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Activation of AMPK by berberine induces hepatic lipid accumulation by upregulation of fatty acid translocase CD36 in mice

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ABSTRACT

Emerging evidence has shown that berberine has a protective effect against metabolic syndrome such as obesity and type II diabetes mellitus by activating AMP-activated protein kinase (AMPK). AMPK induces CD36 trafficking to the sarcolemma for fatty acid uptake and oxidation in contracting muscle. However, little is known about the effects of AMPK on CD36 regulation in the liver. We investigated whether AMPK activation by berberine affects CD36 expression and fatty acid uptake in hepatocytes and whether it is linked to hepatic lipid accumulation. Activation of AMPK by berberine or transduction with adenoviral vectors encoding constitutively active AMPK in HepG2 and mouse primary hepatocytes increased the expression and membrane translocation of CD36, resulting in enhanced fatty acid uptake and lipid accumulation as determined by BODIPY-C16 and Nile red fluorescence, respectively. Activation of AMPK by berberine induced the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and subsequently induced CCAAT/enhancer-binding protein β (C/EBPβ) binding to the C/EBP-response element in the CD36 promoter in hepatocytes. In addition, hepatic CD36 expression and triglyceride levels were increased in normal diet-fed mice treated with berberine, but completely prevented when hepatic CD36 was silenced with adenovirus containing CD36-specific shRNA. Taken together, prolonged activation of AMPK by berberine increased CD36 expression in hepatocytes, resulting in fatty acid uptake via processes linked to hepatocellular lipid accumulation and fatty liver.

1. Introduction

Berberine is an isoquinoline alkaloid found in many plants, including *Coptis chinensis*, and it is a well-known to have multiple pharmacological activities including anti-microbial and anti-diarrhea activity (Amin et al., 1969; Taylor and Greenough, 1989). Many studies have focused on its effects on the metabolic syndrome, such as obesity and type II diabetes mellitus (Leng et al., 2004; Lee et al., 2006). Berberine activates AMPK indirectly by inhibiting mitochondrial respiratory complex I (Turner et al., 2008). In the liver of ob/ob and db/db mice, AMPK activation by berberine resulted in decreased de novo lipogenesis and increased fatty acid oxidation (Kim et al., 2009). Berberine also can inhibit the proteolytic cleavage and nuclear translocation of SREBP in hepatocyte indicating that berberine have triglyceride and cholesterol lowering effect by AMPK activation (Li et al., 2010; Xia et al., 2011). Clinical trials in patients with type 2 diabetes revealed that berberine reduced plasma glucose and hemoglobin A1c and improved the lipid profile with effects comparable to those receiving metformin (Yin et al., 2008; Zhang et al., 2008).

AMP-activated protein kinase (AMPK) was originally identified as a serine/threonine kinase that phosphorylates and inactivates key mammalian enzymes responsible for fatty acid and cholesterol synthesis, namely acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), respectively (Hardie et al., 1989). The role of AMPK in the regulation of physiological energy metabolism has been studied extensively since then and it is now recognized as the master regulator of energy metabolism in the cell. Upon activation, AMPK causes a cellular metabolic switch from lipogenesis to lipolysis and from hepatic glucose synthesis to muscle glucose utilization. Consequently, AMPK improves insulin sensitivity and glucose homeostasis, making it an attractive therapeutic target for metabolic syndromes, including type 2 diabetes (Hardie, 2013).

The primary role of AMPK in cardiac and skeletal muscle contraction is the production of ATP by the uptake of glucose and fatty acids from circulation, which is achieved by enhanced sarcolemmal translocation of GLUT4 and CD36 from the intracellular vesicles, respectively

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(Luiken et al., 2003; Russell et al., 1999). Although the exact mechanisms are unclear, a recent study showed that AMPK phosphorylates and inactivates TBC1D class protein, which is known to block AMPK-induced CD36 translocation (Fentz et al., 2015). However, the effects of this mechanism on CD36 status, fatty acid transport and lipid accumulation in the liver require further investigation. The liver is the primary organ for the storage and distribution of surplus nutrients and thus plays an important role in regulating whole body energy homeostasis. Therefore, the major role of AMPK in the liver is to maintain lipid and glucose homeostasis. Basal low levels of CD36 in normal hepatocytes are increased dramatically in animals fed a high-fat diet and in patients with non-alcoholic fatty liver disease (NAFLD) (Buqué et al., 2012; Memon et al., 1999; Zhou et al., 2008). Moreover, hepatic CD36 upregulation is significantly associated with insulin resistance, hyperinsulinemia and increased steatosis in patients with non-alcoholic steatohepatitis and hepatitis C virus infection (Miquilena-Colina et al., 2011). Importantly, overexpression of CD36 in the liver of normal fed, non-metabolically challenged mice increased hepatic fatty acid transport and induced fatty liver (Koonen et al., 2007).

In the present study, we investigated the effects of AMPK activation by berberine in normal mouse hepatocytes and livers in terms of CD36 expression, fatty acid uptake and lipid accumulation. We found that activation of AMPK by berberine or genetic manipulation in hepatocytes increased the expression and translocation of CD36, which resulted in increased fatty acid uptake and lipid accumulation. In addition, hepatic CD36 expression and triglyceride (TG) levels were increased in normal diet-fed mice administered berberine, which was completely prevented upon CD36 silencing with small-hairpin RNA (shRNA).

2. Materials and methods

2.1. Cell culture and chemicals

Human hepatoma cell line, HepG2 (ATCC, Manassas, VA, USA) were maintained in DMEM (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated FBS (GIBCO BRL) and 1% antibiotic-antimycotic (GIBCO BRL). Mouse hepatocytes were isolated from specific pathogen-free male C57BL/6 mice (20–25 g) by two-step perfusion with calcium and magnesium-free Hanks' salt solution followed by a medium containing collagenase with modifications (LeCluyse et al., 1996). Cells were incubated at 37 °C with the air containing 5% CO_2. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Stock solution of berberine chloride (0.1 M) and AICAR (5-aminoimidazole-4-carboxamide ribonucleotide; 0.1 M) was prepared in DMSO, which was further diluted to the desired concentrations using the cell culture medium.

2.2. Nile red assay and fatty acid uptake assay

Intracellular lipid accumulation was quantified using Nile red, a fluorescent dye that binds to neutral lipids (McMillian et al., 2001). HepG2 cells and primary mouse hepatocytes were seeded in 96 well plates. The cells were stabilized for 24 h and treated with berberine or AICAR for 24 h. On the following day, cells were fixed with 4% paraformaldehyde and stained with 1 μg/ml Nile red solution. The fluorescence was measured with a microplate fluorescence reader (Molecular Devices, Sunny-vale, CA; excitation 488 nm and emission 580 nm) or was observed with an epifluorescence.

Fatty acid uptake was measured using a fluorescent palmitate analog, BODIPY-C16. Stock solutions of BODIPY-C16 (Invitrogen Life Sciences, Carlsbad, CA) were prepared in DMSO. After berberine or AICAR treatment, cells were incubated for 3 min in PBS supplemented with BODIPY-C16 to a final concentration of 100 nM, rinsed with ice-cold PBS, and fixed in ice-cold 4% paraformaldehyde for 10 min. The fluorescence was measured with a microplate fluorescence reader (excitation 500 nm and emission 515 nm) or was observed with an epifluorescence.

2.3. Adenoviral infection and siRNA transfection

A constitutively active mutant of AMPK (Ad-CA-AMPK) and dominant-negative AMPK (DN-AMPK; T172A) plasmid were provided by Dr. Joohun Ha from Kyung Hee University. Ad-CA-AMPK were prepared and purified as described previously (Lee et al., 2003). siRNA for CD36 and C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were infected with adenovirus for 6 h and incubated for 48 h after medium change. Transient transfection with DN-AMPK plasmid, si-C/EBPβ and si-CD36 were performed by using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer's protocol.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described with some modifications (Nelson et al., 2006). Briefly, cell lysate was fixed with 1% formaldehyde in PBS and the cross-linking was stopped with 125 mM glycine at room temperature. Chromatin was sheared by sonication and immunoprecipitated with anti-C/EBPβ antibody or non-immune IgG antibody at 4 °C. Immune complexes were collected with protein A-Sepharose beads (Amersham Biosciences). Total DNA was purified by Chelex 100-based method.

The binding of C/EBPβ to the CD36 promoter was detected by PCR. The sequences for primers over −273 to +19 nt of the mouse FAT/CD36 promoter were: forward, 5′ CTGGCCT CTGACTTACTTGGATGGGA 3′, reverse, 5′ GTCCTACACTGCAGTCCTCACTACATA 3′.

2.5. Subcellular fractionation

Cells were washed with cold PBS and collected in hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 0.2 mM sodium orthovanadate, 1 mM PMSF) containing 1× protease inhibitor cocktail (Roche, Basel, Switzerland). The cell suspension was incubated on ice for 1 h and homogenized by 50 strokes through a Dounce homogenizer followed by incubation on ice for 30 min. Lysates were centrifuged at 1000*g* at 4 °C for 10 min. The pellet, containing the nuclei and cell debris, was discarded and the supernatant (cytosol fraction) was centrifuged at 1,000,000*g* for 40 min to obtain pellets containing membrane fraction. Pellets were resuspended in lysis buffer containing 1% Triton X-100 and were incubated for 15 min. The suspension was sonicated on ice with 3 pulses (9 s) with 5 s intervals followed by 15 min incubation. The sonicated suspension was centrifuged at 1,000,000*g* for 40 min. The supernatant was considered the membrane fraction.

2.6. Immunofluorescence microscopy

For CD36 immunofluorescence microscopy, cells were washed with PBS and fixed with 3.7% paraformaldehyde at 4 °C for 10 min, and washed again with PBS. The cells were then blocked with 1% BSA at room temperature and incubated with anti-CD36 antibody diluted 1:100 in PBS containing 0.5% BSA at 4 °C for 12 h, followed by sequential incubation with Alexa Fluor® 488 dye-labeled secondary antibody. The specimens were mounted using ProLong® Gold Antifade Reagent (GIBCO BRL) after washing and examined with an epifluorescence microscope.

2.7. Animal treatments

Specific-pathogen-free male C57BL/6 mice (6 weeks old) was purchased from Central Lab. Animal Inc. and allowed ad libitum access to standard chow and tap water. They were kept in temperature-controlled, filter-sterilized animal quarters under a 12 h light:12 h dark