the LKB1 phosphorylation site. Secondly, in Hela cells which are known to lack the expression of LKB1 [33-35], AHI no

longer decreased the level of mature SREBP-1/2 (Fig.7E). Thirdly, the reduction of mature SREBP-1/2 by AHI in HepG2 cells can be reversed when LKB1 was silenced (Fig. 7F). These data suggested that AHI affects SREBP-1/2 maturation through LKB1/AMPK.

In HEK293T cells, the interaction of over-expressed SCAP with Sec23 α /Sec24D was reversed (Fig. 7G and H) by either MHY1485 or Compound C treatment. Similarly, the interaction of endogenous SCAP and Sec23 α could also be reserved by MHY1485 or Compound C in HepG2 cells (Fig. 7I). These data proved that AHI blocks the binding of SCAP/SREBPs complex with Sec23 α /Sec24D under the regulation of LKB1/AMPK/mTOR pathway.

3.6 AHI ameliorates diet-induced obesity, hyperlipidemia, hepatic steatosis and insulin resistance in mice

To verify the lipid-lowering effect of AHI *in vivo*, the western-type diet (WD)-fed C57BL/6J mice were treated with vehicle (0.5 % CMC-Na), Lovastatin (30 mg/kg/day), or AHI (30 or 60 mg/kg/day) for 7 weeks. No significant difference in food intake was observed among all groups (Fig. 10A). AHI (30 or 60 mg/kg/day) dose-dependently delayed the body weight increase caused by WD (Fig. 10A). In addition, the fat/lean and fat/body-weight ratios of AHI-treated mice were dramatically decreased (Fig. 10B). Lovastatin reduced the lipid absorption through an increasement of fecal triglyceride and cholesterol in mice (Fig. 10C). However, this effect was not observed in the mice fed with AHI (Fig. 10C). Next, we measured the lipid levels in serum and liver. As shown in Fig. 10E, the serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) were largely reduced and the high-density lipoprotein cholesterol (HDL-C) was markedly increased in AHI treated mice. Additionally, TC level was significantly decreased in the livers of mice treated with

AHI or Lovastatin, while TG level in liver was only reduced in AHI treated groups (Fig. 10D). Meanwhile, AHI treatment also reduced the liver/body weight ratio (Fig. 10F). Oil red O and hematoxylin and eosin (H&E) staining showed that administration of AHI ameliorated excess lipid accumulation in liver (Fig. 10G). Moreover, the cell size of both white adipocyte tissue (WAT) and brown adipocyte tissue (BAT) were reduced in WD-fed mice treated with AHI (Fig. 10G). These results demonstrated that AHI ameliorates hyperlipidemia and reduces lipid accumulation in livers of WD-fed mice.

Next, we tried to detect whether AHI improved insulin resistance. Compared with normal diet-fed mice, the WD-fed mice exhibited impaired glucose tolerance and insulin resistance. Interestingly, the glucose tolerance and insulin resistance were markedly ameliorated in AHI treated WD-fed mice (Fig. 11A and B). Furthermore, AHI also decreased the elevated fasting blood glucose (Fig. 11C). These data indicated that AHI improves glucose tolerance and insulin resistance.

3.7 Inhibition of SREBP processing by AHI through AMPK/mTOR pathway in vivo.

Consistent with *in vitro* results, AHI increased the phosphorylation of AMPK and ACC, decreased the phosphorylation of mTOR and p70s6k (Fig 12A). More importantly, the level of mature SREBPs was reduced in the WD-fed mice treated with AHI (Fig. 12B). Next, we investigated the expression of metabolic genes in liver, BAT and WAT. SREBP-1/2 and their target genes in mice livers were reduced by AHI (Fig. 12C and D). AHI increased the expression of LPL, but did not alter the other lipoprotein metabolism genes (Fig. 12E). The mRNA of carbohydrate metabolism genes, GK, IRS-2 and G-6-PD, were increased by AHI (Fig. 12F). Some other genes involved in lipid metabolic regulation were not down-regulated

by AHI (Fig.12G), indicating the AHI effect on lipid metabolism is through SREBPs. AHI increased the expression of UCP-1, UCP-2, Dio2, and PGC-1 α genes in brown adipose tissues (BAT) (Fig. 12H). In white adipose tissues (WAT), AHI dramatically increased the expression of adiponectin, Glut4, LPL, UCP-1, UCP-2 and TNF- α (Fig. 12I). Meanwhile, the expression of FASN, SCD-1, and HMGCR was decreased in WAT of AHI-treated mice (Fig. 12I). All together, these data indicated that AHI regulates SREBPs target genes and metabolism-related genes in liver, BAT, and WAT, which are responsible for the efficacy of AHI.

Consistent with the *in vitro* data, in WD-fed mice, Compound C reversed the inhibition of SREBPs processing (Fig. 13A) and their target genes expression (Fig. 13B), the reduction of lipid accumulation in blood (Fig. 13C and D) and the amelioration of glucose tolerance and insulin resistance (Fig. 13E-H) caused by AHI. Together, these results indicated that the *in vivo* effects of AHI are mostly ascribed to its activity in inhibiting SREBPs processing through regulating the AMPK/mTOR pathway.

4. Discussion

Over the past few decades, SREBPs pathway and its regulation mechanisms are gradually being recognized. Accordingly, we can speculate that an inhibitor of SREBPs may play a role in the following aspects: (1) Inhibition of SREBPs genes expression; (2) Increasing the interaction between SCAP and Insigs to anchor the precursor SREBPs on ER, such as 25-HC, cholesterol, betulin, and fatostatin [10, 27, 36, 37]; (3) Blocking the migration of the SREBP-SCAP complex from ER to Golgi, such as Xanthohumol [29] and P6-55, a synthetic peptide that can cover the amino acid residues 6-55 of PAQR3 [28]; (4) Inhibition of the two

site-specific proteases S1P and S2P, such as PF-429242 and 1,10-phenanthroline [38, 39]; (5) Preventing mature SREBPs from entering into nucleus; (6) Stimulation of n-SREBPs degradation. These possibilities were carefully investigated, and finally, we found that AHI suppresses the processing of SREBPs through blocking the interaction of SCAP/SREBPs complex with COP II proteins Sec23 α /Sec24D. The complexes energy of AHI and Sec23 (-55.6267 Kcal/mol), Sec24 (-19.5631 Kcal/mol) or SCAP (-48.7126 Kcal/mol) is very low, therefore, we considered AHI did not act directly on Sec23/Sec24 and SCAP according to docking assay.

In this study, we also investigated the inhibition of SREBPs activity by the structural analogs of AHI in *Epimedium*. The inhibition activity on SREBPs of ICT, IDHI and AICT was completely lost (Fig 2B-G). Therefore, we can conclude that 6-isopentene and 5-hydroxyl groups of AHI are necessary for pharmacological activities.

In recent years, an increasing number of reports have shown that the processing of SREBPs is directly regulated by mTORC1 or its downstream kinase p70s6k and PASK (PAS domain containing serine/threonine kinase) [40-42]. There is also evidence showing that after mTOR was inhibited, the translocation of SCAP/SREBP-2 complex from the ER to the Golgi in treated VSMCs (vascular smooth muscle cells) was decreased [43]. AMPK, an upstream regulator of mTOR, is reported to regulate the activity of SREBPs through directly phosphorylating SREBPs [31] or negatively regulating mTOR [44]. Han et al demonstrated that CRTC2 (CREB regulated transcription coactivator 2) mediates the mTOR signaling to modulate COP-II-dependent SREBP-1 processing. CRTC2 competes with Sec23 α to interact with Sec31A, another COP II subunit, thus disrupting SREBP-1 transport [45]. However, the

specific regulation mechanism of how mTOR regulates SREBPs processing is still not fully understood. In this work, we demonstrated that AHI inhibits the interaction of SCAP/SREBPs complex with COP II vesicle through suppressing AMPK/mTOR, this effect is unlikely related to SREBP-1 phosphorylation at Ser372 site. It is possible that there are other phosphorylation sites in SREBPs responsible for the binding affinity to COP II vesicles, or there is another mTOR target which affects the SREBPs processing. In future study, we will perform functional proteomics using biotin labeled probes or photoaffinity labeling techniques to identify the AHI target proteins, these data may help us to better understand the regulation of SREBPs processing.

25-HC, a well-known inhibitor of SREBPs processing, may lead to hepatic steatosis and hypertriglyceridemia due to the induction of fatty acid synthesis through activation of LXR (Liver X receptor) [46, 47]. Statins, the most widely prescribed drugs to treat hypercholesterolemia through inhibiting HMGCR, activates SREBPs and raises liver cholesterol and fatty acid biosynthetic enzymes, which lead to adverse effects [48]. AHI inhibits *de novo* synthesis of fatty acid and cholesterol through restraining the SREBPs activation *in vitro* and *in vivo*. It should be noted that AHI ameliorates obesity, insulin resistance, fatty accumulation in liver and hyperlipemia without serious adverse reactions. Thus, AHI can serve as a leading compound for pharmacological control of metabolic diseases.

In DIO mice, there is a clear insulin resistance phenotype, with the AHI treatment, the insulin resistance is dramatically decreased (Fig 11). On the one hand, it could be attributed to the reduced serum lipid level by AHI, on the other hand, since AHI affects AMPK activity, it

1 485 might also improve insulin signaling via AMPK-S6K pathway [49].
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Conflict of interest

There is a pending patent application of AHI for the treatment of metabolic diseases.

Author contributions

Conceived and designed the work: Xiaojun Xu, Hui-Jun Li and Zu-Guo Zheng;

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Dr. Xiaojun Xu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Fig. 1. AHI reduces *de novo* synthesis of fatty acid and cholesterol by inhibiting SREBPs processing and their target genes. (A) HL-7702/SRE-Luc cells were treated with indicated concentrations of AHI and 25-HC for 4 h, cells were lysed and luciferase activity was measured. (B) HepG2 or HL7702 cells were treated with 25-HC, 10 μ M or 20 μ M AHI for 4 h, whole cell extracts underwent IB with indicated antibodies (left). Statistical analysis: Expression of each protein was adjusted to Actin as the loading control (right). (C) HepG2 or HL7702 cells were treated with increasing concentrations of AHI (0-40 μ M) or Doxorubicin (20 μ M) for 24 h, and cell viability was measured by MTT assay. (D) HepG2 cells were treated with 25-HC, AHI or DMSO for 6 h. After the treatment, the images were captured with confocal microscope. (E) HepG2 cells were treated with indicated concentrations of AHI or 25-HC for 4 h and then were lysed to prepare nuclear and membrane fractions. Immunoblots were performed with indicated antibodies (above). Statistical analysis: Expression of each protein was adjusted to Histone as the loading control (below). (F and G) HepG2 cells were treated with 10 μ M or 20 μ M AHI for 4 h. The expression of various genes was analyzed by Q-PCR. (H and I) The treated cells were fixed and stained with Nile-red (H) or Filipin (I). Quantification of the cellular neutral lipids or cholesterol was analyzed by IPP (Image-Pro Plus). (J and K) HepG2 cells were treated with indicated concentrations of AHI or 2 μ M Lovastatin for 16 h. Acetic acid sodium salt $1\text{-}^{14}\text{C}$ was directly added into the medium and incubated for additional 2 h. All experiments were repeated three times. *** $p < 0.001$ vs DMSO.

Fig. 2. AHI and its analogues extracted from *Epimedium* reduce mature SREBPs. (A)

Schematic representation of construct for SRE-containing promoter luciferase. (B) Structures of AHI and its structural analogues. (C) Different abilities of modulating the activity of SRE-containing promoter by AHI and its analogues. HepG2 cells were treated with 20 μ M AHI or its analogues, 5 μ M 25-HC was used as a positive control. Other conditions are described in Figure 1A. (D) The mature SREBPs were reduced by AHI and its analogues. HepG2 cells was treated with 20 μ M AHI or its analogues. Other conditions are described in Figure 1C. (E) Different glycoside structures of AHI. (F) Different abilities of modulating the activity of SRE-containing promoter by the different glycoside of AHI. HepG2 cells were treated with 20 μ M the different glycoside of AHI. Other conditions are described in Figure 1A. (G) The mature SREBPs were decreased by different glycosides of AHI. HepG2 cells were treated with 20 μ M of the different AHI glycosides. Other conditions are described in Figure 1C. All experiments were repeated three times.

Fig. 3. AHI inhibits SREBPs processing and their target genes in human hepatocytes. (A)

HH cells were treated with increasing concentrations of AHI for 24 h, and cell viability was measured by MTT assay. (B) HH cells were switched to medium D containing 25-HC, or increasing concentrations of AHI. After incubation for 4 h, whole cell proteins underwent IB with indicated antibodies (above). Statistical analysis: Expression of each protein was adjusted to Actin as the loading control (below). (C—E) HH cells were treated with 10 or 20 μ M AHI for 4 h. The cells were then cracked in Trizol reagents, and RNA were extracted. The expression of various genes was analyzed by reverse transcription followed by Q-PCR. All Experiments were repeated three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs DMSO.

Fig. 4. AHI does not promote the degradation and proteolysis of SREBPs. (A and B)

HepG2 cells were transfected with Flag-nSREBP-1 (A) or Flag-nSREBP-2 (B) plasmids and cultured for 24 h. the cells were switched to medium D supplemented with vehicle, or 20 μ M AHI for 4 h, whole cell extracts underwent IB with indicated antibodies (above). Statistical analysis: Expression of each protein was adjusted to Actin as the loading control (below). (C-I) AHI does not promote the degradation of mature SREBPs. HepG2 cells were transfected with Flag-SREBP-1 (C) or Flag-SREBP-2 (E) plasmids and cultured for 24 h. After incubation with 50 μ M cycloheximide for 1 h, the cells were switched to medium A supplemented with 50 μ M cycloheximide plus vehicle, or 20 μ M AHI. (G) HepG2 cells were switched to medium D treated with 200 μ M AEBSF for 1 h, the cells were switched to medium D supplemented with 200 μ M AEBSF plus vehicle, or 20 μ M AHI. After incubation for the indicated periods of time, whole cell extracts underwent IB with indicated antibodies. (D, F, H and I) The signals detected on the membrane in (C), (E), and (G) were quantified. (J) HepG2 cells were switched to medium D treated with 2 μ g/ml Brefeldin A for 1 h, the cells were switched to medium D supplemented with 2 μ g/ml Brefeldin A plus vehicle, or 20 μ M AHI. After incubation for the indicated periods of time, whole cell extracts underwent IB with indicated antibodies (left). Statistical analysis: Expression of each protein was adjusted to Histone as the loading control (right). All experiments were repeated three times. Error bars represent standard deviations. Statistical analyses were done with one-way ANOVA (Tukey's multiple comparisons test). * $p < 0.5$, *** $p < 0.001$.

Fig. 5. AHI inhibits the SCAP/SREBPs complex into COP II vesicles by reducing the interaction of SCAP and Sec23/Sec24. (A) HepG2 cells were depleted of sterols or not by incubating in medium D in presence of 25-HC or AHI for 6 h, cells were stained with primary antibody.

Cell nucleus were stained with Hoechst. The cells were imaged with EVOS FL Auto microscope. (B) HepG2 cells were switched to medium D containing 25-HC or AHI for 4 h, the cells were then subjected to homogenization and cell fractionation by gradient centrifugation. Equal volumes of each fraction were subjected to IB. (C-F) HepG2 cells were transfected with indicated plasmids or not and treated with medium D containing 25-HC or AHI for 4 h, whole cell proteins were underwent IB or IP (above). Statistical analysis: Expression of each protein was adjusted to Actin as the loading control (below). All experiments were repeated three times. *** $p < 0.001$.

Fig. 6. AHI influences AMPK-mTOR-P70S6K pathway by a time- and dose-dependent manner. HepG2 cells were depleted of sterols by incubating in medium D containing AHI for indicated time periods (left) or increasing concentrations of AHI as indicated for 4 h (right). Whole cell extracts were subjected to IB with the antibodies as indicated. Statistical analysis: Expression of each protein was adjusted to Actin as the loading control (below). All experiments were repeated three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs DMSO.

Fig. 7. Inhibition of SREBPs processing by AHI is dependent on LKB-1/AMPK/mTOR pathway. (A) HepG2 cells were incubated with or without MHY1485 or Rapamycin for 1 h, the cells were switched to medium D in the presence of vehicle, or AHI. (B) HepG2 cells were transfected with sh-TSC1 plasmid or not and cultured for 48 h. Cells were switched to medium D containing AHI. (C) HepG2 cells were incubated with or without Compound C for 1 h, the cells were switched to medium D in the presence of vehicle, or AHI. (D) HepG2 cells were transiently transfected with negative control siRNA or siAMPK for 48 h. Cells were switched to medium D with AHI. (E) HeLa and HepG2 cells were incubated in medium D

with vehicle, 25-HC, or AHI. (F) HepG2 cells were transiently transfected with siLKB1 for 48 h. All cells were switched to medium D containing AHI for 4 h, whole cell proteins were underwent IB with indicated antibodies. (G - I) HEK293T cells were transiently transfected with the plasmids as indicated 24 h (G and H), and HepG2 cells (I) were not transfected, both of cells were incubated with or without Compound C or MHY1485 for 1 h, the cells were treated with AHI for 4 h, the cell lysate was used in IB and IP. Statistical analysis: Expression of each protein was adjusted to Actin as the loading control. All experiments were repeated three times. *** $p < 0.001$.

Fig. 8. MHY1485 reverses the inhibiting effects of the activity of SRE-containing

promoter and the SREBP_s targeted genes by AHI. (A) HepG2 cells were treated as in figure 7A. After incubation for 6 h, whole cell proteins were underwent IB with the antibodies as indicated (left). Statistical analysis: Expression of each protein was adjusted to Histone as the loading control (right). (B) HepG2 cells were depleted of sterols through incubation in medium D and incubated with or without 10 μ M MHY1485 for 1 h. and then the cells were switched to medium D in the presence of vehicle, or 20 μ M AHI for 4 h, cells were lysed with reporter lysis buffer, and luciferase activity was measured. (C-D) HepG2 cells were treated as in Figure 8B. The cells then were lysed, and RNAs were extracted. The expression of various genes was analyzed by reverse transcription followed by quantitative-PCR (Q-PCR). All experiments were repeated three times. Error bars represent standard deviations. Statistical analyses were done with one-way ANOVA (Tukey's multiple comparisons test). * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 9. Compound C reverses the SREBPs inhibiting effects by AHI. (A) HepG2 cells

were treated as in Figure 7C. After incubation for 4 h, whole cell proteins were underwent IB with the antibodies as indicated (left). Statistical analysis: Expression of each protein was adjusted to Histone as the loading control (right). (B) AMPK enzyme assay *in vitro*. (C) HepG2 cells were depleted of sterols through incubation in medium D and incubated with or without 5 μ M Compound C for 1 h. and then the cells were switched to medium D in the presence of vehicle, or 20 μ M AHI. After incubation for 4 h, cells were lysed with reporter lysis buffer, and luciferase activity was measured. (D and E) HepG2 cells were treated as in figure 9 B. The cells then were lysed, and RNA were extracted. The expression of various genes was analyzed by reverse transcription followed by quantitative-PCR (Q-PCR). All experiments were repeated three times. Error bars represent standard deviations. Statistical analyses were done with one-way ANOVA (Tukey's multiple comparisons test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 10. AHI improves the development of obesity and reduces the lipid levels in WD-fed mice. (A) Food intake and body weight. (B) The ratio of fat and body weight or lean. (C) Effect of AHI on fecal TG and TC. (D) Effect of AHI on TG and TC in liver. (E) Effect of AHI on blood TG, TC, LDL-C, and HDL-C levels. (F) The weight of liver. (G) Representative gross morphology of the mouse livers, and histological analysis of liver, WAT, and BAT. $n=3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Model. Chow=Normal Diet + Vehicle, Model=Western Diet (WD) + Vehicle, Lova (30mg/kg) =WD + Lovastatin (30mg/kg), AHI (30mg/kg) =WD + AHI (30mg/kg), AHI (60mg/kg) =WD + AHI (60mg/kg).

Fig. 11. AHI improves the glucose intolerance and insulin resistance in WD-fed mice. (A-C) AHI improves glucose tolerance (A) insulin resistance (B), Blood glucose (C) in

WD-fed mice. **p < 0.01, ***p < 0.001 vs Model.

Fig. 12. AHI influences AMPK/mTOR pathway and decreases the expression of SREBPs

and their target genes in WD-fed mice. (A-B) For each group, equal amounts of total

proteins from the livers of 3 mice were subjected to IB with indicated antibodies (above).

Statistical analysis: Expression of each protein was adjusted to Actin as the loading control

(below). (C-I) Total RNA were extracted from different tissues, gene expression in mice liver

(C-G), BAT (H), and WAT (I). n=3, *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 13. Compound C reverses the effects induced by AHI in WD-fed mice. Male

C57BL/6J mice at 8 weeks of age were randomly grouped (6 per group). Mice were allowed

ad libitum access to water and western-type diet (WD). Vehicle, 60 mg/kg/day of AHI was

administered to mice by gastric irrigation once daily. After four-week treatment, mice were

intraperitoneally injected with 15mg/kg/day of Compound C. Vehicle or 60 mg/kg/day of AHI

was still administrated to mice by gastric irrigation once daily for two weeks. Then, mice

were sacrificed and subjected to various analyses. (A) For each group, equal amounts of total

proteins from the livers of 3 mice were subjected to IB with anti-SREBP-1, anti-SREBP-2 or

anti-actin antibodies. The same results were obtained in three separate experiments. (B) For

each group, equal amounts of total RNA from livers of 3 mice were subjected to Q-PCR

quantification as described in methods. Mouse GAPDH was used as the control. Error bars

represent standard deviations. Statistical analyses were done with one-way ANOVA (Tukey's

multiple comparisons test). *p < 0.05, **p < 0.01, ***p < 0.001 vs Model. (C and D) Effect of

AHI on blood triglyceride (TG), cholesterol (TC) levels after 6 weeks treatment. Statistical

analyses were done with one-way ANOVA (Tukey's multiple comparisons test). *p < 0.05 vs

Model. (E-H) Compound C reverses the amelioration of glucose tolerance by AHI in WD-fed mice (E). Quantification of the area under the curve (AUC) (F). Statistical analyses were done with one-way ANOVA (Tukey's multiple comparisons test). * $p < 0.05$, and ** $p < 0.01$ vs Model. Compound C reverses the amelioration of insulin resistance in WD-fed mice. Quantification of the area under the curve (AUC) (G). Statistical analyses were done with one-way ANOVA (Tukey's multiple comparisons test). * $p < 0.05$, ** $p < 0.01$ vs Model (H).

Fig 1

Fig. 2

Fig. 3

Fig. 6

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Table 1 Nucleotide Sequences of Gene-Specific Primers Used for Quantitative Real-Time PCR, Related to the Experimental Procedures

Specifies	Gene name	Sequence of forward and reverse primers(5' to 3')
<i>Mus musculus</i>	GAPDH	TGTGTCCGTCGTGGATCTGA CCTGCTTCACCACCTTCTTGAT
	SREBP-1a	GGCCGAGATGTGCGAACT TTGTTGATGAGCTGGAGCATGT
	SREBP-2	GCGTTCTGGAGACCATGGA ACAAAGTTGCTCTGAAAACAAATCA
	SCAP	ATTTGCTCACCGTGGAGATGTT GAAGTCATCCAGGCCACTACTAATG
	HMGCS	GCCGTGAACTGGGTCGAA GCATATATAGCAATGTCTCCTGCAA
	HMGCR	CTTGTGGAATGCCTTGTGATTG AGCCGAAGCAGCACATGAT
	FDPS	ATGGAGATGGGCGAGTTCTTC CCGACCTTTCCCGTCACA
	SS	CCAACTCAATGGGTCTGTTTCCT TGGCTTAGCAAAGTCTTCCAAC
	LDLR	AGGCTGTGGGCTCCATAGG TGCGGTCCAGGGTCATCT
	ACC	TGACAGACTGATCGCAGAGAAAG TGGAGAGCCCCACACACA
	Fasn	GCTGCGGAACTTCAGGAAAT AGAGACGTGTCACTCCTGGACTT
	SCD-1	CCGGAGACCCCTTAGATCGA TAGCCTGTAAAAGATTCTGCAAACC
	ACOX1	TTTGTTGTCCCTATCCGTGAGA CCGATATCCCCAACAGTGATG
	ApoB	CGTGGGCTCCAGCATTCTA TCACCAGTCATTTCTGCCTTTG
	ApoE	GCTGGGTGCAGACGCTTT TGCCGTCAGTTCTTGTGTGACT

<i>Mus musculus</i>	GK	CCGTGATCCGGGAAGAGAA GGGAAACCTGACAGGGATGAG
	ACL	GCCAGCGGGAGCACATC CTTTGCAGGTGCCACTTCATC
	ACS	GCTGCCGACGGGATCAG TCCAGACACATTGAGCATGTCAT
	G-6-PD	GAACGCAAAGCTGAAGTGAGACT TCATTACGCTTGCACTGTTGGT
	IRS-1	GCGGGCTGACTCCAAGAAC GCTATCCGCGGCAATGG
	IRS-2	GGAGAACCCAGACCCTAAGCTACT GATGCCTTTGAGGCCTTCAC
	PEPCK	CCACAGCTGCTGCAGAACA GAAGGGTCGCATGGCAAA
	ABCA1	CGTTTCCGGGAAGTGTCTA GCTAGAGATGACAAGGAGGATGGA
	ABCG5	TGGATCCAACACCTCTATGCTAAA GGCAGGTTTTCTCGATGAACTG
	Insig-1	TCACAGTGACTGAGCTTCAGCA TCATCTTCATCACACCCAGGAC
	Insig-2a	CCCTCAATGAATGTACTGAAGGATT TGTGAAGTGAAGCAGACCAATGT
	Insig-2b	CCGGGCAGAGCTCAGGAT GAAGCAGACCAATGTTTCAATGG
	HL	GGAGGAATCTGTTCAACTCTCTCG AGAAAGACGATTGCTGGGGG
	SR-B1	TGGACAAATGGAACGGACTC GTGAAGCGATACGTGGGAAT
	LPL	CTTCTTGATTTACACGGAGGT ATGGCATTTCACAAACACTG
	PPAR α	TCTGTGGGCTCACTGTTCT AGGGCTCATCCTGTCTTTG
	Leptin	CGGTTCTGTGGCTTTGG GGTCTGAGGCAGGGAGCA
	adiponectin	GTTGCAAGCTCTCCTGTTCC TCTCCAGGAGTGCCATCTCT
	Glut1	GAGCATCTTCGAGAAGGCAGGTGT GGCCACAATGAACCATGGAATA
	Glut4	AGTGACTGGGACACTGGTCCTT ACATTGTTGGCCAGCATAGC

<i>Mus musculus</i>	PPAR- γ	GGCTGAGGAGAAGTCACACTCTG AAATCTTGTCTGTACACAGTCCTG
	UCP-1	GTGAAGGTCAGAATGCAAGC AGGGCCCCCTTCATGAGGTC
	UCP-2	GCCCCTTCACCTCTTTAGCA CCAAGCACTGGGAAGGTCTAAC
	PCSK9	ACCCTCATAGGCCTGGAGTT CTGTGATGACCTCTGGAGCA
<i>Homo sapiens</i>	SREBP-2	AACGGTCATTCACCCAGGTC GGCTGAAGAATAGGAGTTGCC
	HMGCR	TGATTGACCTTTCCAGAGCAAG CTAAAATTGCCATTCCACGAGC
	LDLR	ACCAACGAATGCTTGGACAAC ACAGGCACTCGTAGCCGAT
	HMGCS-1	CTCTTGGGATGGACGGTATGC GCTCCAACTCCACCTGTAGG
	MVK	GGAGCAAGGTGATGTCACAAC CGGCAGATGGACAGGTATAAGT
	FPPS	TGTGACCGGCAAAATTGGC GCCCCGTTGCAGACACTGAA
	FDFT-1	CCACCCCGAAGAGTTCTACAA TGCGACTGGTCTGATTGAGATA
	LSS	GTACGAGCCCGGAACATTCTT CGGCGTAGCAGTAGCTCAT
	Insig-1	CCTGGCATCATCGCCTGTT AGAGTGACATTCCTCTGGATCTG
	SE	CCTCTTTGTCTTTACGGTTTCC GTCCCAGTGCCTTTGATGTT
	DHCR-7	GCTGCAAAATCGCAACCCAA GCTCGCCAGTGAAAACCAGT
	MSMO-1	TGCTTTGGTTGTGCAGTCATT GGATGTGCATATTCAGCTTCCA
	APOE	GTTGCTGGTCACATTCCTGG GCAGGTAATCCCAAAGCGAC
	ACLY	ATCGGTTCAAGTATGCTCGGG GACCAAGTTTTCCACGACGTT
	PSCK9	ATGGTCACCGACTTCGAGAAT GTGCCATGACTGTCACACTTG

	SREBP-1C	ACAGTGACTTCCCTGGCCTAT
		GCATGGACGGGTACATCTTCAA
<i>Homo sapiens</i>	ACC-1	ATGTCTGGCTTGCACCTAGTA
		CCCCAAAGCGAGTAACAAATTCT
	SCD	TCTAGCTCCTATAACCACCACCA
		TCGTCTCCAACCTTATCTCCTCC
	FASN	CCGAGACACTCGTGGGCTA
		CTTCAGCAGGACATTGATGCC
	FASD-2	GACCACGGCAAGAACTCAAAG
		GAGGGTAGGAATCCAGCCATT
	SCD-2	CTCTGCGAGTGAATTTGGC
		GATCATCGGCTTGGTTGC
	Insig-2	GTGAACTTGATGATTCGAGGAG
		ACATCAGGTGGAAAGAGCGT
	ABCG5	CCTGAGGTTGCCCCGATTT
		GGACAGCAGAGCCACTACACT
	ABCG8	TTTCCAACGACTTCCGAGAC
		GCCTCAGCGATTCTTGATTAT
	ABCA1	TGCTAATTGCCAGACGGAG
		GGGTACTTGCCAAAGGGTG
	CYP7A1	CACCATTCAGCGACTTTCT
		GCCTCAGCGATTCTTGATTAT
	PAQR3	TGTATGCTTTGCTCTGTGGG
		ACACCTGACGCCAGTAGTTATT
	SQS	AAAGAAGGTCCCGCTGTTAC
		GCAAATGTCGCAATCACT

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