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Betulin alleviated ethanol-induced alcoholic liver injury via SIRT1/AMPK signaling pathway

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ABSTRACT

The present study was conducted to investigate the protective effect of betulin, a triterpene from the bark of *Betula platyphylla* Suk, against ethanol-induced alcoholic liver injury and its possible underlying mechanisms. *In vitro*, human hepatic stellate cell line, LX-2 cells were treated with betulin (6.25, 12.5 and 25 μM) prior to ethanol (50 mM) for 24 h. Cell viability was analyzed by methyl thiazolyl tetrazolium assay, protein expressions were assessed by Western blot. *In vivo*, we induced alcoholic liver injury in male C57BL/6 mice, placing them on Lieber-DeCarli ethanol-containing diets for 10 days and then administering a single dose of ethanol (5 g/kg body weight) via gavage. Betulin (20 and 50 mg/kg) were given by gavage every day. *In vitro* results showed that betulin effectively decreased LX-2 cell viability, attenuated collagen-I, α-smooth muscle actin (α-SMA) levels, activated liver kinase B-1 (LKB1) and adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. Betulin suppressed the expression of sterol regulatory element-binding protein-1 (SREBP-1), and genetic deletion of AMPK blocked the effect of betulin on SREBP-1 in ethanol treated LX-2 cells. *In vivo*, betulin attenuated the increases in serum aminotransferase and triglyceride levels in the mice fed with chronic-binge ethanol, while significantly inhibited SREBP-1 expression and activated LKB1-AMPK phosphorylation. Additionally, betulin enhanced the sirtuin 1 (SIRT1) expression mediated by ethanol. Taken together, betulin alleviates alcoholic liver injury possibly through blocking the regulation of SREBP-1 on fatty acid synthesis and activating SIRT1-LKB1-AMPK signaling pathway.

1. Introduction

Alcoholic liver disease (ALD) is a major cause of liver failure worldwide, while alcohol consumption is a leading cause of global morbidity and mortality. The histologic spectrum of ALD includes simple hepatic steatosis (an accumulation of hepatocellular lipid droplets), steatohepatitis (steatosis with inflammation), and cirrhosis [1,2]. Steatosis is the first response of the liver to alcohol abuse. Lipogenic enzymes are upregulated by alcohol through upregulation of sterol regulatory element-binding protein-1 (SREBP-1). The dysregulation of SREBP-1 has been implicated in the pathogenesis of hepatic steatosis [3]. An ever increasing number of evidences now indicate that adenosine monophosphate-activated protein kinase (AMPK) regulates SREBP-1, which is transcriptionally regulated by the nuclear receptors, involved in the control of glucose, lipid, and cholesterol metabolism [4,5]. AMPK plays an important role in regulating hepatic lipidmetabolism and phosphorylates or inactivates acetyl CoA carboxylase (ACC) and 3-hydroxy-3-methyl-glutaryl-CoA (HMGCoA) reductase, thus inhibiting both cholesterol and fatty acid biosynthesis and promoting steatosis [6,7]. Although it is well known that AMPK signaling

Abbreviations: α-SMA, α-smooth muscle actin; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate aminotransferase; FAS, fatty acid synthase; HSCs, hepatic stellate cells; LKB-1, liver kinase B-1; NF-κB, nuclear factor-kappa B; PDTC, pyrrolidinedithiocarbamic acid; SIRT1, sirtuin 1; SREBP-1, sterol regulatory element-binding protein-1; STAT3, signal transducer and activator of transcription 3; TG, triglyceride; TLR4, toll-like receptor 4.

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plays a crucial role in the regulation the action of SREBP-1, the role in hepatocytes treated with ethanol has not been reported. Interestingly, sirtuin 1 (SIRT1) overexpression prevents the upregulation of SREBP-1 on exposure to a high-fat diet [8]. SIRT1, a conserved NAD^+—dependent histone deacetylase, is closely related with various biological processes. SIRT1 controls organism metabolism by the regulation of mitochondrial biogenesis, glucose and lipid homeostasis via activating the nuclear receptors, SREBP and peroxisome proliferatoractivated receptor-γ (PPAR-γ) [9]. SIRT1 also deacetylates transcriptional and related factors, including p53, forkhead box O (FOXO) 1 and nuclear factor-kappa B (NF-κB) for regulating cell-cycle progression, apoptosis and inflammation. Moreover, overexpression or activation of SIRT1 has been demonstrated to be harmful to fibrogenesis [10,11].

Chronic alcohol use can result in fibrosis, which refers to the extracellular accumulation of collagen and other matrix proteins. The major cell type involved in alcohol-induced fibrosis is by far the activated hepatic stellate cells (HSCs). The resident perisinusoidal cell type, have been recognized to be the main producers of excessive extracellular matrix (ECM) [12]. Under continued liver injury, the quiescent HSCs trans-differentiate into fibrogenic myofibroblast like cells, with expression of α-smooth muscle actin (α-SMA) and type I collagen, and secreting profibrogenic mediators, ultimately promoting the progression of liver fibrosis [13]. AMPK activation negatively modulates α1(I) collagen expression in cultured human HSCs and mouse HSCs. Recent studies have highlighted that the activation of AMPK by high-molecular-mass adiponectin, AICAR (a pharmacological activator of AMPK), metformin or adenovirus mediated expression of a constitutively active form of AMPK inhibited proliferation of human immortalized HSCs cell lines and myofibroblasts derived from primary human or rat HSCs [14]. However, better figuring out the molecular mechanisms of AMPK activation in HSCs and reversion to the pathogenesis of liver injury is still beyond our reach.

Betulin (PubChem CID: 72326), lup-20 (29)-ene-3β, 28-diol, also known as betulinol, betuline and betulinic alcohol (Fig. 1A), is a pentacyclic triterpene alcohol with a lupane skeleton. Betulin is readily available from the bark of *Betula platyphylla* Suk in large quantity, exhibit diverse pharmacological activities, including anti-HIV, anti-cancer, and anti-inflammatory activities [15]. Betulin is a pentacyclic triterpene found in many plants and fruits having broad biological activities. It was also reported that betulin can exert an antifibrotic activity by silencing ethanol-activated HSCs [16]. In our previous study, betulin ameliorated acute ethanol-induced fatty liver via Toll-like receptor 4 (TLR4) and signal transducer and activator of transcription 3 (STAT3) [17]. The aims of this current study were to further investigate the protective effect of betulin against ethanol-induced alcoholic liver steatosis and fibrosis through regulation of SIRT1-LKB1-AMPK signaling pathways *in vitro* and *in vivo*.

2. Materials and methods

2.1. Antibodies and reagents

Betulin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and pyrrolidinedithiocarbamic acid (PDTC) obtained from Beyotime (Jiangsu, China). Anti-AMPK (cs-2532), anti-p-AMPK (cs-2531), anti-LKB1 (cs-3047) and anti-p-LKB1 (cs-3482) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-collagen-I (ab-34710), anti-α-SMA (ab-5694), anti-SREBP-1 (ab-3259), anti-SIRT1 (ab-110304) antibodies and anti-β-actin (ab-133626) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-NF-κB p65 (sc-372) and anti-Topo-1 (sc-5342) antibodies, AMPKα1 siRNA (sc-29673) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. All cell culture reagents were from Gibco/Invitrogen (Grand Island, NY, USA).

2.2. Cell culture

LX-2 cells (an immortalized human hepatic stellate cell line) and human Chang liver cells (a type of innocence human hepatic heteroploid cell) was a kind gift from Dr. D.H.Sohn (College of Pharmacy, Wonkwang University, Iksan, Jeonbuk, Korea). The cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 mg/ml streptomycin at 37 °C under 5% CO_2. The cultures were passaged by trypsinization every three days and cells were plated in 100 mm culture dishes at a density of 1×10^6 cells per dish in DMEM. For the experiment, cells were exposed to ethanol with 0.1% FBS in the absence or presence of betulin at the indicated concentrations and time course. Cells in the forth to seventh passages which these numbers refer to those after selection of an immortalized cell population were used in the experiments [18].

2.3. Methylthiazolyltetrazolium (MTT) assay for cell viability

LX-2 cells and normal human Chang liver cells were seeded in 96 well plates at a density of 1×10^4 cells per well. MTT solution (5 mg/ml) was added after betulin treatment for 24 h, and the cells were then incubated for another 3 h. The extent of reduction of MTT to formazan was read at 540 nm using a microplate reader.

2.4. In vitro *small interfering RNA transfection*

To silence AMPK expression, transfection of AMPK-specific and scrambled siRNA into LX-2 cells was performed with HiPerFect Transfection Reagent method (QIAGEN, 301705). After 24 h, siRNA in the medium was substituted with normal DMEM in the absence or presence of betulin. Cells were cultured for another 24 h for evaluating the protein levels of AMPK, SREBP-1 and SIRT1.

2.5. Animal experiment

Male C57BL/6 mice (8 weeks, 20–22 g) were obtained from the Changchun Yisi Laboratory Animal Technology Co., Ltd (Jilin, China) [SPF, SCXK (J) 2003-0008]. The mice were acclimatized to the laboratory environment, maintained at 22 ± 2 °C and 50–60% relative humidity, with 12-h light-dark cycles throughout the experiment. All mice were fed a standard laboratory chow diet *ad libitum*. The experiment was performed in accordance with the guidelines of the Animal Care Committee of Yanbian University [SPF, SCXK (J) 2011-0007]. All mice were randomly divided into the following five groups: normal group, ethanol group, betulin-20 single group, betulin-50 single group, ethanol + betulin-20 group, ethanol + betulin-50 group. Each group contained 8 mice. Betulin (20 and 50 mg/kg) suspended initially in 2% (v/v) Tween 80 and then further in saline. The betulin groups mice were daily gaveged 20 or 50 mg/kg of betulin [17]. Mice were feeding *ad libitum* with control liquid diet or Lieber-DeCarli liquid ethanol diet, which pair-fed isocaloric Lieber-DeCarli liquid diet containing 35% ethanol by caloric content for 10 days. At day 11, mice in ethanol groups were gavaged a single dose of ethanol (5 g/kg body weight), and mice in control groups were gavaged an isocaloric dose of dextrin maltose. The gavage was performed in the early morning, and mice had access to the diets after alcohol gavage [19]. The mice were euthanized; blood and tissue samples were collected 9 h post gavage. Quickly frozen the remaining liver tissues in liquid nitrogen and