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Monascin and ankaflavin act as natural AMPK activators with PPARα agonist activity to down-regulate nonalcoholic steatohepatitis in high-fat diet-fed C57BL/6 mice

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ABSTRACT

Yellow pigments monascin (MS) and ankaflavin (AK) are secondary metabolites derived from *Monascus*-fermented products. The hypolipidemic and anti-inflammatory effects of MS and AK indicate that they have potential on preventing or curing nonalcoholic fatty liver disease (NAFLD). Oleic acid (OA) and high-fat diet were used to induce steatosis in FL83B hepatocytes and NAFLD in mice, respectively. We found that both MS and AK prevented fatty acid accumulation in hepatocytes by inhibiting fatty acid uptake, lipogenesis, and promoting fatty acid beta-oxidation mediated by activating peroxisome proliferator-activated receptor (PPAR)-α and AMP-activated kinase (AMPK). Furthermore, MS and AK significantly attenuated high-fat diet-induced elevation of total cholesterol (TC), triacylglycerol (TG), free fatty acid (FFA), and low density lipoprotein-cholesterol (LDL-C) in plasma. MS and AK promoted AMPK phosphorylation, suppressed the steatosis-related mRNA expression and inflammatory cytokines secretion, as well as upregulated farnesoid X receptor (FXR), peroxisome proliferator-activated receptor gamma co-activator (PGC)-1α, and PPARα expression to induce fatty acid oxidation in the liver of mice. We provided evidence that MS and AK act as PPARα agonists to upregulate AMPK activity and attenuate NAFLD. MS and AK may be supplied in food supplements or developed as functional foods to reduce the risk of diabetes and obesity.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as fatty infiltration of the liver that causes a 5–10% increase in liver weight (Salt, 2004). NAFLD represents a spectrum of conditions characterized microvesicular and macrovesicular hepatic steatosis, which occurs in people who do not consume alcohol in amounts generally considered harmful for the liver (Sanyal, 2002). The spectrum of NAFLD disorders ranges from simple fatty liver (steatosis without liver injury), to nonalcoholic steatohepatitis (NASH) and fibrosis/cirrhosis (Matteoni et al., 1999; Adiels et al., 2006).

AMP-activated kinase (AMPK) has been investigated as a potential therapeutic target for the treatment of type 2 diabetes, mostly because of AMPK-mediated inhibition of gluconeogenesis in the liver during feeding (Shaw et al., 2005). AMPK activity is regulated by nutrients, hormones, calcium, and cellular stress levels (Leclerc and Rutter, 2004). Activation of AMPK is modulated by changes in ATP, ADP, and AMP concentrations as well as phosphorylation at Thr172 by an upstream AMPK kinase (Hawley et al., 1996). AMPK acts primarily by directly affecting the activity of enzymes involved in carbohydrate, lipid, and protein biosyntheses and secondarily by long-term transcriptional control of key components

Abbreviations: ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; ACS, acyl-CoA synthetase; AK, ankaflavin; ALT, alanine aminotranferase; AMPK, AMP-activated kinase; AST, aspartate transaminase; BSA, bovine serum albumin; CPT-1, carnitine palmitoyl transferase I; CV, cardiovascular; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FABP, fatty acid-binding protein; FAS, fatty acid synthase; FAT, fatty acid transporter; FBS, fetal bovine serum; FFA, free fatty acid; FXR, farnesoid X receptor; GLUT, glucose transporter; H&E, hematoxylin and eosin; HAECs, human aortic endothelial cells; HDL-C, high-density lipoprotein cholesterol; HPLC, high-performance liquid chromatography; HUVECs, human umbilical vein endothelial cells; IACUC, Institutional Animal Care and Use Committee; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; LDL-C, low density lipoprotein-cholesterol; MS, monascin; MTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NCBI, National Center for Biotechnology Information; NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor erythroid 2-related factor 2; OA, oleic acid; PCR, polymerase chain reaction; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1-alpha; PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma; PPREs, peroxisome-proliferator-response elements; PTP1B, protein tyrosine phosphatase 1B; SDS, sodium dodecyl sulfate; SPPARMs, selective PPAR modulators; SREBP-1c, sterol regulatory element-binding protein; TC, total cholesterol; TG, triacylglycerol; TGFβ, transformation growth factor beta; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-alpha; TR-FRET, time-resolved fluorescence resonance energy transfer; Tyr, tyrosine; TZDs, thiazolidinediones; VCAM-1, vascular cell adhesion molecule-1.

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of metabolic pathways. AMPK is a major regulator of glucose and lipid metabolism and represents an attractive target for the treatment of hepatic disorders (Viollet et al., 2009). Activation of hepatic AMPK leads to increased fatty acid oxidation with simultaneous inhibition of hepatic lipogenesis, cholesterol synthesis, and glucose production. In addition to short-term effects on specific enzymes, AMPK also modulates the transcription of the genes involved in lipogenesis and mitochondrial biogenesis. The identification of AMPK targets in hepatic metabolism should be useful for developing treatments to reverse metabolic abnormalities related to type 2 diabetes and metabolic syndrome.

There are three peroxisome proliferator-activated receptor (PPAR) subtypes: PPARα, PPARγ, and PPARδ. All three subtypes can modulate transcription by binding to specific peroxisome-proliferator-response elements (PPREs) on target genes. PPARα is highly expressed in the liver, kidney, and skeletal muscle. Fibrates, which are weak PPARα agonists, alter hepatic energy metabolism that, in part, lowers plasma triaceylglycerol (TG) concentrations and slightly increases high-density lipoprotein cholesterol (HDL-C) (Staels and Fruchart, 2005; Tenenbaum et al., 2005). PPARα agonists such as fenofibrate and PPARγ agonists such as pioglitazone activate AMPK to stimulate the pathways that increase energy production, such as glucose transport and fatty acid oxidation, switching off the pathways that consume energy, such as lipogenesis (Chen et al., 2012; Lage et al., 2008).

Monascus-fermented rice is known for its ability to dramatically improve dyslipidemia (Lee et al., 2010). In addition to blood lipid-lowering effects, *Monascus*-fermented rice also has the potential to regulate blood glucose levels (Shi and Pan, 2010), prevent atherosclerosis (Lee et al., 2010), reduce inflammation, act as an anti-oxidant (Hsu et al., 2010a), and attenuate alcoholic fatty liver disease (Cheng and Pan, 2011). Recent studies have shown that monascin (MS) and ankaflavin (AK) isolated from *Monascus*-fermented rice lowers total cholesterol (TC), TG, and low density lipoprotein-cholesterol (LDL-C) levels in blood (Lee et al., 2010) while reducing inflammation (Cheng and Pan, 2011) and ameliorating the capacity for lipolysis in mature 3T3-L1 cells (Hsu et al., 2012a; Jou et al., 2010). The high-fat diet has been reported to result in NAFLD (Kusunoki et al., 2005). Nevertheless, the mechanisms underlying MS and AK on NAFLD *in vitro* and *in vivo* remains unclear. In this study, we examined the oleic acid-induced steatosis effect in FL83B hepatocytes treated with MS and AK. In *in vivo* studies, mice were fed a high-fat diet to induce NAFLD and were then treated with MS and AK to examine their potential therapeutic effects. We demonstrated that both MS and AK increased AMPK activity and beta-oxidation in hepatocytes. We provided evidence that MS and AK act as PPARα agonists to upregulate AMPK activity and attenuate NAFLD.

2. Materials and methods

2.1. Chemicals and reagents

F-12K medium was purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD, USA). Trypsin–EDTA and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), KCl, fenofibrate, NaCl, crystal violet, oil-red O, and oleic acid were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), formaldehyde, and KH_2PO_4 were purchased from Wako Pure Chem. (Saitama, Japan). Chloroform, methanol, and isopropanol purchased from Merck. Co., Inc. (Rahway, NJ, USA). High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Foster City, CA, USA). MK886 (PPARα antagonist) and WY14643 (PPARα agonist) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cytokines assay kits were purchased from Peprotech (Rocky hill, NJ, USA). For MS and AK preparation, the crude extracts of *Monascus*-fermented rice were obtained after filtering and concentrating under reduced pressure, and then coated on silica gel and subjected to dry flash chromatography. Sufficient *n*-hexane was passed through the column to remove the oily hydrophobic materials. Extensive gradient elution was then employed using different ethyl acetate in *n*-hexane ratios to yield numerous fractions. Similar fractions were combined according to thin layer chromatography (TLC), and the solvent was removed under reduced pressure. These fractions were further analyzed by high performance liquid chromatography (HPLC), and then fractions with a similar single peak profile were combined, respectively. Finally, the fraction with the desired compound was concentrated to dryness. Preparation of MS and AK (>95% purity) was confirmed by nuclear magnetic resonance (NMR, Varian Gemini, 200 MHz, FT-NMR, Varian Inc., Palo Alto, CA, USA) and electrospray ionization-mass spectrometry (ESI-MS, Thermo Electron Co., Waltham, MA, USA) analysis. Monascin ($C_{21}H_{26}O_5$) (Supplemental Fig. 1A): ESIMS *m/z* 359 $[M+H]^+$. ^1H NMR ($CDCl_3$, 200 MHz) δ: 0.88 (3H, t, *J* = 6.6, H-19), 1.30 (4H, m, H-17, H-18), 1.43 (3H, s, H-12), 1.61 (2H, m, H-16), 1.84 (3H, d, *J* = 6.0, H-11), 2.49 (1H, m, H-15a), 2.64 (1H, m, H-5a), 2.70 (1H, m, H-15b), 2.99 (1H, m, H-5b), 3.14 (1H, m, H-6), 3.64 (1H, d, *J* = 9.0, H-13), 4.67 (1H, d, *J* = 12.6, H-1a), 5.02 (1H, d, *J* = 12.6, H-1b), 5.26 (1H, s, H-4), 5.85 (1H, d, *J* = 15.4, H-9), 6.47 (1H, dt, *J* = 15.4, 6.0, H-10); ^{13}C NMR (CDCl3, 50 MHz) δ: 12.9 (C-19), 16.7 (C-12), 17.5 (C-11), 21.4 (C-18), 21.8 (C-16), 28.4 (C-17), 30.1 (C-5), 41.9 (C-6), 53.9 (C-13), 62.8 (C-1), 82.2 (C-7), 102.3 (C-4), 113.0 (C-8a), 123.4 (C-9), 134.4 (C-10), 149.8 (C-4a), 159.5 (C-3), 168.5 (C-13a), 188.8 (C-8), 201.5 (C-14). Ankaflavin ($C_{23}H_{30}O_5$) (Supplemental Fig. 1B): ESIMS *m/z* 409 $[M+Na]^+$. ^1H NMR (d_6-acetone, 400 MHz) δ: 0.87 (3H, t, *J* = 6.8, H-21), 1.29 (8H, m, H-17–20), 1.45 (3H, s, H-12), 1.60 (2H, m, H-16), 1.82 (3H, d, *J* = 7.2, H-11), 2.66 (2H, m, H-5), 2.70 (1H, m, H-15a), 2.92 (1H, m, H-15b), 3.15 (1H, m, H-6), 4.27 (1H, d, *J* = 13.2, H-13), 4.68 (1H, d, *J* = 12.4, H-1a), 4.90 (1H, d, *J* = 12.4, H-1b), 5.50 (1H, s, H-4), 6.01 (1H, d, *J* = 15.6, H-9), 6.41 (1H, dq, *J* = 15.6, 7.2, H-10); ^{13}C NMR (d_6-acetone, 100 MHz) δ: 14.2 (C-21), 17.7 (C-12), 18.3 (C-11), 23.2 (C-20), 23.6 (C-16), 29.2 (C-5), 29.9 (C-17, C-18), 32.3 (C-19), 43.4 (C-15), 44.4 (C-6), 55.3 (C-13), 64.2 (C-1), 84.0 (C-7), 104.6 (C-4), 115.1 (C-8a), 125.5 (C-9), 134.6 (C-10), 151.6 (C-4a), 160.2 (C-3), 171.3 (C-13a), 190.6 (C-8), 203.6 (C-14).

2.2. Cell culture

Mouse liver cell FL83B is a hepatocyte cell line isolated from a normal liver taken from a 15 to 17 day old fetal mouse. FL83B cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in F-12K medium containing 10% FBS. The medium was renewed every 2–3 days and subcultured every 4 days.

2.3. Oil red O stain

FL83B hepatocytes (1×10^5 cells) were seeded in 24-well plates and treated with MS (10 μM), AK (10 μM), or WY14643 (10 μM; PPARα agonist) with or without MK886 (10 μM; PPARα antagonist) solved in medium containing BSA (0.5 mM) and oleic acid (OA; 2 mM) as model-1 (Fig. 1A). In model-2, FL83B hepatocytes were treated in medium containing BSA (0.5 mM) and OA (2 mM) for 12 h to induce fatty acid accumulation. And then, MS (10 μM), AK (10 μM), or WY14643 (10 μM) with or without MK886 (10 μM) were added (Fig. 1B). The cells were incubated with 10% formaldehyde for 1 h. Subsequently, oil red O working solution was added to each well and stained for 15 min. The staining photos were taken under the microscope. Data were expressed by resolving the oil red O working solution with 100% isopropanol then as percentage of change in absorbance at 490 nm by using the ELISA reader (Jou et al., 2010).

2.4. Immunoblot analysis

FL83B cells were lysed and the cell lysates were centrifuged (10,000*g* for 10 min) to recover the supernatant. The supernatant was taken as the cell extract. The protein concentration in the cell extract was determined using a Bio-Rad protein assay kit. The samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE). The protein spots were electrotransferred to a polyvinyldiene difluoride (PVDF) membrane. The membrane was incubated with block buffer and then probed with primary antibody overnight at 4 °C. The membrane was washed, shaken in a solution of HRP-linked anti-rabbit IgG secondary antibody. The expressions of proteins were detected by enhanced chemiluminescent (ECL) reagent (Millipore, Billerica, MA, USA).

2.5. Real-time polymerase chain reaction (PCR)

Total RNA from liver tissue and hepatocytes was obtained using the Trizol reagent (Gibco BRL Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Primers were blasted according to National Center for Biotechnology Information (NCBI) primer database to estimate their specificities and then synthesized by MD-Bio, Inc. (Taipei, Taiwan). Primers were shown in Supplemental Table 1. The gene expression level was determined by relative quantitative real-time PCR (CFX Cycler System, Bio Rad Laboratories, Inc., Hercules, CA, USA).