Combination of honokiol and magnolol inhibits hepatic steatosis through AMPK-SREBP-1 c pathway

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Abstract

Honokiol and magnolol, as pharmacological biphenolic compounds of *Magnolia officinalis*, have been reported to have antioxidant and anti-inflammatory properties. Sterol regulatory element binding protein-1 c (SREBP-1 c) plays an important role in the development and processing of steatosis in the liver. In the present study, we investigated the effects of a combination of honokiol and magnolol on SREBP-1 c-dependent lipogenesis in hepatocytes as well as in mice with fatty liver due to consumption of high-fat diet (HFD). Liver X receptor α (LXR α) agonists induced activation of SREBP-1 c and expression of lipogenic genes, which were blocked by co-treatment of honokiol and magnolol (HM). Moreover, a combination of HM potently increased mRNA of fatty acid oxidation genes. HM induced AMP-activated protein kinase (AMPK), an inhibitory kinase of the LXR α -SREBP-1 c pathway. The role of AMPK activation induced by HM was confirmed using an inhibitor of AMPK, Compound C, which reversed the ability of HM to both inhibit SREBP-1 c induction as well as induce genes for fatty acid oxidation. In mice, HM administration for four weeks ameliorated HFD-induced hepatic steatosis and liver dysfunction, as indicated by plasma parameters and Oil Red O staining. Taken together, our results demonstrated that a combination of HM has beneficial effects on inhibition of fatty liver and SREBP-1 c-mediated hepatic lipogenesis, and these events may be mediated by AMPK activation.

Keywords: Honokiol, magnolol, hepatic steatosis, sterol regulatory element binding protein-1 c, AMP-activated protein kinase

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a hepatic component of metabolic syndrome.^{1–3} A spectrum of NAFLD ranges from triglyceride (TG) accumulation in the liver to hepatic steatosis accompanied by inflammation (steatohepatitis), fibrosis, and cirrhosis.^{4,5} Hepatic steatosis is the first stage in the disease continuum of NAFLD. Up to 30% of patients with hepatic steatosis progress to non-alcoholic steatohepatitis, in which 30% of patients also progress to cirrhosis.⁶ Most drugs evaluated for the treatment of NAFLD are those commonly used for the treatment of type 2 diabetes (e.g. metformin, glitazone, statin and insulin), and they exert rather indirect effects through improvement of insulin resistance and glycemia.⁶ There are some therapeutic interventions that likely improve hepatic steatosis but currently lack confirmatory clinical data including metformin, glucagon-like peptide 1 agonist, farnesoid X receptor agonists and angiotensin II type I receptor

blockers.⁶ Therefore, the development of efficacious therapeutics to prevent or treat NAFLD is needed.

Liver X receptors (LXRs), LXR α (NR1H3) and LXR β (NR1H2), are nuclear receptors that regulate the metabolism of lipids such as cholesterol and bile acids.⁷ Among molecules involved in this process, sterol regulatory element binding proteins (SREBPs) have been identified as attractive targets for correcting lipid metabolism. SREBP-1c increases lipogenesis, and its expression is transcriptionally up-regulated by LXR α . The LXR α /SREBP-1c signaling pathway may play a crucial role in the pathogenesis of NAFLD by suppressing key enzymes involved in lipogenesis (e.g. fatty acid synthase [FAS], acetyl-CoA carboxylase [ACC] 1 and glycerol-3-phosphate acyltransferase).

The importance of active constitutive agents in natural products has become increasingly apparent due to their therapeutic properties and abilities. A number of recent studies have focused on the treatment of metabolic syndrome

and its health risks by using naturally occurring phytochemicals. Honokiol and magnolol are two prominent constituents isolated from the medicinal herb *Magnolia officinalis*, which is used for the treatment of some chronic diseases in traditional Chinese medicine.⁸ Some preliminary results have provided a scientific evidence for their uses for the treatment of chronic diseases such as Alzheimer disease based on inhibition of reactive oxygen species (ROS), Ca^{2+} elevation, and apoptosis.⁹ Recent studies have demonstrated that the *M. officinalis* extract or honokiol inhibited ethanol-induced liver disease.^{10,11} Although these studies have reported the protective effects of honokiol or magnolol against cell and organ injury induced by xenobiotic,^{9–12} there is no report showing the effects of honokiol and magnolol on diet-induced fatty liver disease (NAFLD) and metabolic syndrome.

Considering the involvement of SREBP-1c in hepatic steatosis as well as the potential of honokiol and magnolol for preventing liver disease as an antioxidant, this study determined whether or not these beneficial compounds inhibit SREBP-1c-mediated lipogenesis in hepatocytes as well as fatty liver in high-fat diet (HFD)-fed animals. Here, we report that co-treatment of honokiol and magnolol synergistically inhibited the expression of both SREBP-1c and lipogenic genes induced by LXR α through activation of AMPK, thereby protecting the liver from fat accumulation and cellular injury. Our results promote the possibility of a new therapeutic candidate from medical plant to treat hepatic steatosis and metabolic syndrome.

Materials and methods

Chemicals and reagents

For *in vitro* study, honokiol and magnolol were obtained from Sigma-Aldrich Chemicals (St. Louis, MO) and dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 100 mmol/L, and stored at -20°C . For *in vivo* study, honokiol and magnolol were isolated from the methanolic extract of stem bark of *M. officinalis* (Wolsung, Daegu, Korea) using successive silica gel column chromatography. They were verified by HPLC analysis using purchased honokiol and magnolol, and the chemical structure was confirmed by NMR analysis. Compound C was purchased from Calbiochem (San Diego, CA). Antibodies directed against SREBP-1c and AMPK were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against phospho-AMPK, phospho-liver kinase B1 (phospho-LKB1), LKB1, and phospho-ACC were supplied from Cell Signaling Technology (Beverly, MA). Anti- β -actin antibody, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), T0901317 (T090), GW3965, and other reagents were purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Zymed Laboratories (San Francisco, CA). Polyethylene glycol 400 (PEG) was obtained from Yakury Pure Chemical Co. (Kyoto, Japan).

Cell culture

HepG2, Hep3B, and Huh-7 human hepatocyte-derived cell lines were purchased from American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Cultures were maintained at 37°C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2 . FBS, cell culture media, penicillin/streptomycin, and all other reagents used for the cell culture studies were purchased from Gibco (Grand Island, NY).

Cell viability assay

The cells were plated in a 96-well plate to determine any potential cytotoxicity. Cells were serum-starved for 12 h, and then treated with honokiol and magnolol for the next 24 h. After incubation of the cells, viable cells were stained with MTT (0.2 mg/mL, 4 h). The media were then removed, and produced formazan crystals in the wells were dissolved by addition of 200 μL DMSO. Absorbance was measured at 540 nm using ELISA microplate reader (Tecan, Research Triangle Park, NC).

Western blot analysis

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) before lysis. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Thermo Scientific) followed by centrifugation at 15,000 r/min for 15 min, and protein concentration was determined using a bicinchoninic acid (BCA) method.¹³ Protein (30–50 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 5% skim milk in PBS, membranes were incubated with the primary antibodies, followed by the secondary antibody conjugated to horseradish peroxidase (HRP) and detected with an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ). Equal loading of proteins was verified by actin immunoblottings. At least three separate experiments were performed to confirm changes. Band intensities were quantified using IMAGE J 1.42 software (NIH Bethesda).

RT-PCR and real-time RT-PCR analyses

Total RNA was isolated from HepG2 cells using a Trizol reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse-transcribed using reverse transcriptional polymerase chain reaction (RT-PCR) kit (Promega, Madison WI) to obtain cDNA. To determine mRNA level of FAS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), equal amounts of cDNA were subsequently amplified by PCR in a 25 μL reaction volume containing 1X PCR reaction buffer, 200 mmol/L dNTPs, 0.5 pmol specific primers, and 1 unit Taq DNA polymerase (TaKaRa Biotech, Shiga, Japan). GAPDH was used as an internal control of cDNA

amplification. Amplification products were separated on 1.5% agarose gels and results were recorded by an Image Scan & Analysis System (Alpha-Innotech, San Leandro, CA). Band intensities were quantified using IMAGE J 1.42 software. To determine mRNA level of ACC1, stearyl-CoA desaturase-1 (SCD-1), and carnitine palmitoyltransferase-1 (CPT-1), real-time PCR was carried out according to the manufacturer's instruction (7500 real-time PCR system, Applied Biosystems, Foster City, CA). The C_p values for ACC1, SCD-1, and CPT-1 were normalized based on that of GAPDH. A melting curve analysis was done after amplification to verify the accuracy of the amplicon. The following primer sequences were used: human FAS, 5'-GACATC GTCCATTCGTTTGTG-3' (sense) and 5'-CGGATCACC TTCTTGAGCTCC-3' (antisense); human ACC1, 5'-GCT GCTCGGATCACTAGTGAA-3' (sense) and 5'-TTCTGC TATCAGTCTGTCCAG-3' (antisense); human SCD-1, 5'-CCTCTACTTGGAAGACGACATTCGC-3' (sense) and 5'-GCAGCCGAGCTTTGTAAAGAGCGGT-3' (antisense); human CPT-1, 5'-ACAGTCGGTGAGGCCTCTTATGAA-3' (sense) and 5'-TCTTGCTGCCTGAATGTGAGTTGG-3' (antisense); human GAPDH, 5'-AACGACCCCTT CATTGAC-3' (sense) and 5'-TCCACGACATACTCAGC AC-3' (antisense).

Transient transfection and reporter gene assays

HepG2 cells were transiently transfected with TK-CYP7A1-LXREx3-LUC (0.5 μ g) or pGL2-FAS (0.5 μ g) for 6 h in the presence of FuGENE[®] reagent (Roche, Nutley, NJ). The activity of luciferase was measured by adding luciferase assay reagent (Promega).

Animal treatments

C57BL/6 mice at five weeks of age (male, 14–16 g) were provided from Charles River Orient Bio (Seongnam, Korea), acclimatized for one week. Animals were caged under the supply of filtered pathogen-free air, commercial rat chow (Purina, Seoul, Korea) and water *ad libitum* at a temperature between 20°C and 23°C with 12 h light and dark cycles and relative humidity of 50%. All animal procedures received approval from the Institutional Animal Care and Use Committee of the Daegu Haany University and were conducted in agreement with the guidelines of the National Institutes of Health (DHU 2013-096). C57BL/6 mice were started on either a normal diet (ND) or an HFD (60% of kilocalories as fat; Product D12492, Research Diets, New Brunswick, NJ) for eight weeks. After ND and HFD feeding, the mice were randomly distributed based on the body weight into four treatment groups; ND ($n=8$), ND + honokiol + magnolol (HM) ($n=5$), HFD ($n=8$), and HFD+HM ($n=8$). Animals received vehicle or HM dissolved in 40% PEG 400. HM was orally administered four times per week during the last four weeks of diet feeding, and dose of HM was 10 mg/kg for two weeks and 20 mg/kg for consecutive two weeks.

Hematoxylin & eosin or Oil Red O staining

The left lateral lobe of the liver was sliced and tissue slices were fixed in neutral buffered 4% formaldehyde, and then embedded in paraffin. The sectioned liver tissues (3–4 μ m) were stained with hematoxylin and eosin (H&E) for general histopathology. The histopathological profiles of each sample were observed under light microscope (Nikon, Japan). To observe more detailed histopathological changes, the steatohepatitis regions and mean hepatocyte diameters were calculated using an automated image analysis process (DMI; Daegu, Korea) on the restricted view fields according to previous method.¹⁴ The histopathologist was blind to group distribution when this analysis was made.

For Oil Red O (Sigma) staining, 4 μ m sections were cut from frozen optimal-cutting temperature samples, affixed to microscope slides, and allowed to air-dry overnight at room temperature. The liver sections were stained in fresh Oil Red O for 10 min and rinsed in water. The staining photos were taken under the light microscope. The lipid level of liver was estimated by measuring the area of Oil Red O staining in liver tissue by using the IMAGE J image analysis program developed at the National Institutes of Health. In each mouse, the mean for three independent sections was used for the analysis.

Blood chemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, and low-density lipoprotein (LDL) cholesterol levels were analyzed by the Green Cross Reference Lab (Seoul, Korea). Fasting blood glucose was assessed using Accu-Chek Active (Roche Diagnostics, Heidelberg, Germany) after fasting overnight.

Statistical analysis

All data are reported as mean $\pm$ SD. For cell-based assays, data represent at least three separate experiments. One-way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Student's *t* test analysis was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$. For animals, all histomorphometrical values are expressed as mean $\pm$ SD of six mice, one histological field/mouse. A multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test.^{15,16} The Mann-Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different.

Results

Inhibition of LXR α -mediated lipogenesis by honokiol plus magnolol

First, we confirmed the cytotoxic effects of honokiol (Figure 1a) and magnolol (Figure 1b) on a hepatocyte cell line. There were no changes in cell survival rate up to 10 μ mol/L, but treatment of honokiol or magnolol at the higher concentration than 10 μ mol/L showed severe

Figure 1 Structure of honokiol (a) and magnolol (b). (c) The effect of honokiol or magnolol (left) and honokiol + magnolol (right) on hepatocyte viability

cytotoxicity (Figure 1c). Next, the effects of honokiol and magnolol on T090-mediated SREBP-1c induction in HepG2 cells were examined. T090 is a highly potent and non-steroidal synthetic agonist of LXR α and was used here to induce SREBP-1c activation. As expected, T090 treatment for 12 h markedly induced SREBP-1c protein expression in HepG2 cells (Figure 2a). Combined treatment with honokiol and magnolol at 10 μ mol/L each notably inhibited T090-induced upregulation of the SREBP-1c protein level almost down to the level displayed by the control, whereas honokiol or magnolol alone had no effect on SREBP-1c level (Figure 2b). When cells were treated with various concentrations of honokiol plus magnolol (1–10 μ mol/L), a combination of 10 μ mol/L of honokiol and magnolol significantly inhibited SREBP-1c only (Figure 2c). Therefore, combined treatment of 10 μ mol/L HM was used in subsequent experiments. We also confirmed the ability of HM to inhibit SREBP-1c activation in Huh-7 and Hep3B cells, two other human hepatocyte cell lines (Figure 2d). Moreover, HM treatment blocked the ability of GW3965 (another LXR α agonist) to induce SREBP-1c expression (Figure 2e).

LXRs bind to their cognate liver X response elements (LXREs), consisting of two direct hexanucleotide repeats separated by four nucleotides (DR4) in the promoters of target genes such as LXR α , SREBP-1c, cholesterol 7 α hydroxylase (CYP7A1), FAS and ACC.¹⁷ Next, we determined whether or not HM could inhibit LXR α -mediated lipogenesis in reporter gene assay. HepG2 cells were transiently transfected with a luciferase reporter plasmid for LXRE (TK-CYP7A1-LXREx3-LUC). Figure 2f shows that CYP7A1-LXRE-dependent transcription was activated by T090, and this effect was diminished by HM. Our results demonstrated that HM might inhibit LXR α -mediated lipogenesis in hepatocytes.

Inhibition of SREBP-1c target gene induction by honokiol plus magnolol

SREBP-1c activates transcription of ACC, FAS, SCD-1, and others.^{18–21} To elucidate the molecular mechanisms of HM for inhibition of hepatic lipogenesis, we assessed the mRNA expression levels of ACC, SCD-1, and FAS in HepG2 cells. Results showed that HM prevented T090-mediated upregulation of lipogenic gene expression (Figure 3a). This study also determined the effects of HM on the transcriptional activity of SREBP-1c by transfecting a pGL2-FAS luciferase reporter gene construct containing a sterol response element (SRE) in the FAS promoter region into HepG2 cells. Incubation of cells with T090 increased FAS luciferase activity from the construct, which was significantly attenuated by HM (Figure 3b).

Next, we examined whether or not HM affects mitochondrial function. For this, we monitored the transcription level of CPT-1, which promotes mitochondrial fuel oxidation and biogenesis. As shown in Figure 3c, HM significantly increased the mRNA level of CPT-1 in HepG2 cells in a time-dependent manner.

AMPK activation by honokiol plus magnolol

AMPK activation is known to stimulate hepatic fatty acid β oxidation as well as inhibits lipogenesis.²² Therefore, we determined whether or not HM stimulate phosphorylation of AMPK in HepG2 cells. As shown in Figure 4a, HM treatment significantly increased the phosphorylation levels of AMPK and ACC, a downstream substrate of AMPK, beginning from 30 min after HM treatment (Figure 4a). HM treatment also markedly increased the phosphorylation level of LKB1, one of the main upstream kinases of AMPK and a regulator of glucose and lipid metabolisms (Figure 4b).²³

Figure 2 Inhibition of the T0901317-induced SREBP-1 c activation by honokiol plus magnolol. (a) Immunoblotting for SREBP-1 c in HepG2 cells. Immunoblots were performed on the lysates of cells treated with vehicle or honokiol (H), magnolol (M), and honokiol + magnolol (HM) for 1 h, and subsequently exposed to 1 μmol/L T0901317 (T090) for 12 h. (b) Relative protein levels of SREBP-1 c in HepG2 cells. (c) The effect of HM combination on SREBP-1 c induction. HepG2 cells were treated with H and M (1, 3, 5 or 10 μmol/L) for 1 h followed by subsequent exposure to 1 μmol/L T090 for 12 h. (d) Effects of HM on SREBP-1 c induction in human hepatocytes cell lines, Huh-7 and Hep3B. (e) The effect of HM on SREBP-1 c induction by 1 μmol/L GW3965 treatment for 12 h. (f) The relative LXRE luciferase activity. After LXRE transfection, the relative luciferase activity was monitored in the lysates of the cells exposed to different treatment combinations (T090 for 12 h with or without HM pretreatment for 1 h). Data represent the means ± SD of three separate experiments. Statistical significance of differences between each treatment group and the control (* $P < 0.05$; ** $P < 0.01$) or T090 alone (# $P < 0.05$; ## $P < 0.01$) was determined

An inhibitor experiment investigated the role of AMPK in the effects of HM on lipogenesis and fuel oxidation. HepG2 cells were pretreated with Compound C, a specific inhibitor of AMPK, and then treated with HM. Inhibition of AMPK activity by Compound C completely prevented HM from enhancing CPT-1 mRNA expression in HepG2 cells (Figure 4c). Moreover, the inhibitory effect of HM on

SREBP-1 c induction was reversed in the presence of Compound C (Figure 4d).

Effects of honokiol plus magnolol on HFD-induced hepatic steatosis

To investigate an inhibitory effect of HM on fatty liver *in vivo*, we used an animal model in which mice were fed

Figure 3 Effect of honokiol plus magnolol on gene expression of lipogenesis and mitochondrial oxidation. (a) mRNA level of lipogenic gene, ACC and SCD-1 were analyzed by real-time RT-PCR assay (upper), and FAS was by RT-PCR analysis (lower). The relative FAS mRNA level was the band intensity of RT-PCR, which was measured by IMAGE J analysis. HepG2 cells were treated with vehicle or HM (10 + 10 μ mol/L) for 1 h, and continuously incubated with 1 μ mol/L T090 for 12 h. (b) Inhibition by HM of T090-induced, SRE-mediated FAS gene transactivation. FAS luciferase assays performed in the cells exposed to different treatments (T090 for 12 h with or without HM pretreatment for 1 h). (c) Real-time RT-PCR assays for CPT-1 mRNA. HepG2 cells were treated with HM (10 + 10 μ mol/L) for the indicated times. Statistical significance of differences between each treatment group and the control (* P < 0.05; ** P < 0.01) or T090 alone (* P < 0.05; ** P < 0.01) was determined

a HFD (60% fat) for four weeks and then randomly divided into two groups (vehicle or HM) (Figure 5a, left). Body weight gain in mice fed a HFD (17.4 ± 2.2 g) was significant (P < 0.01 versus ND), whereas mice fed a HFD in combination with HM (12.7 ± 2.6 g) showed significantly lower body weights compared to the HFD group (P < 0.01 versus HFD) (Figure 5a, right). To examine the effect of HM on fat accumulation *in vivo*, the livers were stained with Oil Red O, which specifically represents fat accumulation in tissue. HFD feeding for eight weeks caused severe fat deposition, as indicated by increases in Oil Red O staining intensity (Figure 5b, left). Administration of HM significantly reversed the excessive accumulation of fat in livers of mice (Figure 5b, right).

Moreover, mice fed with HFD for eight weeks elevated plasma total cholesterol and LDL cholesterol levels by 1.4-fold and 1.8-fold, respectively. HM treatment significantly reduced total cholesterol and LDL levels by 12.6% (P < 0.01)

and 29.7% (P < 0.05), respectively (Figure 5c, upper). Similarly, elevation of fasting plasma glucose content, which reflects basal insulin sensitivity, was suppressed by HM (Figure 5c, lower left). Consistent with these results, HFD feeding also increased fat weight (2.03 ± 0.42 g), which was significantly reversed by HM treatment (1.41 ± 0.34 g, P < 0.01) (Figure 5c, lower right). These results indicated that oral administration of HM inhibited body weight gain, fatty liver accumulation, as well as plasma markers of metabolic syndrome.

Effects of honokiol plus magnolol on HFD-induced liver injury

To evaluate the effect of HM on dietary stress-induced liver injury, we measured the levels of ALT and AST. Plasma ALT and AST levels were significantly higher in mice fed a HFD as compared to other groups (P < 0.01 and P < 0.05 versus ND, respectively) (Figure 6a). HM administration along

Figure 4 AMPK activation by honokiol plus magnolol, and its role in mitochondrial oxidation and lipogenesis. (a) The activation of AMPK by HM. Phosphorylation of AMPK and ACC were immunoblotted on the lysates of HepG2 cells treated with HM. (b) LKB1 phosphorylation. (c) The effect of AMPK inhibitor, Compound C, on CPT-1 mRNA level. Real-time RT-PCR assays were performed on HepG2 cells treated with 5 μmol/L Compound C for 30 min and continuously incubated with HM (10 + 10 μmol/L) for 12 h. (d) The effect of AMPK inhibition on HM suppression of SREBP-1c. After Compound C treatment (30 min), HepG2 cells were treated with vehicle or HM for 1 h followed by subsequent exposure to 1 μmol/L T090 for 12 h. Statistical significance of differences between each treatment group and the control (* $P < 0.05$; ** $P < 0.01$) or T090 alone (# $P < 0.05$; ## $P < 0.01$) was determined

with a HFD significantly attenuated this HFD-induced elevation of plasma ALT and AST concentrations (Figure 6a). Histopathological examination of liver tissues was also performed by H&E staining. Generally, hyperlipidemia accompanies hepatic damage indicators such as hypertrophic changes in hepatocytes due to lipid droplet deposition.^{24,25} In the present study, hepatic steatosis was also induced as evidenced by deposition of lipid droplets, related severe hypertrophic changes, increased hepatocyte size and histomorphometry; significant ($P < 0.01$) increases in the size of steatosis regions and mean hepatocyte diameters were detected in HFD mice as compared with ND mice. However, these HFD-related steatosis signs were dramatically and significantly ($P < 0.01$) inhibited by HM treatment (Table 1 and Figure 6b). These findings can be considered as evidence that HM has favourable protective effects against HFD-induced steatosis. The percentages of regions displaying steatosis in the hepatic parenchyma of mice fed

ND+HM and HFD increased by 5.19% and 1229.31%, respectively, as compared with ND mice, whereas a 60.75% reduction was detected in mice fed HFD + HM as compared with HFD-fed mice. The mean hepatocyte diameters of mice fed ND + HM and HFD were altered by -0.519% and 119.30%, respectively, as compared with ND mice, whereas a 46.35% reduction was measured in HFD + HM-fed mice as compared with HFD-fed mice (Table 1 and Figure 6b). Moreover, no meaningful changes in hepatic histopathology were found in ND + HM-treated mice as compared with the control, which suggests that HM may not have any direct harmful effect on intact hepatic tissues (Table 1 and Figure 6b).

Discussion

Hepatic steatosis is defined as ectopic accumulation of TGs in hepatocytes, and it develops when free fatty acid uptake

Figure 5 Antisteatotic effect of honokiol plus magnolol in HFD-fed mice. (a) The effect of HM on body weight gain in mice. Male C57BL/6 mice were fed on either a normal diet (ND) or high-fat diet (HFD) for eight weeks. H, M, or HM (10 mg/kg/day) was orally administered to mice four times per week during last four weeks of the diet feeding. (b) Oil Red O staining. The representative pictures show Oil Red O staining of the liver sections (100×) (left). The lipid level of liver was estimated by measuring the area of Oil Red O staining in liver tissue by using the IMAGE J analysis program developed by the National Institutes of Health. Three sections per each sample were randomly measured. (c) Plasma marker of hyperlipidemia and change in the fat weight. At the end of HFD feeding, mice were fasted overnight to measure total cholesterol, low-density lipoprotein (LDL), and fasting glucose levels. Statistical significance of differences between each treatment group and ND (** $P < 0.01$) or HFD alone (* $P < 0.05$; ** $P < 0.01$) was determined. (A color version of this figure is available in the online journal.)

and synthesis in the liver exceeds fatty acid oxidation and excretion into the bloodstream.^{6,26} This may occur as a result of increased hepatic lipogenesis and impaired hepatic fat elimination. To confirm the effects of combined honokiol

and magnolol treatment on lipogenesis in the liver, this study used two approaches: a cell model of hepatocytes treated with LXR α ligands and a mouse model fed a HFD. The results verified that co-treatment of honokiol and

Figure 6 Inhibition of HFD-induced liver injury. (a) Plasma ALT and AST activity. (b) H&E staining in the liver sections. Statistical significance of differences between each treatment group and ND (* $P < 0.05$; ** $P < 0.01$) or HFD alone ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$) was determined. Arrows indicate the lipid droplet. Scale bars = 200 μm . (A color version of this figure is available in the online journal.)

Table 1 Histomorphometrical analysis in HFD-supplied mice livers ^a		
Groups	Percentages of steatosis regions (%/mm ² of hepatic parenchyma)	Mean hepatocyte diameters (μm /hepatocytes)
ND	4.07 $\pm$ 2.92	30.48 $\pm$ 3.97
ND+HM	4.28 $\pm$ 2.55	30.30 $\pm$ 4.47
HFD	54.76 $\pm$ 9.50 ^b	66.84 $\pm$ 10.25 ^b
HFD + HM	21.50 $\pm$ 3.64 ^{b,c}	35.86 $\pm$ 5.13 ^c

ND: normal diet; HFD: high-fat diet; HM: test substance.
^aValues are expressed as mean $\pm$ SD of eight hepatic histological fields.
^b $P < 0.01$ as compared with ND by MW test.
^c $P < 0.01$ as compared with HFD by MW test.

magnolol has a synergistic ability to inhibit SREBP-1c induction as mediated by LXR α , which may result in the treatment of fatty liver disease. Honokiol and magnolol are structural isomers as well as biphenolic components of *M. officinalis*. These compounds have been reported to show various activities such as anti-cancer,^{27,28} anti-epileptic,²⁹ anti-inflammatory,^{30,31} anti-oxidative and hepatoprotective potential,³² and anti-microbial³³ effects. Especially, honokiol has been shown to protect against carbon tetrachloride-induced liver damage in rats³⁴ and prevent renal fibrosis by inhibiting extracellular matrix and pro-inflammatory factors.³⁵ Magnolol also

has antioxidant and hepatoprotective effects on acetaminophen-induced liver damage in rats.³² Nevertheless, some reports have showed that a combination of these compounds has beneficial effects, including chemoprevention, glucose metabolism, and anti-depression. In this study, we also found that co-treatment of honokiol and magnolol at 10 $\mu\text{mol/L}$ has synergistic effects in terms of inhibition of T090-induced SREBP-1c induction in hepatocytes. In another set of experiments using GW3965, a specific LXR α ligand, these combinations had the same inhibitory effects on induction of SREBP-1c protein expression. SREBP-1c is the most important transcription factor in *de novo* lipogenesis in the liver and plays a considerable role in the pathogenesis of NAFLD.^{36,37} SREBP-1c is the most important transcription factor in *de novo* lipogenesis in the liver and plays a considerable role in the pathogenesis of NAFLD.^{21,38} Therefore, the LXR-SREBP-1c pathway may be an attractive target for the treatment of fat accumulation in liver tissue. Our results were in accordance with these previous studies, as LXR directly activated SREBP-1 gene transcription through a LXRE, and subsequent activation of lipogenic genes such as FAS, ACC, and SCD-1 occurred secondary to SREBP-1 gene activation. Moreover, treatment with 10 $\mu\text{mol/L}$ HM significantly inhibited activation of SREBP-1c and induction of lipogenic gene LXR ligands. The real-time PCR and reporter gene results further support that HM suppresses LXR α -mediated SREBP-1c induction. In mice, HFD-induced fat accumulation in liver tissue was also antagonized by administration of HM.

AMPK is a key regulator of lipid metabolism, and the AMPK pathway may involve cholesterol and fatty acid synthesis in the liver.^{39,40} Moreover, activation of AMPK accelerates degradation of SREBP-1c, which supports its inhibitory role against the LXR α -SREBP-1c axis.⁴¹ In this study, treatment with HM activated AMPK in hepatocytes, and induced phosphorylation of LKB1, an upstream kinase of AMPK. Moreover, a pivotal role for AMPK in HM-mediated inhibition of SREBP-1c was determined based on our finding that the AMPK inhibitor (i.e. Compound C) reversed the effect of HM on induction of SREBP-1c by LXR α . A recent study showed that chemical activators of AMPK were able to reduce the activity of LXR α by inhibition of LXR phosphorylation and thus lipogenesis.³⁹ Our results showed that the ability of HM to inhibit activation of SREBP-1c and lipogenesis might be related to its activation of AMPK. This hypothesis is further supported by our result showing that Compound C reversed CPT-1 mRNA induction by HM.

Fatty liver is associated with not only increased synthesis of fat in the liver but also impaired utilization of fatty acids.⁴² It has been shown that mitochondria are the main organelles responsible for balancing overall energy metabolism in cells by generation of ATP.^{43,44} CPT-1 is localized in the membrane of mitochondria, and it regulates mitochondrial β -oxidation.⁴⁵ Our results confirmed that HM induces mRNA expression of CPT-1, which may be dependent on AMPK activation, as assessed by an inhibitor study.

HFD feeding can induce obesity, insulin resistance and fatty liver in animals, similar to human metabolic syndrome.⁴⁶ In this study, we confirmed the successful induction of fat accumulation in the livers of mice fed with a HFD for eight weeks. This was markedly attenuated by HM treatment for four weeks, as indicated by Oil Red O and H&E staining. Moreover, administration of HM significantly inhibited body weight gain induced by HFD. In fact, the increase in body weight results from an imbalance in homeostasis between food intake and energy expenditure.⁴⁷ Administration of HM had no effect on total food intake (data not shown), which means regulator genes involving energy expenditure could have been affected.

Insulin resistance is one of the most important initial symptoms in the progression of metabolic syndrome.⁴⁸ As expected, mice fed with a HFD showed markedly elevated insulin resistance, as assessed by increased plasma fasting glucose levels, which were inhibited by HM treatment. HM also attenuated hyperlipidemia as indicated by reduction of plasma total cholesterol and LDL levels. In addition, other report has showed that honokiol and magnolol could regulate glucose uptake by activating PI3K-dependent Akt *in vitro*.⁴⁹ Therefore, it is likely that HM can treat HFD-induced steatosis partly through inhibition of obesity and insulin resistance.

In conclusion, combinational HM treatment had potentials in both the inhibition of SREBP-1c-related lipogenic genes and the induction of fatty acid oxidation gene. Furthermore, HM has shown the ability to inhibit the acute phase of inflammation in macrophages,⁵⁰ which adds value to its therapeutic use for fatty liver and steatohepatitis. These results provide useful information on

active compounds in traditional medicinal herbs by elucidating their pharmacology and mechanisms of action in the treatment of hepatic steatosis and steatohepatitis.

Author contributions: All authors participated in the review of the manuscript. JHL, YWK, RJZ, and SCK conducted the experiment, analyzed data, and wrote the manuscript. JYJ, EJJ, KHJ, SYM, SKK, SHK, IJC, SJP, and JRL acquired data.

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