Biochemical and Biophysical Research Communications 477 (2016) 440–447

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Alleviation of hepatic fat accumulation by betaine involves reduction of homocysteine via up-regulation of betaine-homocysteine methyltransferase (BHMT)

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ARTICLE INFO

Article history:
Received 12 June 2016
Accepted 15 June 2016
Available online 16 June 2016

Keywords:
Betaine
Homocysteine
BHMT
AMPK
Lipogenesis

ABSTRACT

We investigated the anti-lipogenic effect of betaine in rats fed methionine and choline-deficient diet (MCD). Intake of MCD for 3 wk resulted in a significant accumulation of hepatic lipids, which was prevented by betaine supplementation in drinking water (1%). Phosphorylation of AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), sterol regulatory element-binding protein-1c (SREBP-1c), and liver kinase B1 (LKB1) was inhibited by MCD intake, and these changes were all inhibited by betaine feeding. Meanwhile, betaine supplementation reversed the reduction of methionine and *S*-adenosylmethionine (SAM), and the elevation of homocysteine levels in the liver, which could be attributable to the induction of betaine-homocysteine methyltransferase (BHMT) and methionine adenosyltransferase (MAT). Different cell lines were used to clarify the role of homocysteine on activation of the AMPK pathway. Homocysteine treatment decreased pAMPK, pACC, pSREBP-1c and pLKB1 in HepG2 cells. Metformin-induced activation of AMPK was also inhibited by homocysteine. Treatment with hydroxylamine, a cystathionine β-synthase inhibitor, resulted in a reduction of pAMPK, pACC and pSREBP-1c, accompanied by an elevation of intracellular homocysteine. Betaine treatment prevented the homocysteine-induced reduction of pAMPK, pACC, pSREBP-1c and pLKB1 in H4IIE cells, but not in HepG2 cells. Also the elevation of cellular homocysteine and inhibition of protein expression of BHMT were prevented by betaine only in H4IIE cells which express BHMT. The results suggest that the beneficial effect of betaine against hepatic lipid accumulation may be attributed, at least in part, to the depletion of homocysteine via up-regulation of BHMT in hepatocytes.

1. Introduction

It is well known that accumulation of lipids in hepatocytes plays a crucial role in the development of metabolic diseases which are highly associated with obesity, hyperlipidemia, diabetes, and other related metabolic disorders [1,2]. Accordingly, physiological and pharmacological strategies aimed toward inhibition of lipogenesis and/or enhancement of lipolysis have become of great therapeutic interest. One enzyme that has emerged as a potential target for dissipation of liver fat stores is adenosine monophosphate-activated protein kinase (AMPK) [3].

AMPK is a phylogenetically conserved serine/threonine protein kinase proposed to act as a 'metabolic master switch' that mediates the cellular adaptation to environmental and/or nutritional stress factors [4]. Activation of AMPK leads to phosphorylation of multiple targets in hepatocytes acutely switching off anabolic pathways while switching on alternative catabolic pathways. Among its identified roles, AMPK is implicated in the metabolism of glucose and lipids in association with many additional effects both on expression of related genes and on regulation of specific enzymes. Li et al. [5] reported that AMPK specifically binds to and directly phosphorylates sterol regulatory element-binding protein (SREBP)-1c and SREBP-2. In keeping with this model, AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [6], glycogen synthase [7] and creatine kinase [8], the enzymes that control the synthesis of fatty acids, cholesterol, glycogen and phosphocreatine, respectively. The product of ACC, malonyl-CoA, is both a precursor for the

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http://dx.doi.org/10.1016/j.bbrc.2016.06.080
0006-291X/

Abbreviations

ACC	acetyl-CoA carboxylase
AMPK	adenosine monophosphate-activated protein kinase
BHMT	betaine-homocysteine methyltransferase
CβS	cystathionine β-synthase
CPT1	carnitine palmitoyltransferase 1
ER	endoplasmic reticulum
HA	hydroxylamine
IRE1α	inositol-requiring enzyme 1α
LKB1	liver kinase B1
PEPCK	phosphoenolpyruvate carboxykinase
PPARγ	peroxisome proliferator-activated receptor γ
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SREBP	sterol regulatory element binding protein
TG	triglyceride
UPR	unfolded protein response

biosynthesis of fatty acids and a potent inhibitor of β-oxidation because it inhibits carnitine palmitoyltransferase 1 (CPT1), and thereby prevents the transport of long-chain fatty acids into mitochondrial matrix [9]. Thus when ACC is inactivated by AMPK, cellular concentrations of malonyl-CoA decrease, inhibition of CPT1 is relieved, and more fatty acid is oxidized. In fact, AMPK activation has been shown to increase fatty acid oxidation in liver, heart and skeletal muscle, the primary tissues that oxidize fatty acids [10,11].

Betaine, an oxidative metabolite of choline, is a methyl donor utilized for remethylation of homocysteine to methionine in a metabolic reaction catalyzed by betaine-homocysteine methyltransferase (BHMT). Earlier studies conducted in our laboratory have demonstrated that betaine protects the liver against various toxic substances such as ethanol, lipopolysaccharide, α-naphthylisothiocyanate, and dimethylnitrosamine [12–15]. The liver injury induced by those toxicants is accompanied by an impairment in the sulfur amino acid metabolism, and accordingly, the beneficial effect of betaine is frequently attributed to an improvement of transsulfuration reactions in the liver. It has also been shown that betaine administration is effective in preventing non-alcoholic fatty liver injury both in human and animals [16–19]. In our recent study, betaine supplementation was shown to enhance phosphorylation of AMPK and ACC while reducing the active form of SREBP-1c and liver X receptor α (LXRα) in rats fed a high-fat diet [20]. Similarly, Song et al. [21] reported that betaine administration attenuated hepatic lipogenic capability by increasing phosphorylation of AMPK in mice fed a high-sucrose diet. However, the underlying mechanism involved in the regulation by betaine of the AMPK pathway has not been fully understood. In this study we investigated the effect of betaine on AMPK activation to elucidate the mechanism of anti-steatotic action provided by this substance.

2. Methods

2.1. Animals and treatments

Male Sprague-Dawley rats were purchased from Samtaco Experimental Animals (Osan, Korea). Animals were acclimated to temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled rooms with a 12 h-light/dark cycle for 1 wk before use. Sixteen rats, 8 wk old and weighing 245–270 g, were randomly divided into 4 groups and fed one of the 4 different diets: NC, normal chow diet; CB, NC supplemented with betaine; MCD, methionine and choline-deficient diet; MCDB, MCD supplemented with betaine. NC was purchased from Purina Korea (Seoul, Korea); MCD from Dyets (Bethlehem, PA). Betaine (Sigma-Aldrich, St. Louis, MO) was dissolved at 1% (w/v) in drinking water. Rats consumed the diets *ad libitum* for 3 wk. At the end of the feeding period, rats were euthanized by exsanguination under light ether anesthesia in accordance with the guidelines established by the Animal Care Committee in College of Pharmacy, Seoul National University. The experimental protocol was approved by the Ethical Animal Care and Use Committee of Seoul National University (Approval No. SNU-140326-8).

2.2. Cell culture and chemicals

Human hepatoma HepG2 and rat hepatoma H4IIE cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) or 10% FBS plus 10% new born calf serum, 100 unit/ml of penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a 5% CO_2 incubator. Cells were incubated in serum free DMEM for 12 h before a chemical treatment. Homocysteine, metformin, and hydroxylamine (HA) were purchased from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS).

2.3. Determination of fatty liver

Hepatic triglyceride (TG) concentration was measured using a commercial kit (Sigma-Aldrich). For histopathological evaluation, the liver was excised immediately after sacrifice, sliced at 10 μm, immersed in propylene glycol for 5 min, and stained with Oil Red O for 7 min. After rinsing with 85% propylene glycol and distilled water, the samples were counterstained with hematoxylin for 2 min before microscopic examination.

2.4. Western blotting analysis

The cells were washed with PBS before harvesting in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich). The liver was homogenized in a 4-fold volume of ice-cold 1.15% KCl buffer. After centrifugation, the supernatant was collected from the lysates and protein concentrations were determined using Bradford reagent (Sigma-Aldrich). Equal amounts of protein from each sample were loaded, separated by gel electrophoresis, and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked in 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. The blots were incubated overnight with primary antibodies in 5% bovine serum albumin followed by incubation with secondary antibodies. Polyclonal antibodies against rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and cystathionine β-synthase (CβS) (Santa Cruz Biotechnology, Santa Cruz, CA), BHMT (Everest Biotechnology, Oxfordshire, U.K.), β-actin, pLKB1, pAMPK, pACC and pSREBP-1c (Cell Signaling Technology, Denver, CO) were used as probes. Methionine adenosyltransferase (MAT) was quantified as MATI/III, hepatic forms of MAT, by polyclonal antibody against rat MAT (Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence.

2.5. Biochemical assays

The liver was homogenized in methanol for detection of methionine, or in 1 M $HClO_4$ for detection of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH). Methionine was