Research Paper

Inhibition of Drp1 protects against senecionine-induced mitochondria-mediated apoptosis in primary hepatocytes and in mice

Xiao Yang[a,b], Hua Wang[b], Hong-Min Ni[b], Aizhen Xiong[a], Zhengtao Wang[a], Hiromi Sesaki[c], Wen-Xing Ding[b,*], Li Yang[a,d,**]

[a] The Key Laboratory of Standardization of Chinese Medicines of Ministry of Education, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 2001203, China
[b] Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas 66160, USA
[c] Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205
[d] Center for Chinese Medical Therapy and Systems Biology, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ARTICLE INFO

ABSTRACT

Pyrrolizidine alkaloids (PAs) are a group of compounds found in various plants and some of them are widely consumed in the world as herbal medicines and food supplements. PAs are potent hepatotoxins that cause irreversible liver injury in animals and humans. However, the mechanisms by which PAs induce liver injury are not clear. In the present study, we determined the hepatotoxicity and molecular mechanisms of senecionine, one of the most common toxic PAs, in primary cultured mouse and human hepatocytes as well as in mice. We found that senecionine administration increased serum alanine aminotransferase levels in mice. H & E and TUNEL staining of liver tissues revealed increased hemorrhage and hepatocyte apoptosis in liver zone 2 areas. Mechanistically, senecionine induced loss of mitochondrial membrane potential, release of mitochondrial cytochrome c as well as mitochondrial JNK translocation and activation prior to the increased DNA fragmentation and caspase-3 activation in primary cultured mouse and human hepatocytes. SP600125, a specific JNK inhibitor, and ZVAD-fmk, a general caspase inhibitor, alleviated senecionine-induced apoptosis in primary hepatocytes. Interestingly, senecionine also caused marked mitochondria fragmentation in hepatocytes. Pharmacological inhibition of dynamin-related protein1 (Drp1), a protein that is critical to regulate mitochondrial fission, blocked senecionine-induced mitochondrial fragmentation and mitochondrial release of cytochrome c and apoptosis. More importantly, hepatocyte-specific Drp1 knockout mice were resistant to senecionine-induced liver injury due to decreased mitochondrial damage and apoptosis. In conclusion, our results uncovered a novel mechanism of Drp1-mediated mitochondrial fragmentation in senecionine-induced liver injury. Targeting Drp1-mediated mitochondrial fragmentation and apoptosis may be a potential avenue to prevent and treat hepatotoxicity induced by PAs.

1. Introduction

Pyrrolizidine alkaloids (PAs) are the ester derivatives of necine base and necic acid that are found in more than 6000 plants [1,2]. PAs are potent hepatotoxins that can lead to liver injury, which over 8000 liver injury cases were reported worldwide to be associated with the use of PA-containing products such as herbal medicines [3,4]. In China, hundreds of people developed hepatic sinusoidal obstruction syndrome (HSOS) due to the consumption of Tusanqi (Gynura segetum), a Chinese medicine that contained high amount of PAs [3]. However, no effective therapies are currently available for hepatotoxicity induced by PAs.
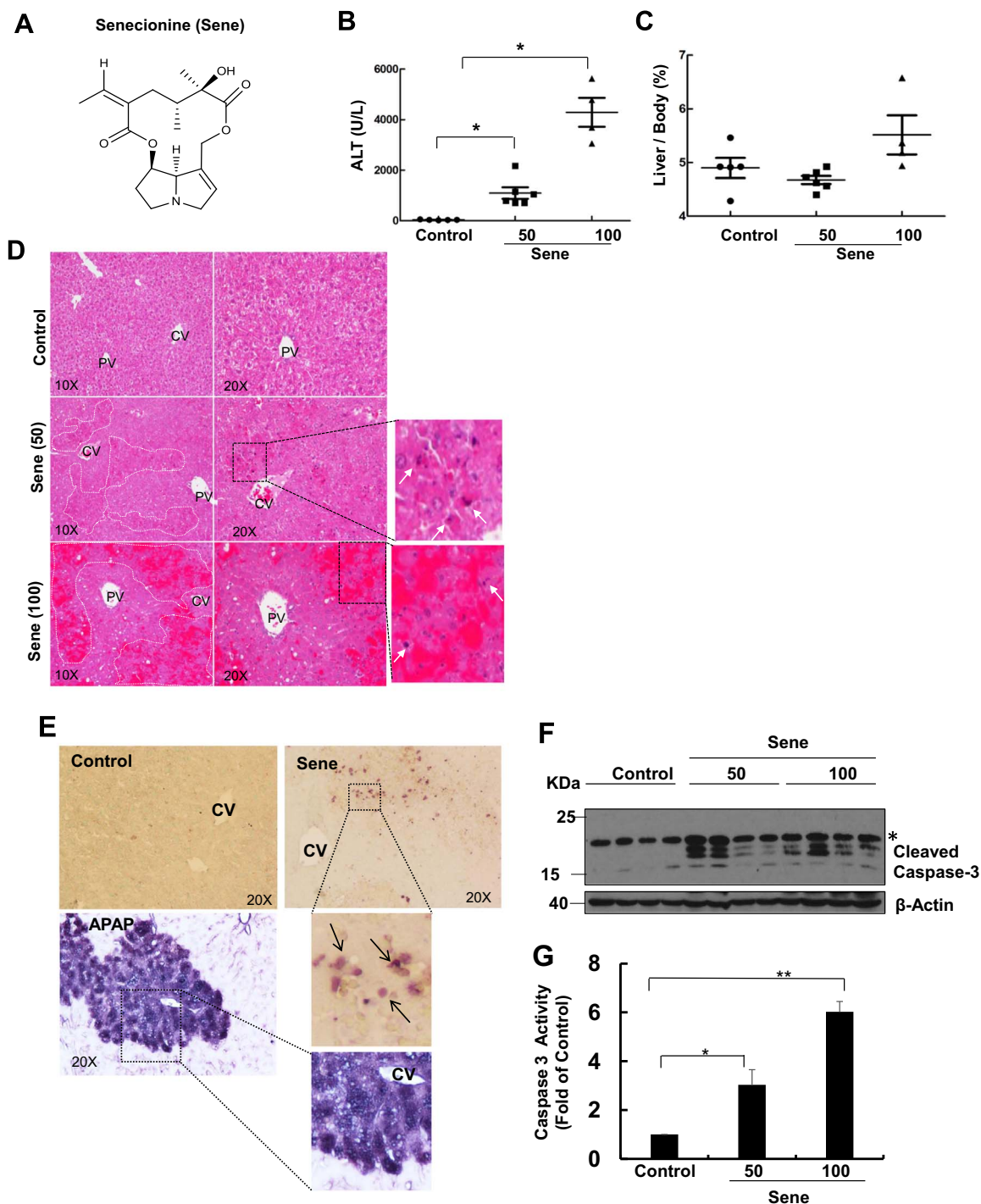
Fig. 1. Sene induces hepatocyte apoptosis and liver injury in mouse livers. The chemical structure of Sene (**A**). Male C57BL/6 J mice were treated with different doses of Sene (50 mg/kg, 100 mg/kg) or vehicle saline control by gavage and mice were sacrificed at 24 h after treatment. Serum ALT levels (**B**) and the ratio of liver to body weight (**C**) were analyzed and data are presented as means ± SE (n=4−6). * $p < 0.05$, one-way ANOVA analysis with Scheffé's post hoc test. Representative images of liver tissue H & E (**D**) and TUNEL staining (**E**). CV: Central vein; PV: Portal vein. Arrows denote apoptotic nuclei and dot line circled areas denote injury zones. APAP-induced TUNEL stained liver tissue used as a positive control for necrosis. Total liver lysates were subjected to western blot analysis (**F**) and caspase-3 activity assay (**G**). *: non-specific band. Data are presented as means ± SE (n=4−6). * $p < 0.05$. Student t-test.

PAs are predominantly metabolized in the liver by cytochrome P450 (CYP) enzymes such as CYP3A to generate reactive metabolites dehydropyrrolizidine alkaloids (DHPAs), which are further hydrolyzed to dehydroretronecine (DHR) [1,2]. DHPAs and DHR are highly reactive metabolites and bind to cellular glutathione (GSH) to form GSH-conjugates, which detoxify DHPAs and DHR. However, DHPAs and DHR can also bind to proteins to form pyrrole-protein adducts to initiate the hepatotoxicity in both parenchymal and non-parenchyma cells such as hepatic sinusoidal endothelial cells (HSEC) [1,2]. The damage to HSEC by PAs due to the depletion of the relative low level of GSH in HSEC often leads to HSOS, which is characterized by hepatomegaly, ascites and hyperbilirubinemia in human [2,5,6].

PAs can induce both apoptotic and necrotic/oncotic cell death in the livers of animals, cultured immortalized human hepatocytes and hepatoma cells [4,7–9]. Mechanistically, increased oxidative stress and mitochondrial pro-apoptotic BNIP3 expression and decreased anti-
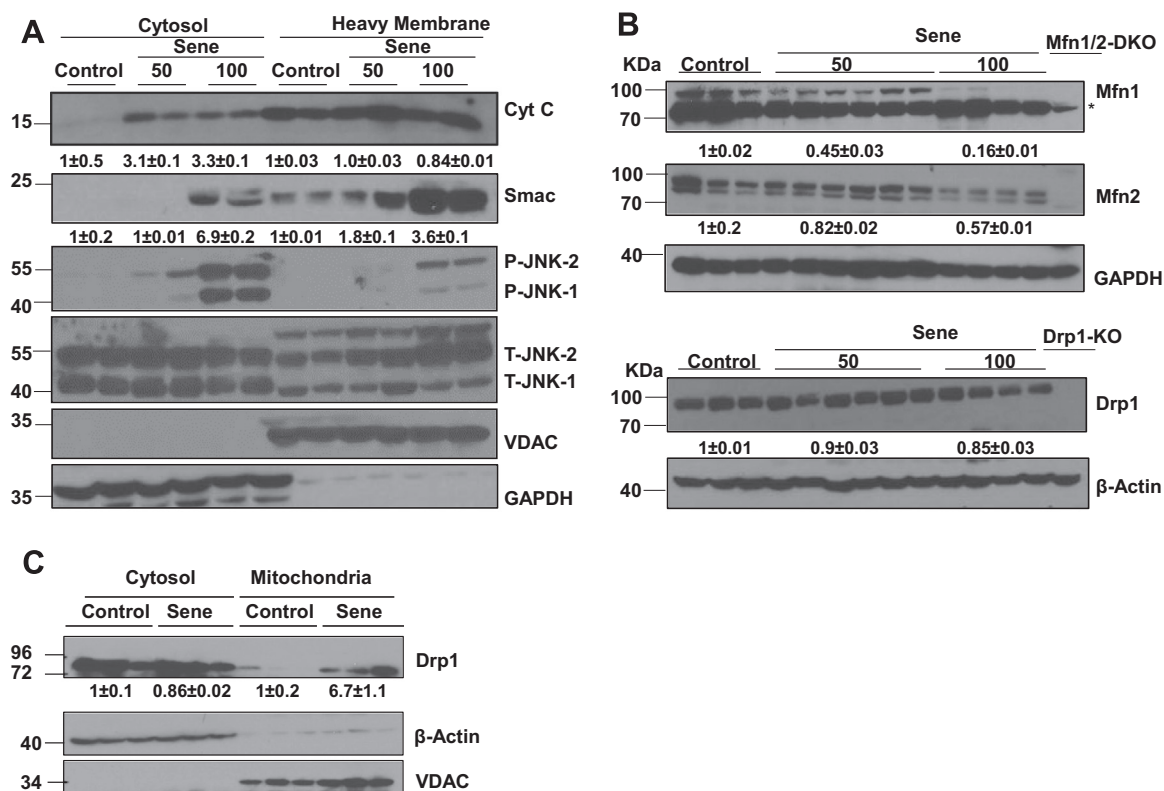
Fig. 2. Sene induces Drp1 mitochondrial translocation, JNK activation and Cyt c release in mouse livers. Male C57BL/6 J mice were treated with Sene (50 mg/kg, and or 100 mg/kg, gavage) or vehicle saline control and mice were sacrificed at 24 h after treatment. Cytosolic and mitochondrial (heavy membrane) fractions (**A & C**) or total liver lysates (**B**) were prepared and subjected to western blot analysis. Total lysate from Mnf1/2 double knockout (DKO) MEFs or liver-specific Drp1 KO mouse livers was used as a positive control. Densitometry analysis data are presented as means ± SE (n=3–6).

apoptotic Bcl-xL expression have been implicated in PA-induced liver injury [7,9,10], suggesting a possible involvement of mitochondrial intrinsic pathway in PA-induced apoptosis in hepatocytes. However, whether PAs could also induce mitochondrial damage in primary hepatocytes and in mouse livers in vivo, and more importantly the underlying mechanisms by which PAs induce mitochondrial damage are unknown.

Mitochondria are central cell death executioners and are highly dynamic organelles that constantly undergo fission and fusion to adapt to changing conditions [11–13]. Mitochondrial fusion in mammals is mediated by the fusion proteins mitofusin 1 (Mfn1), Mfn2 and optic atrophy 1 (OPA1), whereas mitochondria fission is regulated by dynamin-related protein 1 (Drp1) [11–13]. Mitochondrial fusion controls proper distribution of mt-DNA, lipids and proteins across all mitochondria, which is critical for key mitochondrial functions such as energy metabolism, cellular differentiation and proliferation. As an opposite process, mitochondrial fission plays important roles in mitochondrial biogenesis during cellular mitosis and in removal of damaged mitochondria by mitophagy [14,15]. Drp1 is a cytosolic large GTPase protein but it can be recruited to the outer mitochondrial membrane to trigger mitochondria fission [13,16]. Drp1 also interacts with Bax or Bak to disturb the mitochondrial out membrane permeabilization (MOMP) results in the release mitochondrial apoptotic proteins to trigger apoptosis [17]. Mitochondrial division inhibitor-1 (Mdivi), a selective Drp1 inhibitor, inhibits cytochrome c (Cyt c) release and apoptosis by suppressing mitochondrial fragmentation and MOMP [17,18]. Whether PAs would affect Drp1 and mitochondrial dynamics and in turn contribute to PA-induced hepatotoxicity have not been investigated. In the present study, we investigated the role of Drp1 and mitochondrial damage in senecionine (Sene), one of the most common toxic PAs, induced hepatotoxicity in primary cultured mouse and human hepatocytes as well as mouse livers.

2. Materials and methods

2.1. Reagents

Sene was isolated from *Gynura japonica* and the purity was examined as we previously described [19]. Sene was first dissolved in 5% HCl and the pH was adjusted to 6–7 by adding 1 M NaOH, and the volume was adjusted with sterile saline to the appropriate final concentration.

2.2. Animal experiments

We used wild type (WT) male C57BL/6 J mice (Jackson Laboratories, Bar Harbor, ME) in this study. Drp1 Flox/Flox mice (C57BL/6/129) were generated as described previously [20] and were crossed with Albumin-Cre mice (Alb-Cre, C57BL/6) (Jackson Laboratory). All animals received humane care. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Male WT mice, male Alb-Cre positive Drp1 Flox/Flox mice and Alb-Cre negative Drp1 Flox/Flox matched littermates were either given saline (control group) or Sene (50 mg/kg, 100 mg/kg) by gavage and were sacrificed at 24 h after treatment. Liver injury was assessed by the determination of the serum alanine aminotransferase (ALT) activities and Hematoxylin and Eosin (H & E) staining of liver sections [21]. Caspase-3 activities were determined using a fluorescent substrate Ac-DEVD-AFC (Biomol) as we described previously [22]. Total liver lysates were prepared using RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate). Terminal deoxynucleotidy transferase dUTP nick end labeling (TUNEL) staining was performed as described previously [22].
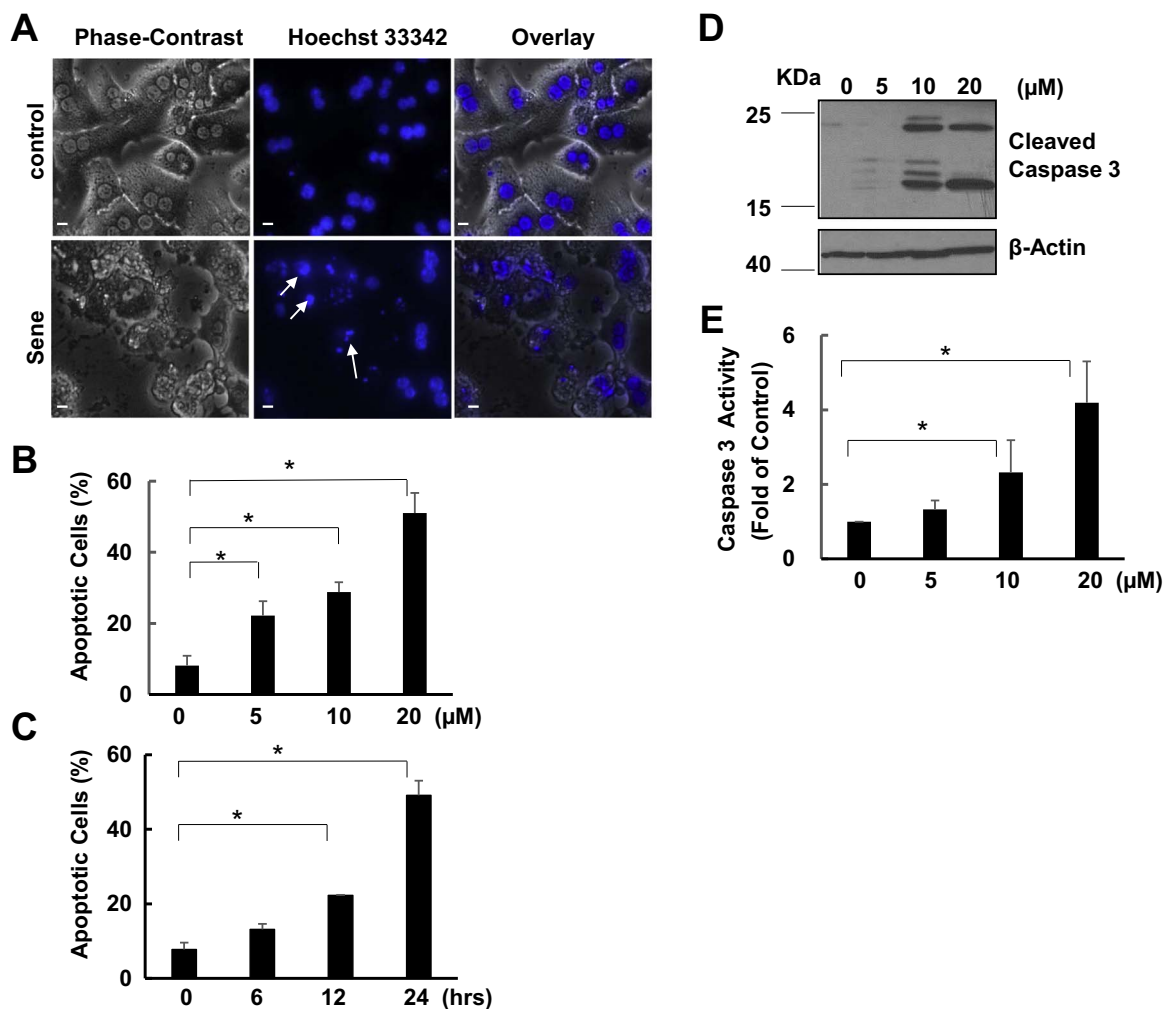
Fig. 3. Sene induces apoptosis in primary cultured mouse hepatocytes. Primary cultured mouse hepatocytes were treated with different concentrations of Sene (0, 5, 10, 20 μM) for 24 h, and cells were stained with Hoechst 33342 (1 μg/ml) for 10 min followed by fluorescence microscopy. Representative images of phase-contrast, Hoechst 33342 nuclei staining and overlapped images are shown (**A**). Arrows denote apoptotic nuclei. Scale bar: 20 μm. Quantification of apoptosis after cells were treated with different concentrations (**B**) and time (**C**) of Sene (20 μM). Data are means ± SE from 3 independent experiments, at least 300 cells were counted in each experiment. * $p < 0.05$. One way ANOVA analysis with Scheffé's post hoc test. Total cell lysates were subjected to western blot analysis for cleaved caspase-3 (**D**) or caspase-3 activity assay (**E**). Data were normalized to the control and are presented as means ± SE (n=3). * $p < 0.05$. One way ANOVA analysis with Scheffé's post hoc test.

2.3. Primary hepatocytes culture

Murine and human hepatocytes were isolated and cultured according to the methods described previously [23]. All cells were maintained in a 37 °C incubator with 5% CO_2. All human liver specimens were obtained in accordance with the University of Kansas Medical Center Human Subjects Committee approved protocol # 13513.

2.4. Statistical analysis

Statistical analysis was performed with Student's *t*-test or one-way ANOVA analysis of variance where appropriate. $p < 0.05$ was considered significant.

Antibodies, Fluorescence Microscopy, Isolation of Subcellular Fractions and Western Blotting and Electron Microscopy were described in Supplemental materials and methods.

3. Results

3.1. Apoptosis, mitochondrial damage and liver injury after Sene treatment in mice

Sene, a 12-membrane macrocyclic diester PA (Fig. 1A), induced

dose-dependent hepatic parenchymal cell injury in mouse livers, as demonstrated by the significant elevate serum alanine aminotransferase (ALT) levels compared with control mice (Fig. 1B). The mice treated with Sene showed less active and lethargic but nevertheless all the mice survived after 24 h treatment even with the high dose of Sene (100 mg/kg). However, the ratio of liver weight to body weight seemed less affected by Sene administration (Fig. 1C). Results from the H & E staining liver tissues revealed that Sene induced marked hemorrhage and apoptotic cell death with fragmented nuclei. Sene-induced liver injury mainly occurred in liver zone 2 regions between portal vein (PV) and central vein (CV) but was more adjacent to CV (Fig. 1D). Increased hepatic red blood cell and neutrophil infiltration as well as condensed nuclear chromatin were also detected by EM studies of liver sections from Sene-treated mouse livers (Supplemental Fig. 1A). Sene-induced parenchymal cell apoptosis was further confirmed by TUNEL staining that showed typical nuclear staining pattern, which is in great contrast with necrotic cell death that showed diffuse cytosolic staining induced by acetaminophen (Fig. 1E). Sene also increased cleavage of caspase-3 and caspase-3 activities in a dose-dependent fashion in mouse livers (Fig. 1F & G).

Damaged mitochondria play critical roles in hepatocyte apoptosis by releasing mitochondrial intermembrane space proteins such as cytochrome C (Cyt C) and Smac. Sene increased the levels of cytosolic
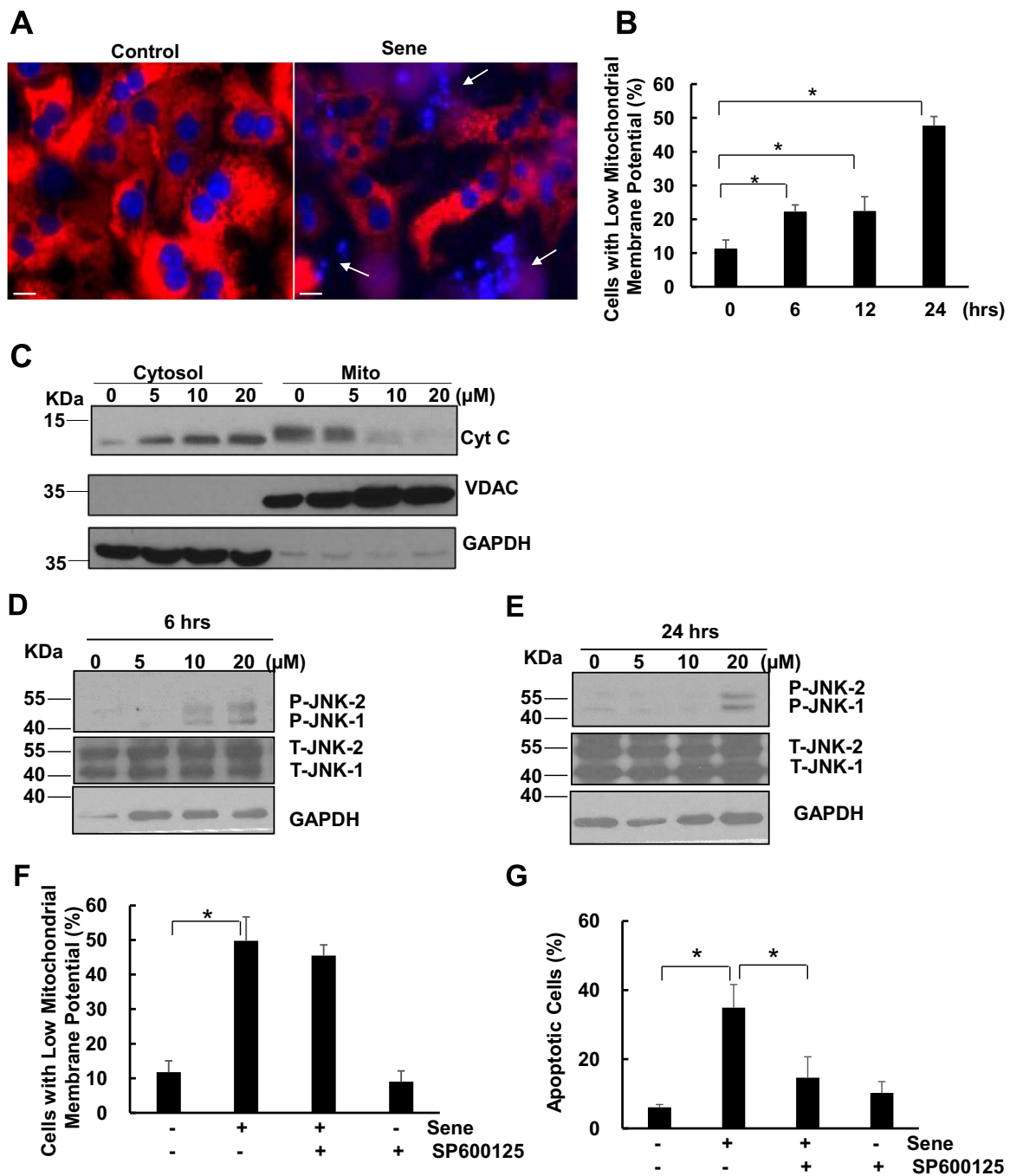
Fig. 4. Sene induces mitochondrial depolarization and JNK activation in primary mouse hepatocytes. Primary cultured mouse hepatocytes were first loaded with TMRM (50 nM) for 15 min and then treated with Sene (20 μM) for different time points. Some cells were further stained with Hoechst 33342 (1 μg/ml) for 5 min followed by fluorescence microscopy. Representative TMRM and Hoechst 33342 stained images are shown (A). Arrows denote the cells with loss of mitochondrial membrane potential (MMP) and apoptotic nuclei. Scale bar: 20 μm. Quantification of the number of cells with low MMP after the cells were treated with Sene (B). Data are means ± SE from 3 independent experiments. More than 300 cells were counted in each experiment from 3 to 4 different fields. * p < 0.05. One way ANOVA analysis with Scheffé's post hoc test. Cytosolic and mitochondrial fractions (C) and total lysates (D & E) were prepared and subjected to western blot analysis. Hepatocytes were treated as in (A) in the absence or presence SP600125 (25 μM) for 24 h. Quantification of the number of cells with low MMP (F) and apoptotic nuclei (G). Data represent means ± SE (n=3). More than 300 cells were counted in each experiment. *p < 0.05. One way ANOVA analysis with Scheffé's post hoc test.

Cyt C and Smac (Fig. 2A). EM studies also showed increased fragmented mitochondria (Sene, 50 mg/kg, Supplemental Fig. 1B) and swollen mitochondria (Sene, 100 mg/kg, Supplemental Fig. 1A) in the liver sections of Sene-treated mice. The c-Jun N-terminal kinase (JNK), the superfamily of mitogen-activated protein   kinases (MAPK), is involved in apoptosis, cell proliferation, differentiation, and inflammation [24]. Sene increased the levels of phosphorylated JNK in the cytosol and mitochondrial fractions as well as levels of total JNK on mitochondria (Fig. 2A). These data suggest that Sene induces JNK

mitochondrial translocation and activation.

Since we found that Sene induced mitochondrial fragmentation and swollen (Supplemental Fig. 1), we next determined the key proteins that regulate mitochondrial fusion and fission. We found that Sene markedly decreased the levels of Mfn1 and Mfn2, two key proteins that are essential for mitochondria fusion (Fig. 2B). While Sene did not cause significant changes on the total levels of Drp1, the key protein that regulates mitochondrial fission, Sene increased the levels of mitochondrial Drp1 (Fig. 2B & C). These results indicate that Sene
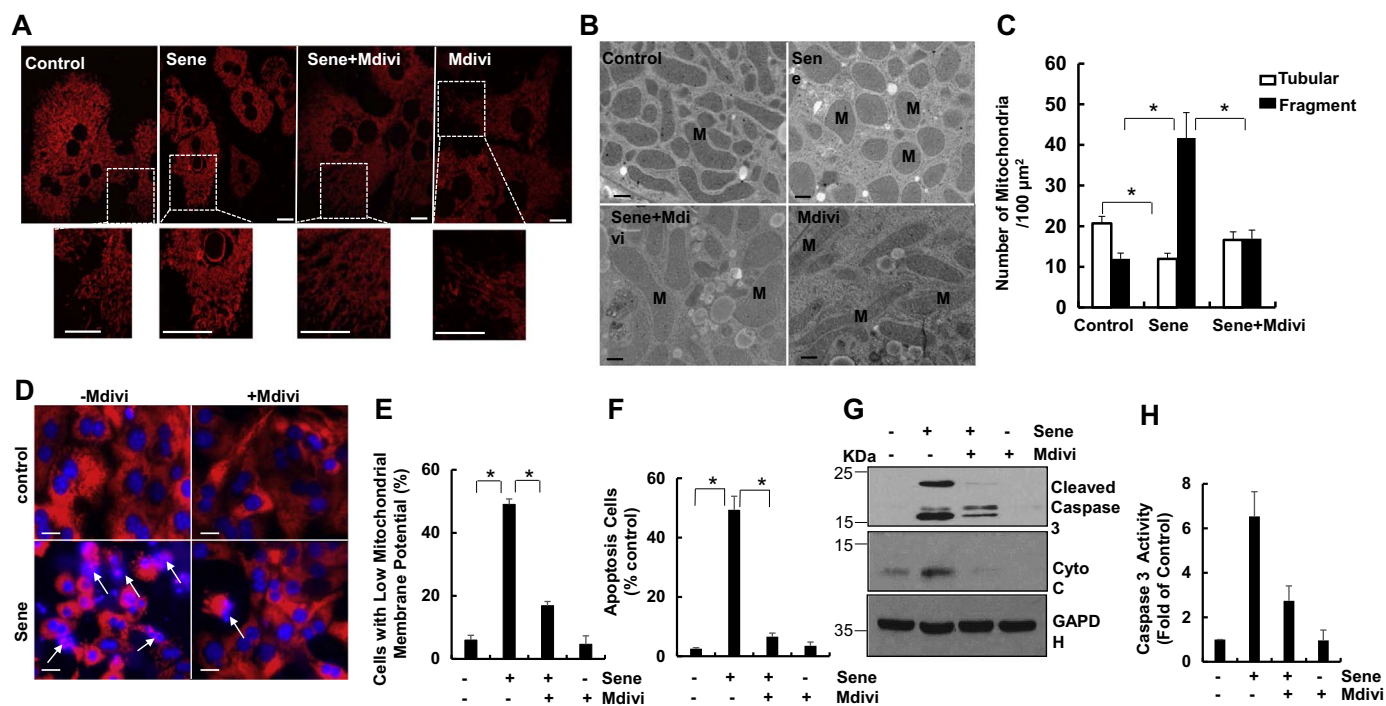
Fig. 5. Mdivi inhibits Sene-induced mitochondrial fragmentation, mitochondrial depolarization and apoptosis in primary mouse hepatocytes. Primary hepatocytes were treated with Sene (20 μM) in the presence or absence of Mdivi (50 μM) for 24 h followed by immunostaining with an anti-Tom20 and confocal microscopy. Representative photographs Tom20 staining are shown (A). Lower panels are enlarged photographs from the boxed areas. Scale bar: 20 μm. Cells were treated as in (A) and further processed for EM studies. Representative EM photographs of hepatocytes are shown (B). M: mitochondria. Bar: 500 nm. Quantification of EM images for tubular and fragmented mitochondria (C) and data (means ± SE) are from at least 10 cell sections per group. * p < 0.05. Student *t*-test. Primary hepatocytes were first loaded with TMRM (50 nM) for 15 min and treated with Sene (20 μM) in the presence or absence of Mdivi (50 μM) for 24 h. Cells were further stained with Hoechst 33342 (1 μg/ml) for 5 min followed by fluorescence microscopy. Representative TMRM and Hoechst 33342 stained images are shown (D). Arrows denote cells with loss of MMP and apoptotic nuclei. Scale bar: 20 μm. Quantification of the number of cells with low of MMP (E) and apoptosis (F) after cells were treated with Sene in the presence or absence of Mdivi. Data are means ± SE from 3 independent experiments. More than 300 cells were counted in each experiment from 3 to 4 different fields. * p < 0.05. One way ANOVA analysis with Scheffé's post hoc test. Cytosolic fractions were subjected to western blot analysis for cleaved caspase-3 (G) or caspase-3 activity assay (H). Data were normalized to the control and are presented as means ± SE (n=3). * p < 0.05. One way ANOVA analysis with Scheffé's post hoc test.

treatment may promote hepatic mitochondrial fission/fragmentation by changing the balance of mitochondrial fission and fusion proteins in mouse livers. Taken together, these data suggest that Sene induces hepatic mitochondrial damage and apoptosis as well as liver injury in mouse livers.

3.2. Sene induces mitochondrial damage, JNK activation and apoptosis in primary mouse hepatocytes

We found that Sene induced plasma membrane blebbing, shrunken cytoplasm and DNA condensation/fragmentation in primary cultured mouse hepatocytes (Fig. 3A), which are typical features of apoptosis. Sene induced apoptosis in a dose-and time-dependent manner (Fig. 3B & C). Sene also increased caspase-3 cleavage and activities in a dose-dependent manner (Fig. 3D & E). ZVAD-fmk, a general caspase inhibitor, significantly inhibited Sene-induced apoptosis (Supplemental Fig. 2). In addition, Sene increased the number of cells with low mitochondrial membrane potential (MMP) as early as 6 h and further increased at 24 h (Fig. 4A & B). Sene also increased levels of cytosolic Cyt c with a concomitant decrease of mitochondrial Cyt c in a dose-dependent manner (Fig. 4C), which is consistent with the results from Sene-treated mouse livers (Fig. 2A). Sene also increased the levels of phosphorylated JNK in a dose- and time-dependent manner (Fig. 4D & E). SP600125, a specific JNK inhibitor, significantly inhibited Sene-induced apoptosis although SP600125 did not affect the number of cells with low MMP induced by Sene (Fig. 4F & G). These data suggest that JNK may promote Sene-induced apoptosis downstream of mitochondrial depolarization. Together, these results indicate that Sene induces mitochondrial damage and JNK activation to trigger caspase-dependent apoptosis in hepatocytes.

3.3. Sene induces mitochondrial fragmentation and apoptosis which is rescued by a Drp1 inhibitor Mdivi

We found that most mitochondria were filamentous (elongated) in control hepatocytes whereas mitochondria were shorter and round shape in Sene-treated hepatocytes as evaluated by the fluorescence microscopy using an anti-Tom20 antibody (Fig. 5A). Mdivi, a specific Drp1 inhibitor that blocks Drp1 GTPase activity [17,25,26], markedly inhibited Sene-induced mitochondrial fragmentation (Fig. 5A). Consistent with the fluorescence microscopy results, EM studies revealed that Sene induced mitochondrial fragmentation, which also suppressed by Mdivi (Fig. 5B). Interestingly, Mdivi also inhibited Sene-induced mitochondrial depolarization, release of mitochondrial Cyt c, caspase-3 activation and apoptosis (Fig. 5C-G & Supplemental Fig. 3). Importantly, Sene also induced apoptosis and caspase-3 activation in primary cultured human hepatocytes, which also inhibited by Mdivi (Supplemental Fig. 4). These data suggest that Sene-induced mito-chondria-mediated hepatocyte apoptosis is conserved from mouse to human. Together, our data implicate that Sene induced Drp1-mediated mitochondrial fragmentation that promotes activation of intrinsic mitochondrial pathway and apoptosis.

3.4. Sene increases specific mitophagy but not general autophagy in hepatocytes

Damaged mitochondria in hepatocytes can be removed by mito-phagy, which serves as a protective mechanism against drug- or alcohol-induced liver injury [27,28]. We next infected hepatocytes with adenovirus GFP-LC3 and determined autophagic flux in Sene-treated hepatocytes. As can be seen in Fig. 6A and Supplemental Fig. 5A,
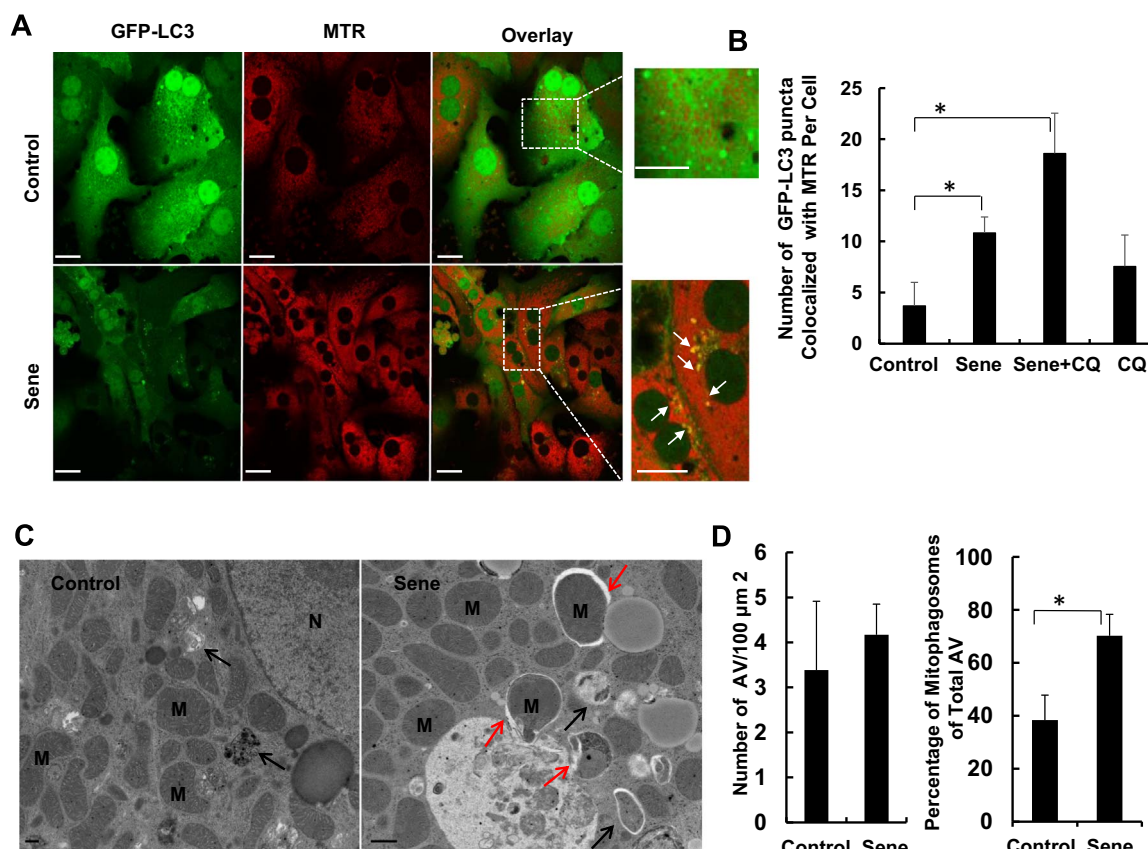
Fig. 6. Sene induces selective mitophagy in primary mouse hepatocytes. Primary mouse hepatocytes were first infected with adenovirus-GFP-LC3 (5 moi) for 24 h and the cells were then treated with Sene (20 μM) in the presence or absence of CQ (20 μM) for 6 h. Cell wered fixed in 4% paraformaldehyde in PBS followed by confocal microscopy. Representative fluorescence images of hepatocytes are shown (**A**). Right panels are enlarged photographs from boxed areas. Arrows denote the colocalization of GFP-LC3 puncta with MTR stained mitochondria. Bar: 20 μm. Quantification of numbers of GFP-LC3 puncta that are colocalized with MTR per cell (**B**). Data are means ± SE from 3 independent experiments, and more than 20 cells were counted in each experiment. * p < 0.05. One way ANOVA analysis with Scheffé's post hoc test. Hepatocytes were treated with Sene (20 μM) for 6 h and fixed in 2.5% glutaraldehyde in Hepes Buffer followed by EM. Representative EM images of hepatocytes are shown (**C**). N: nucleus; M: mitochondria. Red arrows denote mitophagosomes (autophagosomes that envelop mitochondria); black arrows denote regular autophagosomes (AV). Bar: 500 nm. Quantification of EM images (**D**) and data (means ± SD) are from at least 10 cell sections quantified per group. * p < 0.05. Student *t*-test.

control hepatocytes displayed a diffuse GFP-LC3 pattern with few GFP-LC3 puncta, which might reflect the basal autophagy in cultured hepatocytes. While CQ increased the number of GFP-LC3 puncta by blocking the degradation of GFP-LC3 at autolysosomes, the combination of CQ with Sene did not further increased number of GFP-LC3 puncta (Supplemental Fig. 5A & B). Similarly, western blot analysis revealed that CQ increased levels of LC3-II and p62, which were not further increased by the combination of CQ with Sene (Supplemental Fig. 5C). These data suggest that Sene did not increase autophagic flux in hepatocytes. Interestingly, when hepatocyte mitochondria were stained with MTR, we found that the colocalization of MTR stained mitochondria and GFP-LC3 puncta significantly increased in Sene-treated hepatocytes which was further enhanced by CQ (Fig. 6A & B). EM studies also revealed that while Sene did not alter the number of autophagosomes (AV), Sene increased the number of AV that enveloped with mitochondria (Fig. 6C & D). These data clearly suggest that Sene may increase selective autophagy for mitochondria (mitophagy) without affecting the general autophagy. Indeed, we further found that blocking autophagy by CQ exacerbated Sene-induced apoptosis (Supplemental Fig. 5D), supporting a protective role of mitophagy against Sene-induced apoptosis.

3.5. Liver-specific Drp1 knockout mice protect against Sene-induced apoptosis and liver injury

To further test the role of Drp1 in Sene-induced apoptosis and liver injury *in vivo*, liver-specific Drp1 KO mice (Drp1 f/f, Alb Cre+) and

their matched WT (Drp1 f/f, Alb Cre-) mice were treated with Sene for 24 h. Similar to what we found in C57BL/6 J WT mice, Sene treatment increased serum ALT levels, liver zone 2 area hemorrhage and apoptosis as well as TUNEL positive hepatocytes in Drp1 WT mice (Fig. 7**A-D**). All these changes markedly blunted in liver-specific Drp1 KO mice (Fig. 7**A-D**). In addition, Sene also induced casepase-3 activation and JNK phosphorylation in Drp1 WT mice although the levels of phosphorylated JNK varied among these mice. Notably, all of these changes markedly decreased in liver-specific Drp1 KO mice (Fig. 7**E & F**). These data indicate that Drp1 plays a critical role in promoting Sene-induced apoptosis and liver injury.

4. Discussion

In the present study, we found that Sene, one of the most hepatotoxic common PAs that exists in *Senecio vulgaris* and *Gynura segetum* with high contents [29,30], induced apoptosis in primary cultured mouse and human hepatocytes and in mouse livers. Mechanistically, we found that Sene induced mitochondrial depolarization and fragmentation, release of mitochondrial Cyto c, caspase-3 activation and subsequent apoptosis. We further found that Sene-induced mitochondrial fragmentation and damage, which was associated with mitochondrial Drp1 translocation. Pharmacological inhibition or genetic deletion of Drp1 in hepatocytes suppressed Sene-induced mitochondrial damage and hepatotoxicity.

In the present study, mice were treated with 50 mg/kg and 100 mg/kg of Sene, respectively. The doses that we chose were very comparable
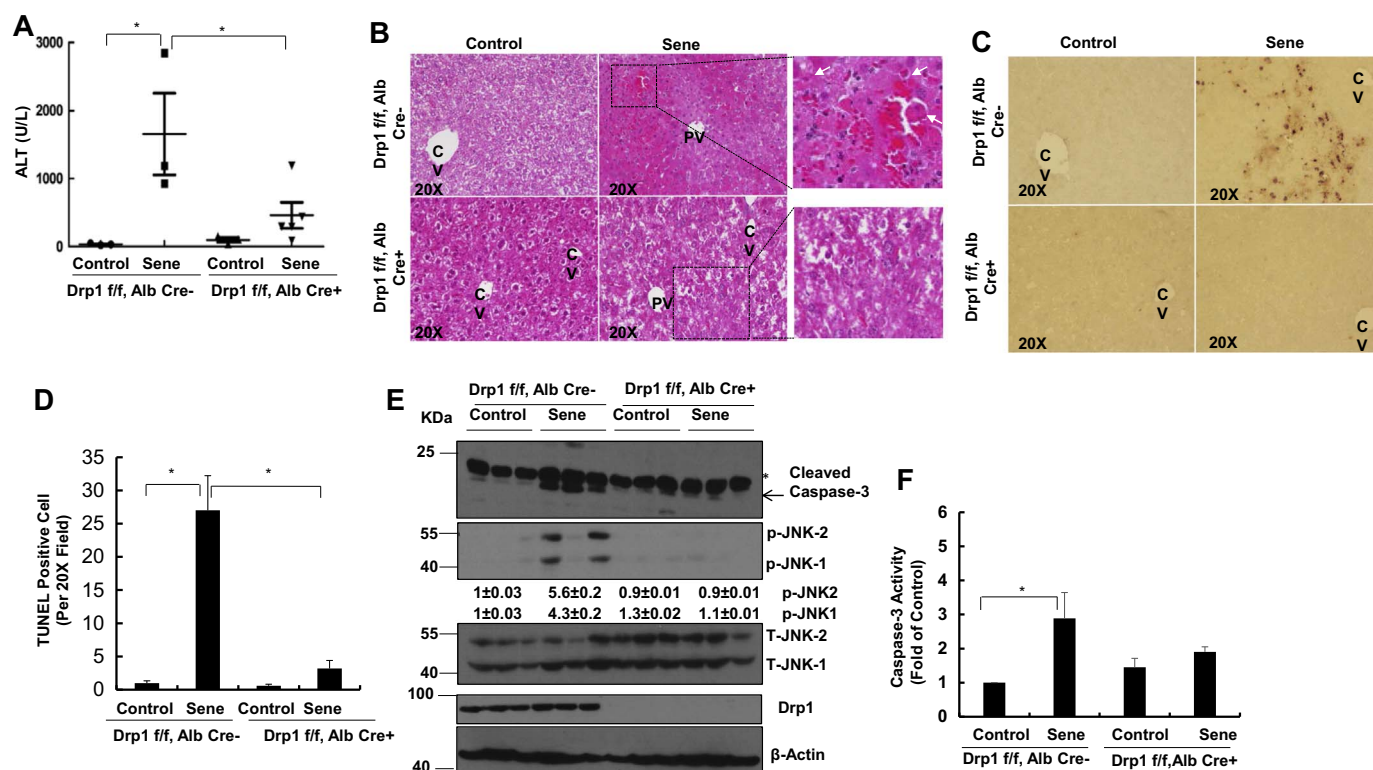
Fig. 7. Liver-specific Drp1 KO mice are resistant to Sene-induced apoptosis and liver injury. Liver-specific Drp1 KO mice (Drp1 f/f, Alb Cre+) and matched WT mice (Drp1 f/f, Alb Cre-) were treated with Sene (50 mg/kg) or saline control by gavage and mice were sacrificed at 24 h after treatment. Serum ALT levels (**A**) were analyzed and data are presented as means ± SE (n=4–6). * p < 0.05, one-way ANOVA. Representative images of liver tissue H & E (**B**) and TUNEL staining (**C**) are shown. CV: Central vein; PV: Portal vein. Right panels are enlarged photographs from boxed areas. Arrows denote apoptotic nuclei. Quantification of TUNEL positive nuclei (**D**) and data are means ± SE (n=3). More than 5 different 20X fields were counted from 3 mice in each group. * p < 0.05, one-way ANOVA analysis with Scheffé's post hoc test. Total liver lysates were subjected to western blot analysis (**E**) and caspase-3 activity assay (**F**). *: non-specific band. Data are presented as means ± SE (n=3–6). * p < 0.05. One way ANOVA analysis with Scheffé's post hoc test.

with the published papers in PA-induced liver injury. For example, DeLeve *et al.* successfully established an HSOS model on rats by single oral gavage a dose of 160 mg/kg of monocrotaline (one type of PAs) [31]. In a more recent study, a dose of 90 mg/kg monocrotaline by gavage was used to induce HSOS in rats [32]. Moreover, it should also be noted that mice/rats have different metabolic rates and tolerance on xenobiotics. The purpose of this study was to establish a mouse model of Sene-induced hepatotoxicity and determine the molecular mechanisms by which Sene induced liver injury in mice, and correlated these mechanisms with humans. The findings that Sene also induced mitochondrial damage and apoptosis in human hepatocytes may support a strong relevance of our studies to Sene-induced liver injury in humans.

The majority of Drp1 resides as soluble dimers and tetramers in the cytosol, which are recruited to mitochondrial fission sites by several outer mitochondrial membrane receptor proteins, including fission 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamic protein of 49 and 51 kDa (MiD49/51) [14]. Once on mitochondria, Drp1 polymerizes into ring-like structures around the endoplasmic reticulum-mitochondria contact sites, where it constricts and segregates mitochondria to induce mitochondrial fission [14,15]. Drp1 self-assembly promotes Bax/Bak-mediated MOMP and Cyt c release, which inhibited by Mdivi [17]. We found that Sene induced mitochondrial depolarization, fragmentation, Cyt c release, caspase-3 activation and apoptosis in primary cultured hepatocytes, all these events suppressed by Mdivi. More importantly, Sene also induced mitochondrial Cyt c release, caspase-3 activation and apoptosis as well as liver injury in mouse livers, which were also suppressed in liver-specific Drp1 KO mice. These results from both pharmacological inhibition and genetic deletion of Drp1 suggest that Drp1-mediated mitochondrial fission plays a critical role in Sene-induced activation of mitochondrial

intrinsic pathway and apoptosis that contribute to liver injury. Whether Mff and Mid49/51 would play a role in regulating Sene-induced mitochondrial fragmentation and apoptosis remains to be determined. In addition, posttranslational modification of Drp1 including phosphorylation, ubiquitination, SUMOylation and S-nitrosylation is also important in regulating Drp1 mitochondrial translocation [15]. For instance, CdK1/cyclin B and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylate Drp1 and promote Drp1 mitochondrial translocation and mitochondrial fission during cell mitosis and chronic β-adrenergic receptor activation in heart, respectively [33,34]. Whether Sene induced Drp1 posttranslational modifications to trigger its mitochondrial translocation in hepatocytes remains to be determined. Notably, in addition to inducing Drp1 mitochondrial translocation, Sene also decreased hepatic levels of Mfn1 and Mfn2, two essential mitochondrial outer membrane fusion proteins. These results suggest that Sene could impair both mitochondrial fusion and fission machinery proteins, which seems to ensure mitochondrial fission/fragmentation after exposure to Sene. However, Sene-induced apoptosis and liver injury markedly suppressed by Mdivi and in liver-specific Drp1 KO mice, respectively. These observations suggest that Drp1 may play more important roles in regulating intrinsic mitochondrial apoptotic pathway activation and liver injury than that of Mfn1 and Mfn2. Future investigations to use liver-specific Mfn1 and Mfn2 KO mice would help to clarify the role of mitochondrial fusion in Sene-induced mitochondrial damage and hepatocyte apoptosis.

Increasing evidence indicates that autophagy can help to remove damaged mitochondria to protect against various liver injury including alcohol, drug, sepsis, inflammation, viral infection and hepatic ischemia and reperfusion [21,35]. Indeed, the number of GFP-LC3 positive autophagosomes that colocalized with MTR stained mitochondria by confocal microscopy and the number of double membrane autophago-
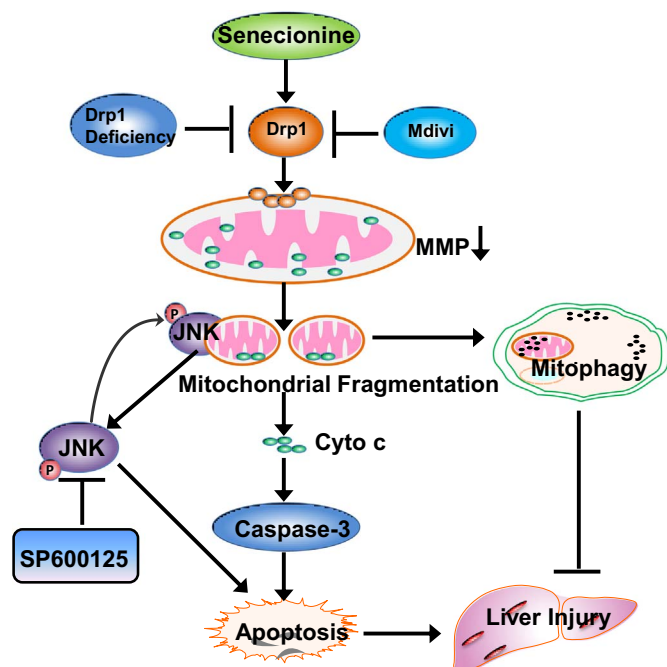
Fig. 8. Proposed model for Drp1-mediated mitochondrial fragmentation and mitochondrial damage in Sene-induced apoptosis and liver injury. In hepatocytes, Sene induces Drp1 mitochondrial translocation to trigger mitochondrial fragmentation, loss of MMP, increased mitochondrial Cyt c release and apoptosis. Sene also induces mitochondrial JNK translocation and phosphorylation. Pharmacological inhibition of JNK inhibits Sene-induced apoptosis downstream of Sene-induced mitochondrial damage. Damaged mitochondria may be removed by mitophagy as an adaptive response to protect against Sene-induced apoptosis. Pharmacological inhibition or genetic deletion of Drp1 inhibits Sene-induced mitochondrial damage, apoptosis and liver injury.

somes that enveloped with mitochondria by EM studies, all increased in Sene-treated hepatocytes. Intriguingly, Sene did not increase general autophagic flux in primary hepatocytes. These results suggest that Sene selectively increased mitophagy without affecting general non-selective autophagy in hepatocytes. CQ, the lysosomal inhibitor that blocks autophagic degradation, exacerbated Sene-induced apoptosis, further supporting a protective role of mitophagy in Sene-induced hepatotoxicity. Selective mitophagy is mediated by several mitophagy receptor proteins including p62/SQSTM1, NBR1, NDP52, Optineurin and NIX [11,36]. Whether Sene-induced selective mitophagy involves one or more of these receptor proteins remain to be determined.

Liver is known to have a remarkable metabolic zonation, which generally is divided into three zones: periportal (zone 1), intermediate (zone 2) and perivenous/ or centrilobular zone (zone 3). These unique liver zonations form functional gradients for oxygen concentrations, hormonal factors as well as activities of metabolic enzymes including CYPs [37]. We found that Sene-induced apoptosis and hemorrhage mainly occurred in the liver zone 2 and adjacent areas to centrilobular area (zone 3). Since metabolic activation of PAs by CYPs is critical for PA-induced hepatotoxicity, it is likely some of the CYPs that are more potent to metabolize Sene may be enriched in zone 2 and adjacent to zone 3 that may account for the unique zonated damage induced by Sene. Future experiments to identify these metabolic enzymes for Sene in these specific zone areas will be very interesting.

Another interesting finding in the present study was that Sene activated JNK in hepatocytes and in mouse livers. It has been shown that JNK activation contributes to both hepatocyte apoptosis and necrosis in various liver injury models [38]. We also found that pharmacological inhibition of JNK attenuated Sene-induced apoptosis in hepatocytes. Interestingly, Sene-induced JNK activation markedly suppressed in liver-specific Drp1 KO mouse livers, suggesting that Drp1 may promote Sene-induced JNK activation. One of the mechanisms to activate JNK is through the production of reactive oxygen

species (ROS) in mitochondria [38]. It is well known that Drp1 activates MOMP via Bax/Bak and MOMP promotes mitochondrial ROS production [17,39]. While it remains to be studied, we speculate that the levels of mitochondrial ROS may be decreased in Drp1 KO hepatocytes compared with wild type hepatocytes after Sene treatment, which may partially contribute to Sene-induced JNK activation.

In conclusion, we demonstrated that Sene-induced apoptosis in zone 2 hepatocytes in mouse livers, which is associated with mitochondrial Drp1 translocation, mitochondrial fragmentation and damage. The possible cellular events and signaling pathways that contribute to Sene-induced apoptosis and liver injury are summarized in Fig. 8. Pharmacological inhibition of Drp1 may be a novel therapeutic approach for treating Sene-induced liver injury.

Conflict of interest

Nothing to claim.

Funding

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.02.020.

References

[1] M.G. Neuman, et al., Hepatotoxicity of Pyrrolizidine Alkaloids, J. Pharm. Pharm. Sci. 18 (4) (2015) 825–843.

[2] M. Chojkier, Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids, J. Hepatol. 39 (3) (2003) 437–446.

[3] J.Y. Wang, H. Gao, Tusanqi and hepatic sinusoidal obstruction syndrome, J. Dig. Dis. 15 (3) (2014) 105–107.

[4] B.L. Stegelmeier, et al., Pyrrolizidine alkaloid plants, metabolism and toxicity, J. Nat. Toxins 8 (1) (1999) 95–116.

[5] G. Lin, et al., Hepatic sinusoidal obstruction syndrome associated with consumption of Gynura segetum, J. Hepatol. 54 (4) (2011) 666–673.

[6] L.D. DeLeve, et al., Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes: the role of glutathione and relevance to hepatic venoocclusive disease, Hepatology 23 (3) (1996) 589–599.

[7] L. Ji, et al., Involvement of Bcl-xL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes, Toxicol. Appl Pharm. 231 (3) (2008) 393–400.

[8] P.P. Fu, et al., Pyrrolizidine alkaloids–genotoxicity, metabolism enzymes, metabolic activation, and mechanisms, Drug Metab. Rev. 36 (1) (2004) 1–55.

[9] B.L. Copple, et al., Modes of cell death in rat liver after monocrotaline exposure, Toxicol. Sci. 77 (1) (2004) 172–182.

[10] L. Ji, et al., Protective mechanisms of N-acetyl-cysteine against pyrrolizidine alkaloid clivorine-induced hepatotoxicity, J. Cell Biochem 108 (2) (2009) 424–432.

[11] H.M. Ni, J.A. Williams, W.X. Ding, Mitochondrial dynamics and mitochondrial quality control, Redox Biol. 4 (2015) 6–13.

[12] L. Pernas, L. Scorrano, Mito-morphosis: mitochondrial fusion, fission, and cristae remodeling as key mediators of cellular function, Annu. Rev. Physiol. 78 (2016) 505–531.

[13] R.J. Youle, A.M. van der Bliek, Mitochondrial fission, fusion, and stress, Science 337 (6098) (2012) 1062–1065.

[14] P. Mishra, D.C. Chan, Mitochondrial dynamics and inheritance during cell division, development and disease, Nat. Rev. Mol. Cell Biol. 15 (10) (2014) 634–646.

[15] P. Mishra, D.C. Chan, Metabolic regulation of mitochondrial dynamics, J. Cell Biol. 212 (4) (2016) 379–387.

[16] S. Frank, et al., The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis, Dev. Cell 1 (4) (2001) 515–525.

[17] A. Cassidy-Stone, et al., Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization, Dev. Cell 14 (2) (2008) 193–204.

[18] K. Naylor, et al., Mdv1 interacts with assembled dnm1 to promote mitochondrial

division, J. Biol. Chem. 281 (4) (2006) 2177–2183.

[19] Y.Q. He, et al., Identification of the UDP-glucuronosyltransferase isozyme involved in senecionine glucuronidation in human liver microsomes, Drug Metab. Dispos. 38 (4) (2010) 626–634.

[20] Y. Kageyama, et al., Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain, EMBO J. 33 (23) (2014) 2798–2813.

[21] H.M. Ni, et al., Activation of autophagy protects against acetaminophen-induced hepatotoxicity, Hepatology 55 (1) (2012) 222–232.

[22] H.M. Ni, et al., Caspase inhibition prevents tumor necrosis factor-alpha-induced apoptosis and promotes necrotic cell death in mouse hepatocytes in vivo and in vitro, Am. J. Pathol. 186 (10) (2016) 2623–2636.

[23] H.M. Ni, et al., Critical role of FoxO3a in alcohol-induced autophagy and hepatotoxicity, Am. J. Pathol. 183 (6) (2013) 1815–1825.

[24] D.N. Dhanasekaran, E.P. Reddy, JNK signaling in apoptosis, Oncogene 27 (48) (2008) 6245–6251.

[25] S.W. Park, et al., A selective inhibitor of drp1, mdivi-1, increases retinal ganglion cell survival in acute ischemic mouse retina, Invest. Ophthalmol. Vis. Sci. 52 (5) (2011) 2837–2843.

[26] N. Zhang, et al., A selective inhibitor of Drp1, mdivi-1, acts against cerebral ischemia/reperfusion injury via an anti-apoptotic pathway in rats, Neurosci. Lett. 535 (2013) 104–109.

[27] J.A. Williams, et al., Parkin regulates mitophagy and mitochondrial function to protect against alcohol-induced liver injury and steatosis in mice, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (5) (2015) G324–G340.

[28] J.A. Williams, et al., Chronic Deletion and Acute Knockdown of Parkin Have Differential Responses to Acetaminophen-induced Mitophagy and Liver Injury in Mice, J. Biol. Chem. 290 (17) (2015) 10934–10946.

[29] A. Xiong, et al., Metabolomic and genomic evidence for compromised bile acid homeostasis by senecionine, a hepatotoxic pyrrolizidine alkaloid, Chem. Res. Toxicol. 27 (5) (2014) 775–786.

[30] L. Fang, et al., Mass-spectrometry-directed analysis and purification of pyrrolizidine alkaloid cis/trans isomers in Gynura japonica, J. Sep. Sci. 37 (15) (2014) 2032–2038.

[31] L.D. DeLeve, et al., Characterization of a reproducible rat model of hepatic veno-occlusive disease, Hepatology 29 (6) (1999) 1779–1791.

[32] K. Nakamura, et al., Sorafenib attenuates monocrotaline-induced sinusoidal obstruction syndrome in rats through suppression of JNK and MMP-9, J. Hepatol. 57 (5) (2012) 1037–1043.

[33] N. Taguchi, et al., Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission, J. Biol. Chem. 282 (15) (2007) 11521–11529.

[34] S. Xu, et al., CaMKII induces permeability transition through Drp1 phosphorylation during chronic beta-AR stimulation, Nat. Commun. 7 (2016) 13189.

[35] M.J. Czaja, et al., Functions of autophagy in normal and diseased liver, Autophagy 9 (8) (2013) 1131–1158.

[36] W.X. Ding, X.M. Yin, Mitophagy: mechanisms, pathophysiological roles, and analysis, Biol. Chem. 393 (7) (2012) 547–564.

[37] T. Oinonen, et al., Zonation of cytochrome P450 enzyme expression in rat liver. Isozyme-specific regulation by pituitary dependent hormones, Biochem. Pharm. 51 (10) (1996) 1379–1387.

[38] E. Seki, D.A. Brenner, M. Karin, A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches, Gastroenterology 143 (2) (2012) 307–320.

[39] D.B. Zorov, M. Juhaszova, S.J. Sollott, Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release, Physiol. Rev. 94 (3) (2014) 909–950.