ASPP2 attenuates triglycerides to protect against hepatocyte injury by reducing autophagy in a cell and mouse model of non-alcoholic fatty liver disease

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Abstract

ASPP2 is a pro-apoptotic member of the p53 binding protein family. ASPP2 has been shown to inhibit autophagy, which maintains energy balance in nutritional deprivation. We attempted to identify the role of ASPP2 in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). In a NAFLD cell model, control treated and untreated HepG2 cells were pre-incubated with GFP-adenovirus (GFP-ad) for 12 hrs and then treated with oleic acid (OA) for 24 hrs. In the experimental groups, the HepG2 cells were pre-treated with ASPP2-adenovirus (ASPP2-ad) or ASPP2-siRNA for 12 hrs and then treated with OA for 24 hrs. BALB/c mice fed a methionine- and choline-deficient (MCD) diet were used to generate a mouse model of NAFLD. The mice with fatty livers in the control group were pre-treated with injections of GFP-ad for 10 days. In the experimental group, the mice that had been pre-treated with ASPP2-ad were fed an MCD diet for 10 days. ASPP2-ad or GFP-ad was administered once every 5 days. Liver tissue from fatty liver patients and healthy controls were used to analyse the role of ASPP2. Autophagy, apoptosis markers and lipid metabolism mediators, were assessed with confocal fluorescence microscopy, immunohistochemistry, western blot and biochemical assays. ASPP2 overexpression decreased the triglyceride content and inhibited autophagy and apoptosis in the HepG2 cells. ASPP2-ad administration suppressed the MCD diet-induced autophagy, steatosis and apoptosis and decreased the previously elevated alanine aminotransferase levels. In conclusion, ASPP2 may participate in the lipid metabolism of non-alcoholic steatohepatitis and attenuate liver failure.

Keywords: ASPP2 • NAFLD • triglyceride • autophagy

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition that varies in severity from lipid accumulation to steatohepatitis and even cirrhosis. NAFLD is associated with insulin resistance and obesity [1, 2] and induces a wide spectrum of liver damage [3]. At present, NAFLD occurs in one-third of the adult population and is increasingly observed in children in developed countries [4]. Although the pathogenesis of NAFLD is unclear, NAFLD has been linked to lipid accumulation and lipid metabolism dysfunction [5, 6].

Lipid metabolism is critical in the progression of NAFLD, and autophagy plays an important role in lipid metabolism. In addition to its role in the elimination of intracellular pathogens and the removal of damaged organelles [7], autophagy has multiple pathophysiological and physiological functions in NAFLD. Autophagy can be induced by free fatty acids in vitro [8], and insulin resistance has been associated with autophagy impairment in NAFLD [9, 10]. However, autophagy appears to play a paradoxical role in cell survival and death. In
chemotherapy, the autophagic response plays a protective role in impeding eventual cell death [11–13]. However, recent studies have indicated that when autophagy was inhibited, there was decreased apoptosis [14, 15].

Significant evidence has demonstrated that p53 plays a major role in the pathogenesis of NAFLD [16, 17]. Zoltan et al. reported that p53 plays a central role in NAFLD, and the partial inhibition of p53 activity markedly diminished hepatic steatosis in high-fat-diet-fed mice [18]. Tomit reported that p53 knockout mice fed a methionine- and choline-deficient (MCD) diet for 8 weeks developed less steatosis compared with their wild-type littermates [17].

P53-2 (ASPP2) is a pro-apoptotic regulator that enhances apoptosis by binding to p53 [19, 20]. ASPP2 is involved in many biological pathways [21] and has been implicated in the pathogenesis of NAFLD. This study aimed to elucidate whether ASPP2 can decrease triglyceride (TG) accumulation and to clarify the relationship between ASPP2, apoptosis and autophagy in NAFLD. Our results suggest that the overexpression of ASPP2 could decrease the accumulation of TGs and inhibit autophagy and apoptosis.

Materials and methods

Cell culture and treatment

The human hepatoblastoma cell line HepG2 was grown in DMEM containing 10% foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). The HepG2 cells were divided into four groups. The control groups consisted of HepG2 cells treated with or without 400 µM oleic acid (OA) for 24 hrs; these cells were pre-incubated with GFP-adenovirus (GFP-ad) for 12 hrs. In the experimental groups, the HepG2 cells were pre-incubated with ASPP2 adenovirus (ASPP2-ad) or ASPP2 siRNA for 12 hrs and then treated with 400 µM OA for 24 hrs. FuGENE HD transfection reagent (Promega, Madison, WI, USA) was used for the transfection of ASPP2 siRNA.

Lipid extraction

The cells were collected and washed with PBS. Small interfering RNAs (siRNA) specific to ASPP2 (target sequence: 5-AAGTTGCTGAGCAGGA GAAAC-3) and a nonspecific control were synthesized by Genepharmer. ASPP2 siRNA was transfected into HepG2 cells by using the FuGENE HD transfection reagent (Promega, Madison, WI, USA) for 20 min. at 37°C to visualize the intracellular lipid droplets. Images were acquired with a transmission electron microscope.

Oil Red O staining and the determination of the intracellular lipid accumulation

The HepG2 cells were incubated for 24 hrs with 400 µM OA. The cells were cultured on glass cover slips and stained with Oil Red O (5 mg/ml; St. Louis, USA) for 20 min. at 37°C to visualize the intracellular lipid droplets. Images were acquired with a Nikon Eclipse 80i (Nikon, Tokyo, Japan). The intracellular lipid droplets were measured using Image J software.

Mice and diets

Male BALB/c mice (Academy of Military Medical Sciences, China) were randomly assigned to the experimental group (MCD) or the control group (MCS). The mice in the MCD and MCS groups were fed an MCD Diet and MCS diet respectively. The diets were purchased from Nantong (China). All studies were approved by the Ethical Committee of the You An Hospital Center, affiliated Hospital of Capital Medical University, Beijing, and all animal care complied with the applicable health guidelines. ASPP2+/− mice (BALB/c) will be used for the transfection of ASPP2 siRNA.
Liver tests

The serum ALT, AST, TG and CHOL levels were quantitated with an AU400 automatic biochemical analyzer (Shinjuku, Tokyo, Japan, Japan).

Patients and immunofluorescence

Moderate NAFLD patients were recruited from You An hospital, Beijing. As approved by the Ethics Committee of You An Hospital, Beijing, normal liver tissues were obtained from liver transplant donors as the healthy control group; informed consent was obtained from all patients. Immunofluorescence was observed as previously described.

Data analysis

All data are expressed as the mean ± SEM and represent at least three independent experiments. Statistical significance between groups was determined by using Student’s t-test. Differences were considered statistically significant at confidence levels of $P < 0.05$ (*) or $P < 0.01$ (**).

Results

ASPP2 overexpression reduced the level of TG and autophagy in OA-treated HepG2 cells

To investigate the role of ASPP2 in the process and mechanism of NAFLD, we constructed a NAFLD model by using HepG2 cells as previously described [1]. The results showed that the OA- and GFP-ad (ad)-treated group significantly induced TG accumulation compared with the Ad treatment alone (Fig. 1A-a and -b). Significantly fewer lipid droplets were observed in the ASPP2-ad treated group (Fig. 1A-c). The intracellular TG and lipid levels were further quantified as shown in Figure 1B and C. These levels confirmed that the accumulation of lipids and TGs induced by OA treatment was significantly decreased by ASPP2 overexpression. Recent studies have shown that autophagy plays a critical role in NAFLD and that ASPP2 inhibits autophagy [22]; therefore, we examined whether the elimination of excessive intracellular lipids was associated with ASPP2-mediated autophagy. The level of autophagy was analysed via the expression of microtubule-associated protein light chain 3 (LC3). In this study, we identified the level of autophagy based on the number of GFP-LC3 puncta and the LC3-I/LC3-II ratio, which are both markers of autophagy. The percentages of HepG2 cells that contained GFP-LC3 puncta were determined in eight non-overlapping fields. Each experiment was repeated three times. Immunofluorescence and western blot (WB) analyses showed that the levels of autophagy in the OA plus Ad-treated group were higher than those in the Ad-treated control group (Fig. 1D-a, -b and F-lanes 1 and 2 and H). However, the levels of autophagy observed in the OA plus ASPP2-ad group were lower compared with the OA plus Ad-treated group (Fig. 1D-b and -c, F-lanes 2 and 3, and H). The OA- plus ASPP2-ad-treated group had increased expression of ASPP2 compared with the OA- plus GFP-ad-treated group (Fig. 1G). Electron microscopy (EM) was useful for observing the autophagy and lipid contents of the cells. To further confirm that the ASPP2 decreased the levels of lipids and autophagy, we performed an EM assay. The experiment revealed a large number of autophagic lysosomes and lipids in the OA-treated group, with only a small number of autophagic vacuoles and low lipid levels in the ASPP2-ad group (Fig. 1I). This result supports the findings in Figure 1A-G, which indicate that ASPP2 overexpression can significantly reduce the level of autophagy, as well as the lipid accumulation in OA-treated HepG2 cells.

ASPP2 overexpression can significantly reduce the apoptosis of OA-treated HepG2 cells

Apoptosis, which promotes inflammation and interferes with hepatocyte function, plays an important role in the progression of NAFLD [23]. ASPP2 selectively stimulates the p53 (and p73/p63) transactivation of target genes, in addition to mediating p53-independent functions and inhibiting cell growth. Importantly, the ASPP2 allele targeted in mouse models confirms that ASPP2 is a tumour suppressor [24]. In this study, we aimed to identify the apoptotic role of ASPP2 overexpression in the progression of NAFLD. We detected cell death by using the M30 antibody, which can specifically recognize the k18 fragments produced by caspase 3 cleavage during the apoptotic process. In our HepG2 experiments, cell death was significantly lower after the OA stimulus and pre-incubation with ASPP2-ad compared with after the OA stimulus and pre-incubation with GFP-Ad alone (Fig. 2A). Upon counting the M30-positive cells (200 cells/well in three random wells), we found that the number of cells stained positive for M30 (8.8 ± 3.3%) in the OA plus GFP-Ad-treated group was higher compared with the OA plus ASPP2-Ad group (3.7 ± 1.5%, Fig. 2B).

We then measured the levels of LDH released from the OA plus GFP-Ad and OA plus ASPP2-Ad groups (i.e., the levels released into the culture medium) to identify necrosis and late apoptosis in the differently treated HepG2 cells. The levels of LDH in the culture medium did not significantly differ between the OA plus GFP-Ad and OA plus ASPP2-Ad-treated HepG2 cells (Fig. 2C). We then confirmed this finding by examining the ultrastructure of the HepG2 cells by using TEM (Fig. 2D); the numbers of apoptotic bodies in the OA plus
ASPP2-Ad-treated HepG2 cells were significantly lower compared with the OA plus GFP-Ad-treated HepG2 cells. Therefore, ASPP2 might protect HepG2 cells from OA-induced early apoptosis.

**Reduced autophagy by ASPP2 contributed to OA-treated HepG2 survival**

In the results described earlier, ASPP2 overexpression reduced the autophagy and apoptosis of OA-treated HepG2 cells. We wondered whether the autophagy reduced by ASPP2 was associated with the death of the OA-treated HepG2 cells. First, we detected the level of ASPP2 expression by using WB analysis of an untreated control, an OA plus ASPP2 siRNA control, and an ASPP2 siRNA control in HepG2 cells. ASPP2 expression was induced by OA treatment and knocked down by ASPP2 siRNA (Fig. 3A upper row and Fig. 3B). The ratio of LC3-II/LC3-I was 1.2-fold higher in the OA plus ASPP2 siRNA treatment group compared with OA alone, which was statistically significant (Fig. 3A middle row and Fig. 3C). Immunofluorescence examination of GFP-LC3 puncta and ASPP2 expression also confirmed that the number of GFP-LC3 puncta was significantly higher in the OA plus ASPP2 siRNA-treated
HepG2 cells (Fig. 3D-c versus -b and Fig. 3E). In a cell apoptosis assay based on M30 immunofluorescence staining, the percentage of M30-positive cells in the OA plus control siRNA-treated HepG2 cells (10 ± 1%) was lower compared with the OA plus ASPP2 siRNA-treated HepG2 cells (16.3 ± 1.5%; Fig. 3F and G). These results further confirmed that ASPP2 can significantly reduce the level of autophagy and has a protective role in OA-treated HepG2 cells.

The ASPP2-mediated inhibition of autophagy contributes to the TG reduction in a mouse NASH model

Our study with OA-treated HepG2 cells showed that ASPP2 decreased the level of TGs and inhibited autophagy. We further investigated this result in a mouse model. The NAFLD model of BALB/c mice is described in the supplementary data. Murine livers experience mild to moderate steatosis after 10 days on a MCD diet, and the lipid droplets in the hepatocytes presented as a mixture of large and small bubbles (Fig. 4A and B). In a clinical setting, small-bubble lipid drops are reversible in fatty liver patients. Therefore, the mice fed a MCD diet for 10 days were used to determine the effect of ASPP2 on the TG levels and autophagy.

The GFP-LC3 expression vector and ASPP2-Ad were injected into the MCD mice via the tail vein. Forty-eight hours later, liver ultrasounds indicated echo enhancement in the GFP-ad group, which showed light to moderate fatty degeneration. Interestingly, the liver echo was weaker in the ASPP2 group compared with the GFP-ad group (Fig. 4C). Immunofluorescence staining and WB analysis showed that the number of GFP-LC3 puncta and the LC3-II/LC3-I ratio in the ASPP2-Ad group were significantly lower compared with the GFP-ad group (Fig. 4D–H). The EM data also indicated that ASPP2 overexpression inhibited autophagy. The autophagic vesicles were much larger and contained several varieties of organelles in the GFP-ad group compared with the ASPP2-ad group (Fig. 4I).

ASPP2 overexpression contributes to cell survival by reducing liver failure and inhibiting apoptosis

We demonstrated that ASPP2 overexpression reduced the level of TG by inhibiting autophagy and apoptosis in vitro and in vivo. Some papers have reported that autophagy affects the level of TGs in alcoholic fatty livers [25]. The level of TGs has also been shown to impact hepatic function. Furthermore, it is unknown whether liver function is influenced by autophagy and apoptosis in ASPP2-induced NAFLD. We further assessed the effects of ASPP2 overexpression on liver function in NALFD mice that were fed a MCD diet. The serum ALT and CHOL were lower in the ASPP2-ad group compared with the GFP-ad group (Fig. 5A and C), while the TG content was lower not only in the serum but also in the liver of the ASPP2-ad group compared with the GFP-ad group (Figs. 4J and 5B).

Because hepatocytes may have been killed during MCD administration, M30 was used to detect early apoptosis [26–28]. Interest-
ingly, the incidences of early apoptosis were 6.6 ± 4.5% and 14.5 ± 7.6% in the ASPP2-ad and GFP-ad groups, respectively (Fig. 5D and E). Small apoptotic bodies were observed under EM in the GFP-ad group, but not in the ASPP2-ad group (Fig. 5F). Taken together, these data suggest that ASPP2 overexpression may contribute to cell survival by the alleviation of liver failure and the inhibition of apoptosis.

Decreased ASPP2 expression in NAFLD

Our findings indicate that ASPP2 overexpression attenuates the levels of TGs in the HepG2 cells treated with OA and in the BALB/c mice fed a MCD diet. Thus, we investigated ASPP2 expression in the clinical specimens of fat liver tissue from fatty liver patients. It is important to elucidate the role of ASPP2 in NAFLD. We observed the expression of ASPP2 in normal liver tissue and in liver tissue obtained from fatty liver patients. Immunofluorescence showed that ASPP2 expression was reduced in the fatty liver tissue compared with the control tissue (Fig. 6A and B). Western blot data demonstrated that ASPP2 is lower and the LC3-II/I ratio is higher in the NAFLD samples compared with the normal liver tissue (Fig. 6C–E), which further suggests that ASPP2 may play a role in NAFLD. The mechanism for this role requires further study.

Discussion

With the growing incidence of obesity, sedentary lifestyles, and unhealthy dietary patterns, NAFLD has become a major health burden. At present, no effective method is available for comprehensively treating NAFLD. Continuous cell apoptosis is an indicator of NAFLD [23]. Recent studies have indicated that simple steatosis in NAFLD is reversible [24]. Autophagy changes play a role in the pathogenesis of NAFLD, but the relationship between autophagy and NAFLD remains

Fig. 3 ASPP2 siRNA promoted autophagy and apoptosis. (A) The detection of autophagy using western blotting. (B and C) The relative protein levels of ASPP2 and LC3II/LC3I were normalized with β-actin. (D and E) The detection of autophagy by using immunofluorescence observation. (F and G) The detection of apoptosis using anti-M30. Original magnification (400×); *P < 0.05; **P < 0.01.
controversial. Yuka indicated that NAFLD is associated with autophagic dysfunction. Hepatic steatosis has been found to suppress autophagic proteolysis [25]. However, studies by Ma demonstrated that mice with hepatocyte-specific autophagy deficiency were protected from fat accumulation in the liver (induced by a high-fat diet); these authors also observed decreased expression of specific genes involved in lipid metabolism [29]. Our finding is consistent with Ma’s results.

ASPP2 can regulate apoptosis in both p53-dependent and -independent pathways [30]. p53 can regulate autophagic activity by inducing LC3 expression [31]. N-terminal ASPP2, a known activator of p53, shares structural similarities with LC3 [32]. N-terminal ASPP2 inhibits autophagy. It is unknown whether ASPP2 regulates the level of autophagy through p53-dependent or -independent pathways.

To understand the functional role of ASPP2 in the regulation of an OA/MCD-induced model of NAFLD, we investigated whether ASPP2 overexpression could influence the TG content and cell survival by regulating autophagy. We found that ASPP2 overexpression contributed to cell survival, which was mediated by reducing the TG content and level of autophagy in cell and murine NAFLD models. Intracellular lipids are reportedly degraded in lysosomes when hepatocytes undergo autophagy [33]. In general, autophagy has been considered to play a protective role [34], but recent studies have reported that autophagy can play a dual role in cell protection and cell toxicity [35]. Ost reported the overactive autophagy was associated with large numbers of cytosolic lipid droplets [36]. Hagiwara demonstrated hyperactive autophagy under pathological conditions, including liver inflammation in a rat model of diabetes mellitus [37]. Komatsu described extensive liver autophagy during the catabolism of cytoplasmic components to free fatty acids [38], and diets rich in fatty acids promote fatty liver development [39], which indicates that excessive autophagy might induce fatty liver development. Our findings indicate that ASPP2 contributes to TG reduction by inhibiting autophagy. ASPP2 might reduce the level of TGs through its inhibition of hyperactive and toxic autophagy, which is consistent with the finding that hepatic steatosis is not exacerbated by hepatocyte-specific autophagy deficiency. Instead,
autophagy blockade might accelerate the progression of non-alcoholic steatohepatitis. No inflammation occurs in the early stage of NAFLD.

Fatty degeneration is sometimes reversible during the early stages of NAFLD. NAFLD is characterized by the accumulation of lipids. Schleicher developed a mathematical model for the fate of fatty acids

Fig. 5 ASPP2 protects the liver from injury by inhibiting apoptosis. (A–C) The serum ALT levels, TG content, and CHOL content. (D and E) The detection of apoptosis by using an anti-M30 antibody. Original magnification (400×). (F) The detection of apoptosis in electron micrographs; *P < 0.05; **P < 0.01.

Fig. 6 ASPP2 expression was decreased in the NAFLD liver. (A and B) Immunofluorescence images showing ASPP2 expression in the control and fatty liver. (C) The ASPP2 and LC3 levels detected by using western blot analysis. (D and E) The relative protein levels of ASPP2 and LC3II/LC3I were normalized by using β-actin; *P < 0.05; **P < 0.01.

Fatty degeneration is sometimes reversible during the early stages of NAFLD. NAFLD is characterized by the accumulation of lipids.
that are stimulated by hepatic lipid dynamics in hepatocytes. The model demonstrates reversion of the steatotic state back to the healthy state after reductions in the fatty acid uptake to below the threshold at which steatosis started [40]. Our findings demonstrated that fatty degeneration resulted in the appearance of a mixture of large and small bubbles in murine livers within 10 days of the MCD diet; the fatty degeneration of a small-bubble mixture can be reversed in the clinical setting. These data were obtained 10 days into our MCD mouse model and may represent the threshold for reversing steatosis. The ASPP2 overexpression group (with pre-incubation of ASPP2-ad by tail vein injection) had reduction in steatosis, which is consistent with the finding that hyperactive autophagy is a possible cause of hepatocyte death in anorexia nervosa patients [46]. Taken together, these findings suggest that ASPP2 overexpression might represent a novel strategy for hepatocyte survival by decreasing the level of TGs in the treatment of NAFLD.

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Conflicts of interest

The authors declare that they do not have conflicts of interest to disclose for this manuscript.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The construction of a fatty liver model of BALB/c mice.

Figure S2 The construction of a fatty liver model of BALB/c mice.

Figure S3 The autophagic levels in mice.

Data S1 The construction of a fatty liver model in BALB/c mice.

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