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Phillyrin attenuates high glucose-induced lipid accumulation in human HepG2 hepatocytes through the activation of LKB1/AMP-activated protein kinase-dependent signalling

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ABSTRACT

Phillyrin, an active constituent found in many medicinal plants and certain functional foods, has anti-obesity activity *in vivo*. The aim of our study was to provide new data on the molecular mechanism(s) underlying the role of phillyrin in the prevention of high glucose-induced lipid accumulation in human HepG2 hepatocytes. We found that phillyrin suppressed high glucose-induced lipid accumulation in HepG2 cells. Phillyrin strongly inhibited high glucose-induced fatty acid synthase (FAS) expression by modulating sterol regulatory element-binding protein-1c (SREBP-1c) activation. Moreover, use of the pharmacological AMP-activated protein kinase (AMPK) inhibitor compound C revealed that AMPK is essential for suppressing SREBP-1c expression in phillyrin-treated cells. Finally, we found that liver kinase B1 (LKB1) phosphorylation is required for the phillyrin-enhanced activation of AMPK in HepG2 hepatocytes. These results indicate that phillyrin prevents lipid accumulation in HepG2 cells by blocking the expression of SREBP-1c and FAS through LKB1/AMPK activation, suggesting that phillyrin is a novel AMPK activator with a role in the prevention and treatment of obesity.

1. Introduction

The liver is the major organ responsible for the conversion of excess dietary carbohydrates into triglycerides in mammals. Glucose can be metabolised in the liver to provide substrates such as acetyl CoA for fatty acid synthesis. Next, fatty acids are incorporated into triglycerides, which function as a long-term energy reservoir. The absorption of carbohydrates in the diet is concomitant with increases in the concentration of substrates such as glucose but also with changes in the concentration of insulin. Previously, it was thought that insulin was the main regulator of glycolytic and lipogenic gene expression. However, using cultured primary hepatocytes it has been shown that nutrients themselves play an important role in the regulation of gene expression, independently of insulin (Girard, Ferre, & Foufelle, 1997; Towle, Kaytor, & Shih, 1997), and it has been proposed that two signalling pathways elicited in response to dietary carbohydrates play a synergistic role in regulating lipogenic gene expression (Vaulont, Vasseur-Cognet, & Kahn, 2000). The induction of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) expression requires both glucose metabolism and insulin (Dentin et al., 2004). Control of the *de novo* lipogenic pathway occurs largely through effects on sterol regulatory element-binding proteins (SREBPs). SREBPs are transcription factors (SREBP-1a, -1c, and -2) that are important in cholesterol and fatty acid (Horton, Goldstein, & Brown, 2002; Horton et al., 2003; Shimomura, Shimano, Korn, Bashmakov, & Horton, 1998).

AMP-activated protein kinase (AMPK) is a metabolic master switch, mediating adaptation of the cell to variations in the nutritional environment (Hardie, 2003). Conditions that deplete cellular ATP and elevate AMP levels, including glucose deprivation and hypoxia, result in AMPK activation (Kemp et al., 2003). For many years, the upstream kinase(s) responsible for phosphorylation of the critical Thr172 phosphorylation site on the α-subunit were unknown, until recent studies revealed that both liver kinase B1 (LKB1) (Hawley et al., 2003; Woods et al., 2003) and calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ) (Hurley et al., 2005; Woods et al., 2005) can activate AMPK by

Abbreviations: ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKKβ, calcium/calmodulin-dependent protein kinase kinase-β; FAS, fatty acid synthase; HG, high glucose; LKB1, liver kinase B1; LXR, liver X receptors; SREBP, sterol regulatory element-binding protein.

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phosphorylation at Thr172 in mammalian cells. AMPK activation results in the phosphorylation and inhibition of ACC activity (Park et al., 2002) and the loss of inhibition of carnitine palmitoyltransferase I, leading to an increase in fatty acid oxidation. A recent study demonstrated that activated AMPK interacts with and phosphorylates SREBP-1c and -2 directly. The phosphorylation of SREBP-1c at Ser372 by AMPK is necessary for inhibiting proteolytic processing, nuclear translocation, and the transcriptional activity of SREBP-1c. Inhibition represses SREBP-1c target gene expression in hepatocytes exposed to high glucose, leading to reduced lipogenesis and lipid accumulation (Li et al., 2011).

Forsythia suspensa (Thunb.) Vahl is widely distributed in China, Korea and Japan; the species belongs to the family Oleaceae. *Forsythia fructus* prepared from the dried mature fruit of *F. suspensa*, *F. koreana*, or *F. viridissima*, has strong anti-allergy and anti-inflammatory effects (Choi et al., 2007; Kim et al., 2003). The butanol fraction of an aqueous extract of *F. koreana* inhibited nitric oxide production in murine macrophage-like RAW 264.7 cells (Kim et al., 2000). Phillyrin, one of the major active constituents of *F. suspensa* and *F. koreana*, plays an important role in the anti-inflammatory (Diaz Lanza et al., 2001; Lee et al., 2011) and anti-oxidant (Gulcin, Elias, Gepdiremen, & Boyer, 2006) effects. An *in vivo* study showed that phillyrin exerted anti-obesity effects in nutritive obesity mice (Zhao, Li, Yang, An, & Zhou, 2005). In fact, although phillyrin is commonly used as an important ingredient in the food, beverage, and cosmetic industries (Sheng, Li, & Li, 2012), the mechanism by which phillyrin attenuates lipogenesis remains unknown.

In this study, we tested the hypothesis that phillyrin's anti-obesity effects are mediated by attenuating lipid accumulation in human HepG2 hepatocytes. We found that phillyrin inhibited lipid accumulation inside HepG2 cells significantly by suppressing *FAS* gene expression through the modulation of SREBP-1c activation. Furthermore, we found that phillyrin strongly increased the phosphorylation of AMPK at Thr172, leading to the inhibition of SREBP-1c activation in human HepG2 hepatocytes. Finally, the use of LKB1-deficient HeLa cells and LKB1 siRNA revealed that the phillyrin-induced activation of AMPK is regulated by the upstream kinase LKB1.

2. Materials and methods

2.1. Materials

Compound C and STO-609 were purchased from Calbiochem (La Jolla, CA). Nile red was purchased from Sigma Chemical Co. (St. Louis, MO). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corp. (Cleveland, OH), and a lactate dehydrogenase (LDH) release detection kit was obtained from Roche Applied Science (Indianapolis, IN). A protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). An enhanced chemiluminescence (ECL) system and polyvinylidene difluoride (PVDF) membrane were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The plasmid pCMV-β-gal was purchased from Clontech (Palo Alto, CA), and Lipofectamine™ 2000 was obtained from Invitrogen Co. (Carlsbad, CA). Oligonucleotide polymerase chain reaction (PCR) primers were custom-synthesized by Bioneer Co. (Daejeon, South Korea). Antibodies against p-AMPKα (Thr172), p-ACC (Ser79), p-LKB1 (Ser428), LKB1, and secondary antibodies (HRP-linked anti-rabbit and anti-mouse IgG) were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies against AMPKα, FAS, SREBP-1, Lamin B1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and reagents were of analytical grade.

2.2. The purification procedure and the identification of phillyrin

The dried fruits of *F. suspensa* (1 kg) were extracted with MeOH (2 × 2 L) at room temperature. The filtrate was dried under reduced pressure to afford crude MeOH extract (74 g), which was suspended in water and partitioned with EtOAc, and *n*-BuOH, sequentially. The EtOAc-soluble fraction (18 g) was divided into 26 fractions by silica gel column chromatography (70–230 mesh, 900 g) eluting with a gradient of *n*-hexane/acetone (from 100:0 to 0:100). Phillyrin (24 mg) was purified from fraction F24 (1.05 g) by preparative high-performance liquid chromatography (HPLC) (Shim-Pack Prep-ODS column, 20 × 250 mm; Shimadzu, Kyoto, Japan) using a gradient of MeOH–H_2O (from 20:80 to 50:50, over 60 min). The structural identification was carried out by spectroscopic analyses as follows:

Phillyrin: white powder; m.p. 155–157 °C, $[\alpha]_D^{25}$ + 44.8° (*c* = 2.0, MeOH). UV $(EtOH)_{max}$ (log ε): 229, 278 nm. FAB-MS *m/z*: 535 $[M+H]^+$. ^1H NMR (250 MHz, pyridine-d_5): δ 7.59 (1H, d, *J* = 8.3 Hz, H-5), 7.23 (2H, s, H-2, 2′), 7.00 (2H, t, *J* = 8.3 Hz, H-6, 6′), 6.94 (1H, d, *J* = 8.0 Hz, H-5′), 5.72 (1H, s, Glc-1), 4.91 (1H, d, *J* = 5.8 Hz, H-7′), 4.63(1H, d, *J* = 6.7 Hz, H-7), 4.49 (1H, dd, *J* = 12.5, 2.5 Hz), 4.34 (1H, m, Glc-6), 4.32 (1H, m, Glc-3), 4.30 (1H, m, Glc-2), 4.29 (1H, m, Glc-4), 4.08 (1H, m, Glc-5), 4.07 (1H, d, *J* = 9.7 Hz, H-9a), 3.96 (1H, t, *J* = 8.5 Hz, H-9b), 3.81 (1H, m, H-9b), 3.72 (9H, s, C-3, 3′, 4′-OCH_3), 3.54 (1H, t, *J* = 8.5 Hz, H-9′), 3.37 (1H, t, *J* = 8.5 Hz, H-8′). ^{13}C NMR (63 MHz, pyridine-d_5): δ 150.1 (C-3), 149.3 (C-3′), 149.1 (C-4′), 147.6 (C-4), 136.0 (C-1), 132.0 (C-1′), 119.0 (C-6), 118.3 (C-6′), 116.0 (C-5), 112.1 (C-5′), 110.9 (C-2), 110.2 (C-2′), 102.0 (Glc-1), 87.8 (C-7), 82.2 (C-7′), 78.8 (Glc-5), 78.5 (Glc-3), 74.8 (Glc-2), 71.1 (C-9, Glc-4), 70.0 (C-9), 62.2 (Glc-6), 55.7 (C-3, 3′, 4′-OCH_3), 55.1 (C-8), 50.4 (C-8′).

A purity of 98% was determined by HPLC analysis [Waters ODS-3 column (250 × 4.6 mm, 5 μm; Waters Corp., Milford, MA), mobile phase: linear gradient of MeOH/H_2O (30:70 → 43:57, over 39 min; 43:57 → 70:30, over 55 min, flow rate: 1 mL/min, UV detection at 280 nm].

2.3. Cell culture and treatment

Human HepG2 hepatocytes and human cervical carcinoma HeLa cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing normal glucose (5.5 mM D-glucose), supplemented with 10% heat-inactivated foetal bovine serum (FBS; Invitrogen). Cells were cultured in a humidified 5% CO_2 incubator at 37 °C in complete medium supplemented with 10% FBS to 80% confluency for use in the assays. Phillyrin, compound C, and STO-609 were dissolved in dimethyl sulfoxide (DMSO), and working concentrations of phillyrin (1–100 μM), compound C (10 μM), and STO-609 (10 μM) were added directly to serum-free culture medium. The final concentration of DMSO did not exceed 0.1% (v/v) and did not affect cell viability. A cell model of the high glucose-induced accumulation of hepatic lipids was prepared by exposing HepG2 cells to a high concentration of glucose (30 mM) for 24 h. Briefly, HepG2 cells were quiesced in serum-free DMEM overnight and incubated in DMEM containing a normal (5.5 mM) or high (30 mM) concentration of D-glucose. The designation "normal glucose" refers to medium containing 5.5 mM D-glucose, while "high glucose" refers to medium supplemented with 30 mM D-glucose.

2.4. Measurement of cell viability and cytotoxicity

Cells were cultured at 37 °C in medium containing 10% FBS at a density of 4×10^4 cells/500 μL in 48-well plates. After 24 h, the growth medium was replaced with serum-free medium and the