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Full paper

Akebia saponin D alleviates hepatic steatosis through BNip3 induced mitophagy

Li-li Gong, Song Yang, Wen Zhang, Fei-fei Han, Ya-li Lv, Zi-Rui Wan, He Liu, Yang-jie Jia, Xuan Ling-ling, Li-hong Liu*

Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

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ABSTRACT

Akebia Saponin D (ASD) is the most abundant constituent of the rhizome of Dipsacus asper Wall. The prior studies have shown that ASD alleviates hepatic steatosis targeted at the modulation of autophagy and exerts hepatoprotective effects through mitochondria. However, it is still unclear which signal transduction pathway that ASD increase autophagy and protect the mitochondria. The purpose of this paper was to explore the mechanisms through which ASD alleviates hepatic steatosis. ASD significantly reduced lipid accumulation in BRL cells. Furthermore, ASD significantly increased the mitophagy acting as increase the colocalization between mitochondria and punctate EGFP-LC3. ASD treatment increased the expression of BNip3, phospho-AMPK, prevented oleic acid (OA) induced LC3-II and phospho-mTOR expression. These effects were similar to the effects cotreatment with rapamycin. ASD treatment could not attenuate the expression of BNip3 blocked by chloroquine (CQ) or siRNA-mediated knockdown of BNip3. These results suggest that Akebia saponin D alleviates hepatic steatosis targeted at BNip3 mediated mitophagy. Activation of BNip3 via ASD may offer a new strategy for treating NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to the accumulation of hepatic steatosis not due to excess alcohol consumption. The prevalence of NAFLD is up to 30% in developed countries and nearly 10% in developing nations, making NAFLD the most common liver condition in the world.[1,2] The hallmark of NAFLD is the intracellular accumulation of lipids, resulting in the formation of lipid droplets within hepatocytes.[3] Glick et al. have shown that BNip3 is required to prevent excess lipid accumulation in the liver and loss of BNip3 leads to steatohepatitis.[4]

The Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNip3) is a BH3-only protein and plays a key role in the pathogenesis of many diseases. Although previous studies have reported the mechanism and functioning of BNip3, the controversy still exist. On one hand it was shown to be pro-apoptotic protein in some studies where as others have established its non-independent nature to induce cell death.[5] A role for BNip3 in promoting cell death has been elucidated by evidence that overexpression of BNip3 is not sufficient to kill cells[6] but rather promotes mitophagy through interactions with LC-3.[7] BNip3 localizes to the outer mitochondrial membrane, where it functions in mitophagy and mitochondrial dynamics. It was suggested that autophagy may be the primary role of BNip3 given the fact that knockdown of BNip3 in hypoxic cells reduces the ATP cellular levels that was consistent with BNip3 dependent autophagy, contributing to energy balance in cells. Defects in lipid utilization via mitochondrial oxidation may also contribute to hepatic lipid build-up.[1] BNip3 protein levels are up regulated during the hepatic fasting response, and loss of BNip3 leads to impaired fatty acid β-oxidation and the development of sever liver steatosis. Therefore, activation of BNip3 could constitute a therapeutic approach against hepatic steatosis.

Akebia Saponin D (ASD, also named Asperosaponin VI) (Supplemental Fig. 1) is the most abundant constituent of the rhizome of Dipsacus asper Wall, which has long been used for the enhancement of liver and kidney activity and treatment of lower back pain and bone fractures. In recent years, it was reported that ASD was a potential treatment drug for cancer,[8] Alzheimer's

* Corresponding author. Beijing Chaoyang Hospital, Capital Medical University, 8 Gongren Tiyuchang Nanlu, Beijing, 100020, China.
E-mail addresses: liulihong@bjcyh.com, hongllh@126.com (L.-h. Liu).
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disease,[8–10] cardiovascular disease[11–13] and bone fractures.[14,15]Our previous studies have shown that ASD decreased hepatic lipid accumulation on ob/ob mice[16] and against rotenone-induced mitochondria toxicity in liver BRL cells.[17] The preliminary results have shown that ASD alleviates hepatic steatosis targeted at the modulation of autophagy. However, it is still unclear which signal transduction pathway that ASD increase autophagy and protect the mitochondria. Therefore, this study aims to gain an insight into the mechanisms through which ASD alleviates hepatic steatosis.

2. Materials and methods

2.1. Drugs, reagents and plasmids

ASD was purchased from Sigma–Aldrich (St. Louis, MO, USA). Bodipy 493/503 was acquired from Invitrogen (Carlsbad, Calif, USA). Oleic acid (OA), rapamycin, chloroquine (CQ), Compound C and bovine serum albumin (BSA) were gained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) were obtained from Hyclone (Logan, Utah, USA). Immobilon PVDF membrane was purchased from Millipore (Billerica, MA, USA). Antibodies against LC3-II, p-AMPK, AMPK, p-mTOR, mTOR, BNip3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Plasmids EGFP-LC3 was a generous gift from Prof. Jian Lin (Peking University). Microtubule associated protein light chain 3 (LC3) is a ubiquitin-like protein that binds to autophagosomes and subsequently EGFP tagged LC3 was used to track and follow the fate of autophagosomes in the cell.

2.2. Cell culture and treatment

BRL cells, a cell line established from Buffalo rat liver, have been widely used for liver biological and chemical studies. BRL cells were purchased from the Cell Center of Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with10% FBS at 37 °C with 5% CO_2. OA (20 mM) was made as described previously.[16] Briefly, OA (20 mM) in 0.01 N NaOH was incubated at 70 °C for 30 min, and then mixed with 10% BSA in PBS at a 1:9 M ratio of OA to BSA. The OA-BSA conjugate was administered to the cultured cells. BSA was used as a vehicle control. ASD was dissolved in PBS to provide a 100 mM stock solution. PBS was used as the vehicle control. BRL cells were pretreated with ASD (100 μM) for 1 h, followed by incubation with 200 μM OA or vehicle control for 24 h in order to prevention the effect that induced by OA. Next, we incubated BRL cells with AMPK inhibitor Compound C (10 μM) for 1 h before treatment with ASD and investigated the effect of these inhibitors. Rapamycin (25 ng/mL), chloroquine (CQ) (50 nM) were used as negative and positive control of autophagosome accumulation.

2.3. Bodipy staining of lipid droplet

BRL Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and stained with Bodipy 493/503 (10 μg/mL; Invitrogen) for 1 h in darkness. The cells were visualized under the Olympus fluorescence microscopy.

2.4. Monitoring of mitophagy

BRL cells were cultured in DMEM supplement with 10% FBS at 37 °C in an atmosphere of 5% CO_2. About 1×10^6 cells were plated into confocal dish 24 h before transfection. Plasmids EGFP-LC3 was used to detect autophagy. Cells were transiently transfected with the EGFP-LC3 plasmid for 24 h and then treated with OA (200 μM) or ASD (100 μM) for an additional 24 h. Cells were then loaded with 50 nM MitoTracker Red (Life-Molecular Probes) in PBS for 30 min at 37 °C. Under conditions favoring mitophagy, numerous mitochondria could be seen colocalized with lysosomes. The cells were observed under confocal microscopy without fixation.

2.5. RNA interference

The set of siRNAs targeting BNip3 were the following: (forward) 5′-CCACAGCUUUGGUGAGAAATT-3′ and (reverse)5′-UUUCUCACCAAAGCUGUGGTT-3′. The transfection of the siRNAs targeting BNip3 or a control siRNA into the BRL cells were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended conditions.[18] The siRNA transfection mix containing a final siRNA concentration of 100 nM was added to the BRL cells for 24 h. Total RNA was extracted from cell culture lysates using TRIzol reagent (Invitrogen) following manufacture's instruction. Total RNA concentration was measured in duplicate using NanoDrop ND-1000 Spectrophotometer. cDNA was synthesized using M-MLV Reverse TranscriptaseKit (promega).

2.6. Real-time PCR analysis

Real-time PCR (RT-qPCR) has been widely used for quantification of relative gene expression. Target gene expression was normalized by stably-expressed internal reference gene (GAPDH). Real-time PCR was performed with gene specific primers and SYBR Premix Ex Taq (Takara, Japan) in Cobasz 480 real-time PCR machine (Roche). Target sequences were amplified by using the following conditions: 2 min at 95 °C, and 45 cycles of 10 s at 95 °C, 20 s at 60 °C and 20 s at 68 °C. All reactions were performed in triplicate. The expression levels of the genes of interest were presented as the relative levels to the mRNA level of the control gene.

2.7. Immunoblotting

Cells were lysed in protein lysis buffer (Applygen, Beijing, China) and the protein concentration was determined using the bicinchoninic acid assay (BCA assay). Proteins were resolved by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk and incubated overnight at 4 °C with the primary antibodies (1:1000) (BNip3, LC3, p-PI3K, PI3K, p-AMPK, AMPK, p-mTOR, mTOR, Cell Signaling Technology, USA). The membranes were washed and then incubated IRDye 680LT secondary antibody (Licor, USA) for 1 h at room temperature. After washing, immunoreactive bands were visualized with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.8. Statistical analysis

The results were expressed as means ± S.E.M as indicated. The results were evaluated by the One-way ANOVA analysis using SPSS 21 (IBM Corporation, Chicago, IL, USA). $P < 0.05$ was considered statistically significant in all calculations.

3. Results

3.1. ASD reduced lipid accumulation in BRL cells

To visualize lipid exchange, we accumulated the Bodipy 493/503 in the lipid droplets (LDs) of BRL cells. As shown in Fig. 1, upon loading cells with oleic acid (OA), the LDs were both significantly brighter and larger in cells. In contrast, the accumulation of LDs in BRL cells was markedly reduced when treated with ASD (100 μM). LDs are barely detectable in vehicle control.

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