AUTOPHAGY AND RELATED TRANSCRIPTION FACTORS IN LIVER AND GUT DISEASES

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AUTOPHAGY AND RELATED TRANSCRIPTION FACTORS IN LIVER AND GUT DISEASES

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Editorial: Autophagy and Related Transcription Factors in Liver and Gut Diseases

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Editorial on the Research Topic

Autophagy and Related Transcription Factors in Liver and Gut Diseases

The cell biologist Yoshinori Ohsumi received the 2016 Nobel Prize in Medicine for his early identification and characterization of the autophagy machinery, in particular, AuTophaGy-related (Atg) genes, in yeast. Macroautophagy (hereafter, autophagy) is a cytoprotective pathway for sequestration of cellular components (such as misfolded proteins, damaged organelles, and excessive lipids) into autophagosomal vesicles, followed by clearance *via* the lysosomal system (Galluzzi et al., 2017). Autophagy is specifically upregulated upon exposure to various stressors such as oxidative and endoplasmic reticulum stress, thus aiding in the prevention of various pathologies. Therefore, autophagy dysregulation may be involved in inflammatory, metabolic, toxic, and infectious diseases and cancer (Kroemer et al., 2010; Eid et al., 2013; Horibe et al., 2017). Most organelles also seem to have selective programs of autophagy, including mitochondria, lipid droplets, endoplasmic reticulum, and even lysosomes. Selective autophagic removal of damaged mitochondria, or mitophagy, is an anti-apoptotic mechanism induced and specifically upregulated in response to various damaging agents such as binge ethanol exposure or drug-induced liver injury in animal models (Otsuki et al., 1994; Youle and Narendra, 2011; Lemasters, 2014; Eid et al., 2016; Eid et al., 2019). Autophagy can be regulated not only at the gene level, but its final performance can be modulated by lysosomal lipid composition. For instance, accumulation of lipids (e.g., cholesterol) in lysosomes has been shown to impair the fusion of autophagosomes (containing disrupted mitochondria) with lysosomes, contributing to the perpetuation of damaged mitochondria, which sensitizes to acetaminophen hepatotoxicity (Baulies et al., 2015). On the other hand, autophagic clearance of lipid droplets is referred to as lipophagy (Singh and Cuervo, 2012). Various transcription factors such as transcription factor EB (TFEB), Nrf2, HIF, and Foxo3a play important roles in the regulation of autophagy and mitophagy-related proteins such as LC3, cathepsins, and Parkin (Sardiello, 2016; Horibe et al., 2017; Eid et al., 2019). The focus of this Research Topic is to

highlight the involvement of these transcription factors in the regulation of liver and gut diseases through autophagy pathway as these are potential therapeutic targets for the restoration of autophagy and in the management of these diseases.

This Research Topic compiles nine articles, including four reviews and five original research contributions. The interesting review by Su et al., on *Mitophagy in Hepatic Insulin Resistance: Therapeutic Potential and Concerns*, focuses on advances in the understanding of relationship between mitophagy and hepatic insulin resistance and the potential value of mitophagy in the treatment of hepatic insulin resistance and metabolic syndrome (*via* clearance of damaged mitochondria and subsequent reduction of lipid accumulation). This observation is supported by an elegant study demonstrating that loss of Parkin-mediated mitophagy promoted further β-cell failure under pathological stress conditions including STZ exposure and leptin receptor defects (Hoshino et al., 2014).

Recent advances with incretin-associated drugs have opened new avenues in the management of diabetes. In another interesting review article, Kanasaki et al. analyzes distinct molecular mechanisms of autophagy regulation by glucagon, GLP-1, and DPP-4 inhibitor. In addition, they also discuss the potential contribution of these regulatory pathways in the induction of beneficial autophagy-upon bariatric surgery, which have implications in the treatment of diabetic diseases (Adeghate et al., 2019).

Lipophagy, a process controlled by the autophagy master regulator, TFEB, is key to maintaining a healthy liver. The third review by Yang et al. discusses the different lipophagic responses in rodent hepatocytes after exposure to acute and chronic ethanol. They showed that these responses are controlled by subcellular TFEB localization. They suggest that natural products and drugs such as caffeine/coffee, resveratrol, corosolic acid, zinc, carbamazepine, and rapamycin may activate autophagy/lipophagy for preventing or even aiding in the treatment of alcohol-induced fatty liver. In addition, they stress that the specific upregulation of TFEB by certain small molecules (related to digoxin, ikarugamycin, and alexidine dihydrochloride) may be of therapeutic value in the treatment of human fatty liver disease (Wang et al., 2017).

In another review article, Zhang L. et al. elegantly summarize the current understanding on the use of herbal medicine extracts and natural products for activation of hepatic autophagy, thus helping in the prevention and treatment of non-alcohol fatty liver diseases (NAFLD). A specific focus is set on mechanisms by which autophagy can target the main events in the pathogenesis of NAFLD, including hepatic steatosis, inflammation, oxidative stress, and apoptosis.

The research article by Fan et al. provides novel data supporting a protective role for methylprednisolone (MP) in an experimental autoimmune hepatitis (AIH) model, possibly mediated by the Akt/mTOR signaling pathway. MP seems to ameliorate apoptosis and promote autophagy in hepatocytes in *in vitro* and *in vivo* mouse model. They suggest a potential use of MP to treat AIH. Their study provides interesting insights into the mechanisms underlying the effect of MP on hepatocytes.

The interesting study by Guo et al. explores the effects of 6-bromo-indirubin-3′-oxime (6BIO), a potent inhibitor of glycogen synthase kinase-3 (GSK-3), on the aging rodent liver. They found that 6BIO mitigates oxidative stress, improves lipid metabolism, enhances autophagy, and significantly retards liver aging *via* modulating the GSK-3β and mTOR pathways. They suggest that 6BIO could be a potential agent to protect the liver in the field of anti-aging pharmacology.

Hepatitis C virus (HCV) dysregulates lipid metabolism to accomplish several steps of its life cycle (Paul et al., 2014; Strating and van Kuppeveld, 2017). Vescovo et al. investigates the impact of mevastatin (a cholesterol-lowering agent isolated from *Penicillium citinium*) on HCV replication and autophagy in MMHD3 non-transformed hepatocytes harboring sub-genomic HCV replicons, specifically in relation to the extracellular lipid uptake. In contrast to the previous studies in transformed human cell lines, they observed drastic upregulation of intracellular cholesterol in MMHD3 cells upon mevastatin treatment, which is associated with enhanced lipophagy and HCV replication. However, these effects are reversed when cells are cultured in delipidated serum, which establishes the fact that suppression of extracellular lipid uptake is as important as inhibiting cholesterol biosynthesis in suppressing HCV replication. This study may have implications in the development of treatment modalities targeting cholesterol levels to limit HCV replication.

Fan et al., in their original research article, report on isoorientin-mediated suppression of APAP-induced hepatotoxicity in mice *via* activation Nrf2 anti-oxidative pathway and the involvement of AMPK/Akt/GSK3β signaling. This hepatoprotective effect of isoorientin could be mediated by autophagy activation, as reported by others (Muhammad et al., 2018; Lv et al., 2019). The last article by Zhang et al. conclude that salvianolic acid B inhibits activation of human primary hepatic stellate cells through downregulation of MEF2 (myocyte enhancer factor 2) signaling pathway, resulting in subsequent amelioration of stellate cell-mediated hepatic fibrosis. However, we cannot rule out the possible involvement of autophagy in the hepatoprotective effect of salvianolic acid B as it has been reported that autophagy may be required for stellate cell activation and hepatic fibrosis in alcohol liver disease (Eid et al., 2013).

The field of autophagy research is growing at a rapid pace and the discoveries revealing novel roles for the autophagy pathway in diverse pathologies are making it a very attractive target for pharmacological intervention. Strategies are being envisaged for therapeutic upregulation and/or suppression of autophagy and/or specialized processes like lipophagy or mitophagy. The collection of articles in this Research Topic, including original research and reviews, are aimed at summarizing some of these ideas within the specialized field of gastrointestinal/hepatic pharmacology and beyond.

AUTHOR CONTRIBUTIONS

All the authors contributed to this editorial work.

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ORIGINAL RESEARCH
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Isoorientin Ameliorates APAP-Induced Hepatotoxicity via Activation Nrf2 Antioxidative Pathway: The Involvement of AMPK/Akt/GSK3β

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Oxidative stress has been highlighted as therapeutic targets for acetaminophen (APAP)-induced hepatotoxicity. Isoorientin (Iso), a well-known flavonoid-like compound, has been shown to have antioxidant potential. However, the effect of Iso on APAP-induced liver injury has not yet been elucidated. The present study investigated the hepatoprotective effect of Iso and its underlying mechanism. C57BL/6J mice were used to evaluate the hepatoprotective effect of Iso *in vivo* and HepG2 cells were utilized to further decipher the mechanisms of Iso -induced Nrf2 activation. We found that Iso treatment significantly reduced APAP-induced hepatotoxicity by reducing the lethality, histopathological liver changes, and alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in serum. These effects were accompanied by decreased malondialdehyde (MDA) formation and myeloperoxidase level (MPO), and by decreased superoxide dismutase (SOD) and glutathione (GSH) depletion. Moreover, Iso induced Nrf2 activation and translocation as well as upstream AMPK/Akt/GSK3β activation. Furthermore, Iso effectively alleviated mitochondrial dysfunction by reducing c-jun N-terminal kinase phosphorylation and translocation, Bax mitochondrial translocation, and apoptosis-inducing factor and cytochrome c release. Further mechanistic investigations revealed that the activation of Nrf2 by Iso via the AMPK/Akt/GSK3β pathway contributed to the hepatoprotective activity of Iso *in vitro*. In addition, the Iso-mediated inhibition of APAP-induced the lethality, histopathological changes and mitochondrial dysfunction observed in WT mice was nearly absent in Nrf2$^{-/-}$ mice. In summary, Iso ameliorated APAP-induced hepatotoxicity by activating Nrf2 via the AMPK/Akt/GSK3β pathway.

Keywords: acetaminophen APAP, hepatotoxicity, Isoorientin Iso, Nrf2, oxidative stress

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INTRODUCTION

Isoorientin (3′,4′,5,7-tetrahydroxy-6-C-glucopyranosyl flavone; Iso), a common C-glycosyl flavone in the human diet, and the chemical structure of Iso is shown in **Figure 1A**. It can be isolated from several plant species, such as *Phyllostachys pubescens, Patrinia spp.*, buckwheat and corn silks (Yuan et al., 2016). It has been shown that Iso not only exerts anti-inflammatory effects by inhibiting lipopolysaccharide-stimulated cyclooxygenase-2(COX-2) and cytokine production,

but also exhibits antioxidant potential (Anilkumar et al., 2017). Previously, we found that Iso treatment exhibits a significant hepatoprotective effect against tertiary-butyl hydroperoxide (t-BOOH)-induced oxidative damage in liver cells and further protects against carbon tetrachloride (CCl_4)-induced oxidative damage in rats (Orhan et al., 2003). Oxidative stress causes injury in cells by generating reactive oxygen species (ROS), and excessive ROS in the liver can cause extensive liver injury (Chan et al., 2006). Based on the significant pathophysiological role of antioxidation/detoxification system dysfunction in the development of liver diseases, antioxidant agents can be an effective strategy for fighting oxidative stress that causes liver damage and diseases (Lv et al., 2018). Therefore, the ability of Iso to act as an antioxidant with anti-inflammatory activity renders this compound a prime candidate to be a hepatoprotective agent.

In response to oxidative stress caused by ROS, cells have developed adaptive, dynamic processes to maintain cellular redox homeostasis and reduce oxidative damage through a series of antioxidant molecules and detoxifying enzymes (Dunaway et al., 2018). The major pathway that responds to reactive species and redox potential is the Nrf2/ARE pathway, which activates phase II detoxification enzymes at the transcriptional level (Ahmed et al., 2017). Nrf2-deficient animals are more susceptible to organ injury induced by toxic stimuli such as acetaminophen (APAP), benzo[a]pyrene, diesel exhaust and other oxidative stresses due to decreased antioxidant protection (Raghunath et al., 2018). APAP overdose is the leading cause of drug-induced acute liver failure in many developed countries (Budnitz et al., 2011). Given the public concern regarding APAP hepatotoxicity, great efforts have been made to understand the mechanisms of the toxic effects. Mitochondrial oxidative stress and mitochondrial dysfunction are considered to be the predominant cellular processes in APAP hepatotoxicity (Jaeschke et al., 2012). Accordingly, inhibition of oxidative stress and mitochondrial dysfunction may play an essential role in attenuating APAP-induced acute liver injury (AILI). Considering the importance of oxidative stress in APAP-induced hepatotoxicity, we speculate that Nrf2 activators might protect against this toxicity.

In recent few decades, several studies have demonstrated the ability of natural products to counteract oxidative stress and exhibit hepatoprotective activity by modulating the Nrf2/ARE pathway (Kumar et al., 2014; Iranshahy et al., 2018). In view of the importance of free radical stress as a major contributor to the toxicity of APAP, we speculated that Iso has the potential to protect the liver against APAP-induced toxicity. In the current study, we evaluated the effects of Iso on Nrf2 activation and translocation, explored the role of Nrf2 activation in the expression of major antioxidant enzymes, and identified a novel signaling pathway for the regulation of Nrf2 activation. The results of this study using Nrf2 knockout (KO) animal and cell models clearly demonstrate that the ability of Iso to protect hepatocytes from APAP toxicity is dependent on Nrf2 activation.

MATERIALS AND METHODS

Reagents and Chemical

Isoorientin, purity > 98%) was obtained from the Chengdu Pufei De Biotech Co., Ltd. 3-(4,5-Dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Ly294002 (Akt inhibitor) and DCFH-DA were purchased from the Sigma Chemical Co. (St. Louis, Mo, United States). FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences. (San Jose, CA, United States).

Antibodies directed against Nrf2 and HO-1 were purchased from Abcam (Cambridge, MA, United States). Anti-phospho-c-Jun NH2-terminal kinase (JNK) antibody and β-actin were obtained from SUNGENE BIOTECH (Tianjin, China). Antibodies against Cytochrome c, Bax, Bcl-2, AMPK, phosphor-AMPK, AKT, phosphor-AKT, GSK-3β and phosphor- GSK-3β were purchased from Cell Signaling (Boston, MA, United States). The horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG were purchased from proteintech (Boston, MA, United States). Additionally, ALT, AST, MDA, MPO, GSH, and SOD test kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and Ethics Statement

Wild-type and $Nrf2^{-/-}$ (knockout) C57BL/6 mice were purchased from Liaoning Changsheng Technology Industrial, Co., Ltd. (Certificate SCXK2010-0001; Liaoning, China) and The Jackson Laboratory (Bar Harbor, ME, United States), respectively. All animals were housed in Specific pathogen free-facility. All animal experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals*, which was published by the *United States National Institute of Health*. This study was reviewed and approved by the *Animal Welfare and Research Ethics Committee at Jilin University*.

Experimental Design and Animal Procedures

Mice were randomly separated into the following four groups (n = 10/group): control (saline); Iso (50 mg/kg); APAP only (900 mg/kg or 400 mg/kg); and APAP (900 mg/kg or 400 mg/kg) + Iso (50 mg/kg). The mice were administered Iso (50 mg/kg) i.p. two times for 12 h each time. Then, 1 h after the last dose of Iso, they were exposed to a lethal dose of APAP (900 mg/kg) to observe the mortality rate or treated with APAP (400 mg/kg) for 6 h to evaluate pathological change and biochemical analysis.

Isolation and Culture of Primary Mouse Hepatocytes

C57BL/6 mice were used in this study. This study was reviewed and approved by the Animal Welfare and Research Ethics Committee at Jilin University. Surgical procedures were conducted under isoflurane-induced anesthesia in an isolated system. Isolated mouse hepatocytes were obtained using a modified collagenase perfusion method (Arbo et al., 2016). Briefly, a cannula was inserted in the hepatic portal vein and

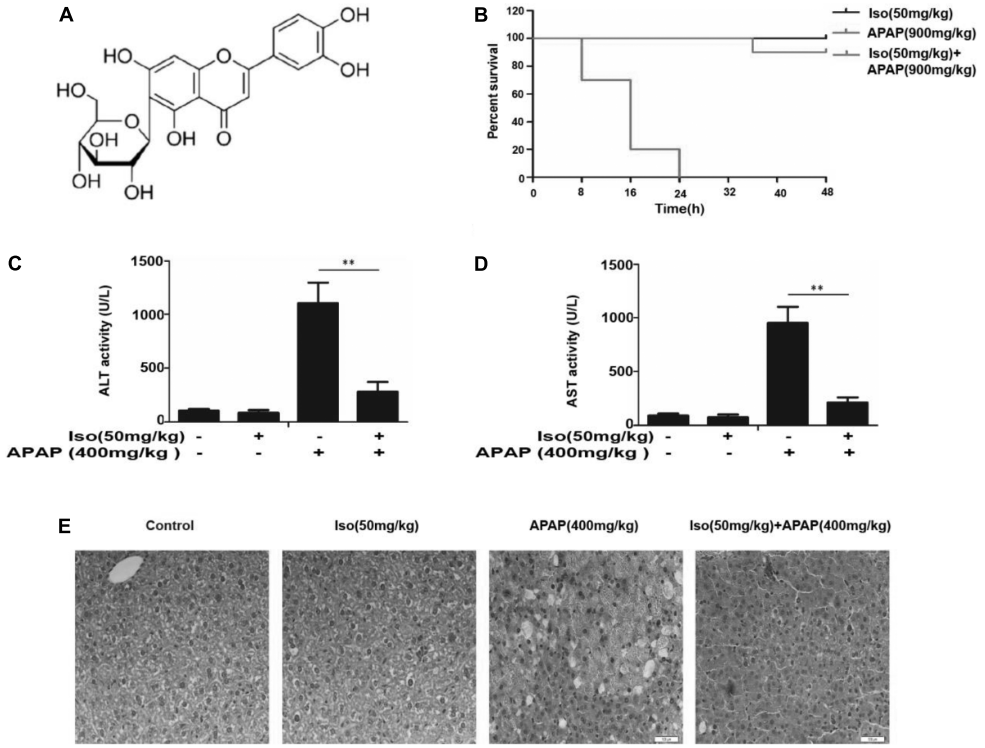

FIGURE 1 | Iso protected against APAP-induced fulminant hepatic failure. **(A)** Chemical structure of Iso. **(B)** Mice were treated with Iso (50 mg/kg i.p.) twice at 12 h intervals. The survival rate of mice was determined by treatment with APAP (900 mg/kg) at a lethal dose 1 h after the last dose of Iso (50 mg/kg). The survival rates of the mice were observed within 48 h after APAP treatment. The percentage of surviving mice is shown for each time point. $n = 10$ in each group. **(C,D)** One hour after the last dose of Iso, APAP (400 mg/kg) was administered for 6 h ($n = 5$/group). We harvested serum for analysis of ALT and AST levels. **(E)** Representative liver histological sections were stained with hematoxylin and eosin (H&E) (400 × magnification). The data represent the average of three independent experiments. All data are presented as the means ± SEM ($n = 5$ in each group). $^{**}p < 0.01$ versus the control group.

the liver was perfused initially with an EGTA-buffer at 37°C, at a constant flow of 5 mL/min. Subsequently, perfusion was continued with a solution of collagenase supplemented by its co-factor calcium until complete digestion. The liver capsule was then gently disrupted to release isolated liver cells into a suspension buffer. The liver cell suspension was subsequently filtered through 100-μm cell strainer and purified through three cycles of low-speed centrifugations (300 rpm for 3 min at 4°C). Cell viability was estimated by the trypan blue exclusion test and was always higher than 80%. A suspension of 0.5×10^6 viable cells/mL was subsequently seeded in 6- or 96-well plates previously coated with collagen (40 μg/mL), in complete culture medium. Cells were incubated overnight at 37°C, with 5% CO_2, to allow cell adhesion before drug exposure.

Histopathology Assessment

After bleeding, a portion of fresh liver tissues were fixed in Neutral-buffered formalin, embedded in paraffin and cut into 3 μm sections. The tissue sections were subsequently stained with hematoxylin and eosin (H & E) for microscopic examination.

Biochemical Assay

The serum/cell supernatant ALT and AST concentrations were measured using an assay kit according to the manufacturer's instructions. Mouse liver tissues GSH, MPO, MDA and SOD levels according to the manufacturer's instructions. All results were normalized by the total protein concentration in each sample.

Cell Culture and MTT Analysis

A hepatoma-derived HepG2 cell line, bought from the China Cell Line Bank (Beijing, China), was cultured in DMEM medium supplemented with 10% FBS, 100 U/mL of penicillin, 100 U/mL of streptomycin and 3 mM glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Moreover, HepG2 cells (1×10^4 cells/well) were treated with Iso (5, 10, and 20 μM) for 1 h, and exposed to APAP (15 mM) for 20 h. After treatment, cells were incubated with MTT (5 mg/mL) for additional 4 h. Then, the supernatant was removed and the formed blue formazan was dissolved in DMSO. The optical density was measured at 570 nm and Cell viability was normalized as the percentage of control.

CRISPR/Cas9 Knockout

HepG2 cells were grown in 24-well plates for 16 h, then transfected with a plasmid expressing Cas9 with Nrf2 sgRNA or AMPK sgRNA and a plasmid carrying a puromycin resistant gene using Viafect transfection reagent (Promega). After 36 h, 2 mg/ml puromycin was added to selected cells. Two days later,

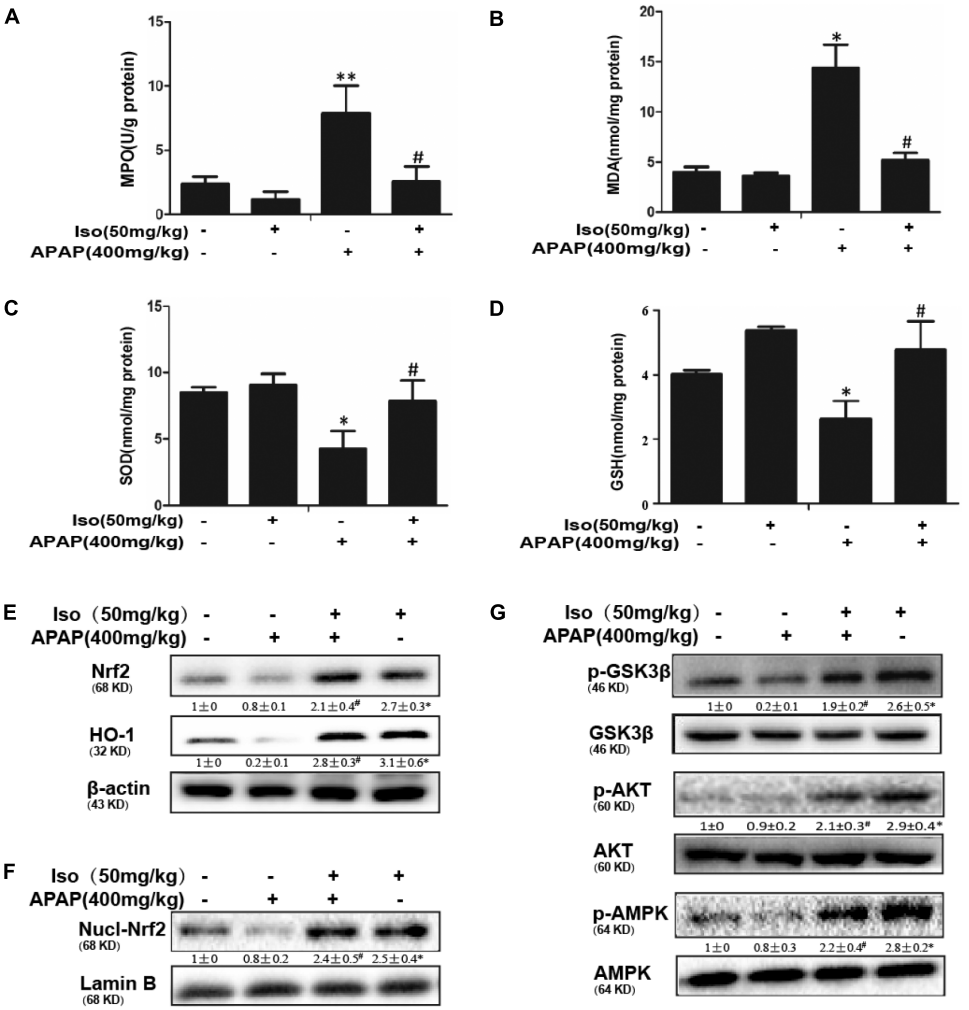

FIGURE 2 | Effect of Iso treatment on the levels of APAP-mediated oxidative stress, Nrf2 activation, and AMPK, Akt and GSK3β phosphorylation in mice. Mice were treated with Iso (50 mg/kg i.p.) twice. One hour after the last dose of Iso, mice received APAP (400 mg/kg). Liver tissues were obtained from the mice 6 h after the APAP challenge for the measurement of MPO and MDA formation and SOD and GSH activity. Furthermore, protein samples were extracted from liver tissue homogenates and analyzed by Western blotting. **(A,B)** Effects of Iso on APAP-mediated MPO and MDA content in liver. **(C,D)** Effects of Iso on APAP-mediated SOD and GSH content in liver. **(E,F)** Effects of Iso on APAP-induced expression and nuclear translocation of Nrf2 and HO-1 protein expression in liver. **(G)** Effects of Iso on APAP-induced AMPK, Akt and GSK-3β phosphorylation in liver. The data represent the average of three independent experiments. Data are presented as the means ± SEM (n = 5 in each group). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the APAP group.

cells were seeded in 96-well plates at a density of 1 cell per well. Gene editing efficiency after clonal expansion was determined by immunoblotting. DNA sequencing was employed to verify that gene editing was successful.

Intracellular ROS Measurement

HepG2 cells were seeded into 96-well plates (1×10^4 cells/well) for 24 h, and recovered in serum-free DMEM for 6 h. Next, the cells were subjected to different dosages of Iso (5, 10, or 20 μM) for 18 h, and APAP (15 mM) was added to each well for 3 h. Then the cells were incubated with 50 mM of DCFH-DA for 40 min and DCF fluorescence intensities were assessed by a multi-detection reader at excitation and emission wavelengths of 488 and 535 nm, respectively.

Quantification of Apoptotic and Necrosis Cells

HepG2 cells were seeded into 12-well plates (5×10^5 cells/well) for 24 h incubation, and then were treated with Iso (5, 10, or 20 μM) 1 h prior to APAP treatment. After 18h, cells were washed twice with ice-cold PBS, and subjected to Annexin V and propidium iodide staining. The percentage of apoptosis and necrosis were determined using flow cytometry (LSR II Flow Cytometer; BD Biosciences, SanJose, CA, United States).

Western Blot Analysis

The total protein samples from liver tissues were homogenized using RIPA lysis buffer containing protease and phosphatase inhibitors. Cells were plated into 6-well plates and treatment

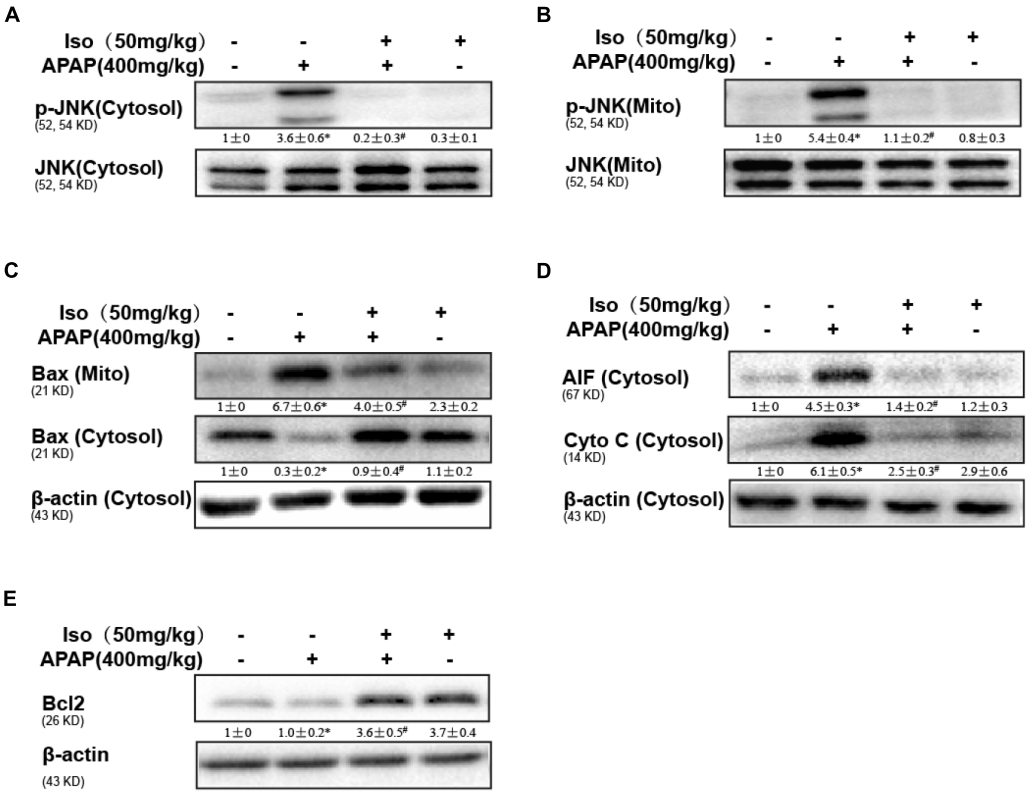

FIGURE 3 | Protective effect of Iso against mitochondrial dysfunction. Mice were treated with Iso (50 mg/kg i.p.) twice. One hour after the last dose of Iso, the mice received APAP (400 mg/kg). **(A,B)** Western blotting was used to detect the levels of phosphorylated JNK in mitochondrial and cytosolic liver fractions at 6 h post-APAP. **(C,D)** Mitochondrial Bax and cytosolic Bax, AIF and cytochrome c were measured by immunoblot analysis at 6 h post-APAP injection. **(E)** Bcl2 in liver tissue lysates was assayed by Western blotting. The results show the average of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the APAP group.

with different concentrations of Iso for the indicated durations. Nuclear and cytoplasmic fractions of cells and liver tissues were obtained using an NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL, United States) according to the manufacturer's instructions. Mitochondria Isolation Kit (Sigma-Aldrich, United States) according to the manufacturer's instructions. A BCA protein assay kit (Beyotime, China) was employed to measure protein concentrations. Equal amounts of proteins were separated by 10% SDS-poly-acrylamide gel, electrophoretically transferred to PVDF membranes and blocked with 5% BSA. Then, the membranes were washed and probed with corresponding primary antibodies and subsequently by secondary antibodies. Protein bands were visualized by ECL. The gray densities of protein bands were normalized by employed β-actin density as an internal control.

Statistical Analysis

All data referenced above were expressed as the means ± SEM and analyzed using SPSS19.0 (IBM), and One-way analysis of variance (ANOVA) was employed for comparisons between the experimental groups. Statistical significance was defined as $p^{*} < 0.05$ or $p^{**} < 0.01$.

RESULTS

Iso Alleviated APAP-Induced Acute Liver Failure (ALF) *in vivo*

As shown in **Figure 1B**, the survival rate of the APAP (900 mg/kg) plus Iso (50 mg/kg)-treated mice were much higher than that of the APAP (900 mg/kg)-treated mice. Furthermore, the serum ALT and AST levels were significantly increased in APAP (400 mg/kg)-treated mice, whereas Iso (50 mg/kg) reduced these increases (**Figures 1C,D**). Histopathological evaluation of the liver further demonstrated the protective effects of Iso against APAP-induced liver damage, including the amelioration of hepatocyte necrosis and hemorrhage (**Figure 1E**).

Iso Ameliorated APAP-Induced Oxidative Stress Injury and Induced Nrf2 Transcriptional Activation *in vivo*

As presented in **Figure 2**, compared to those in the control group, the levels of MDA and MPO were significantly increased in the APAP group, whereas the GSH content and SOD activity were significantly decreased in the APAP group. Our results showed that Iso pretreatment significantly decreased

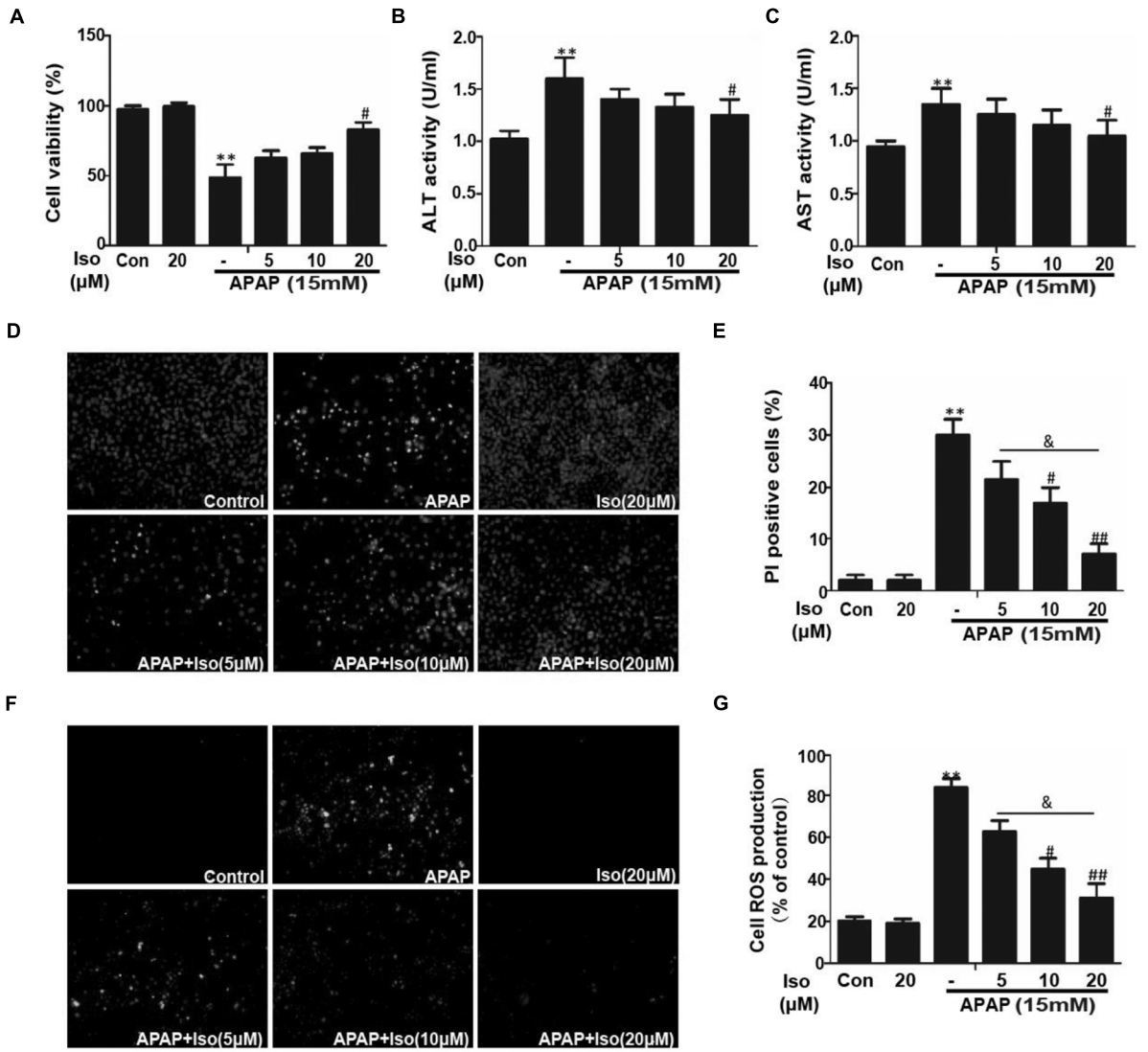

FIGURE 4 | Effect of Iso exposure on APAP-induced cytotoxicity, ALT and AST activities, apoptosis, and oxidative stress in HepG2 cells. **(A)** HepG2 cells were treated with various concentrations of Iso (5, 10, and 20 μM) for 1 h, and the cells received APAP (15 mM) for 24 h. Cell viability was evaluated using an MTT assay. **(B,C)** HepG2 cells were incubated with APAP (15 mM) and various concentrations (5, 10, and 20 μM) of Iso. Cell supernatants were collected after 24 h of incubation to determine ALT and AST activities in the supernatant. **(D,E)** HepG2 cells were treated with various concentrations of Iso (5, 10, and 20 μM) for 1 h, and the cells received APAP (15 mM) for 24 h, and then stained with Hoechst/PI for 15 min. The fluorescence was immediately detected by fluorescence microscope. **(F,G)** HepG2 cells were treated with Iso (5, 10, and 20 μM) for18 h, treated with APAP (15 mM) for 3 h, and then stained with 50 μM DCFH-DA for 40 min. The fluorescence was immediately detected by fluorescence microscope and a multidetection reader. The results show the average of three independent experiments. $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the APAP group. $^{\&}p < 0.05$ versus the Iso (20 μM) plus APAP group.

the levels of MPO and MDA (**Figures 2A**,**B**) and increased the levels of GSH and SOD (**Figures 2C,D**), which play vital roles in protecting against APAP-induced oxidative stress. Moreover, APAP challenge mildly suppressed Nrf2 and HO-1 protein expression and Nrf2 translocation, whereas these changes were completely reversed by Iso pretreatment (**Figures 2E,F**). We further examined the upstream proteins AMPK, AKT and GSK3β, which are involved in the regulation of the Nrf2 activation. As shown in **Figure 2G**, Iso treatment significantly increased the phosphorylation of AMPK, AKT and GSK3β.

Iso Ameliorated APAP-Induced Mitochondrial Dysfunction *in vivo*

Oxidative stress-induced mitochondrial dysfunction is regarded as a crucial factor in APAP-induced liver injury, so we examined whether Iso pretreatment could inhibit APAP-induced mitochondrial dysfunction. As shown in **Figures 3A–D**, APAP (500 mg/kg) significantly induced Bax mitochondrial translocation, the release of AIF and cytochrome c, and JNK activation, contributing to mitochondrial dysfunction; however, these effects were effectively inhibited by Iso pretreatment

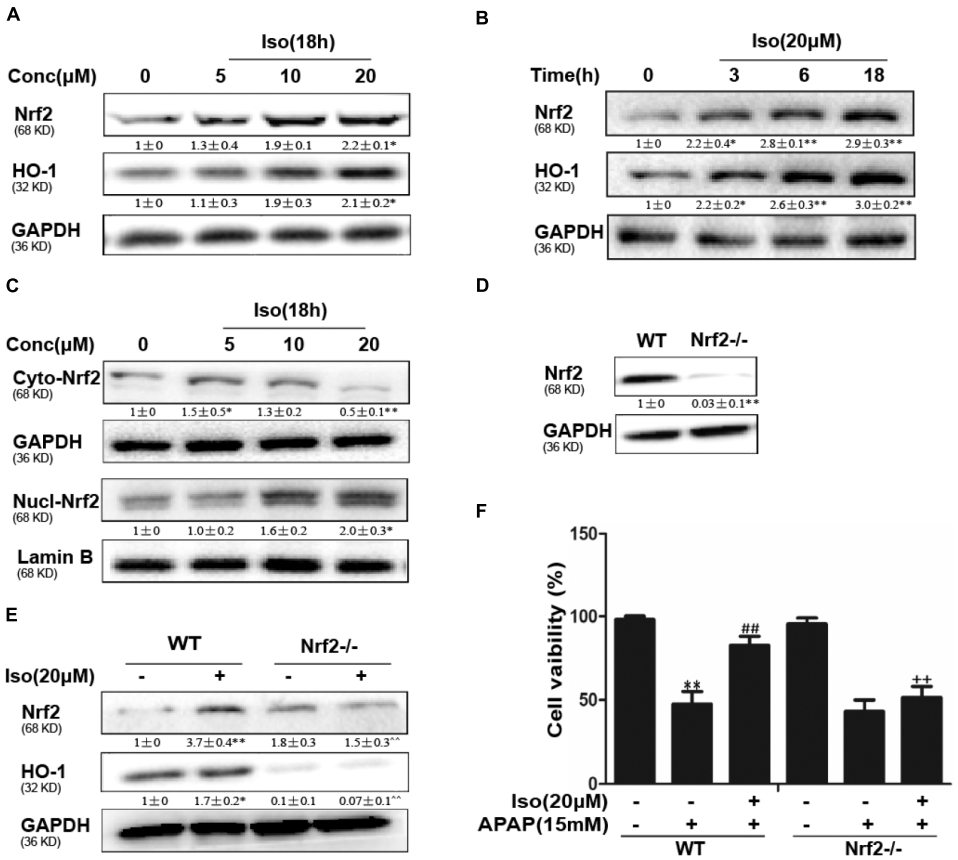

FIGURE 5 | Effect of Iso treatment on Nrf2 expression and nuclear translocation and Nrf2-mediated defense against APAP-stimulated cytotoxicity in HepG2 cells. **(A–C)** HepG2 cells were treated with Iso for the indicated time, and Western blot analysis was used to determine the protein levels. **(D,E)** HepG2 WT and HepG2 Nrf2$^{-/-}$ cells were grown with or without Iso (20 μM) for 18 h; Nrf2 and HO-1 expression was detected by Western blot analysis. **(F)** HepG2 WT and Nrf2$^{-/-}$ cells were treated with Iso (20 μM) for 1 h before APAP (15 mM) treatment for 24 h; cell viability was determined with an MTT assay. All the data represent the average of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the WT control group; $^{\#\#}p < 0.01$ versus the WT APAP group; $^{++}p < 0.01$ versus the WT APAP plus Iso group.

(50 mg/kg). In addition, Bcl-2 was activated by Iso pretreatment, which may be responsible for the hepatoprotective effects of Iso (**Figure 3E**).

Effect of Iso on APAP-Induced Cell Viability, Cell Death and Oxidative Stress in HepG2 Cells

Cell viability of HepG2 cells co-treated with APAP and Iso were increased compared to control group treated with only APAP. And, when cells were co-treated with Iso, APAP-mediated cytotoxicity was attenuated in a dose-dependent manner (**Figure 4A**). Primary hepatocytes also have the same result (**Supplementary Figure 1C**). It is known that ALT and AST are released from damaged hepatocytes. Iso significantly decreased activities of ALT and AST in HepG2 cells due to APAP damage (**Figures 4B,C**). As shown in **Figures 4D,E**, APAP (15 mM) caused high cellular mortality and apoptosis, an effect that was inhibited by Iso pretreatment in a dose-dependent manner. Moreover, APAP (15 mM) exposure caused severe oxidative stress, as evidenced by increased ROS generation, whereas Iso markedly suppressed ROS production in a dose-dependent manner (**Figures 4F,G**).

Iso Activated Nrf2 and Increased Antioxidant Enzyme Expression *in vitro*

The data in **Figures 5A,B** show that Iso significantly upregulated Nrf2 and HO-1 protein expression *in vitro* in a dose-dependent manner and in a time-dependent manner, respectively. Primary hepatocytes also have the same result (**Supplementary Figure 1A**). Moreover, Iso induced Nrf2 translocation in a dose-dependent manner (**Figure 5C**). An increasing body of evidence indicates that Nrf2 plays a key role in HO-1 regulation. Therefore, a plasmid expressing Cas9 with Nrf2 sgRNA was used to knock out Nrf2 expression in HepG2 cells. The results of Western blot analysis showed that the upregulation of Nrf2 and HO-1 expression induced by Iso was markedly suppressed in Nrf2$^{-/-}$ cells compared to control cells (**Figures 5D,E**). Furthermore, the protective effect of Iso against APAP-induced cell death in the HepG2 WT cells was abolished in the HepG2 Nrf2$^{-/-}$ cells (**Figure 5F**).

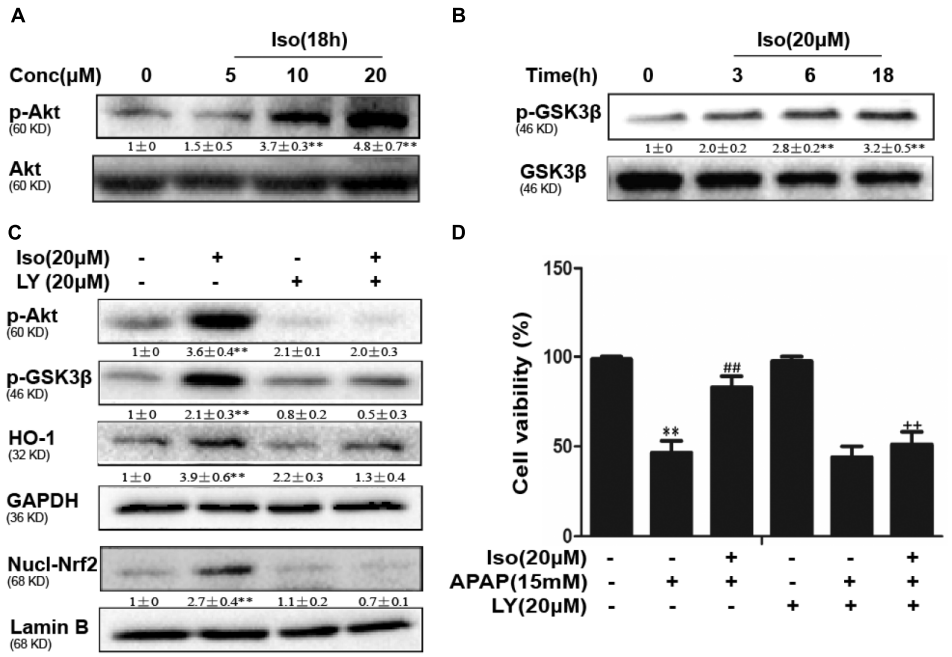

FIGURE 6 | Effect of Iso exposure on AKT-mediated GSK3β phosphorylation and the critical role in Iso-mediated protection against APAP-induced cytotoxicity in HepG2 cells. **(A,B)** HepG2 cells were incubated for 18 h with Iso, and the levels of AKT and GSK3β phosphorylation were determined by Western blot analysis. **(C)** HepG2 cells were pretreated with LY294002 (20 μM) for 18 h and then treated with Iso for 18 h; Western blot analysis was used to determine the levels of AKT and GSK3β and the extent of Nrf2 nuclear translocation. **(D)** The cells were pretreated with LY294002 (20 μM) for 1 h before treatment with Iso. After 1 h, the cells were received APAP for 24 h, and cell viability was measured with an MTT assay. The results show the average of three independent experiments. $^{**}p < 0.01$ versus the control group; $^{\#\#}p < 0.01$ versus the APAP group; $^{++}p < 0.01$ versus the APAP plus Iso group.

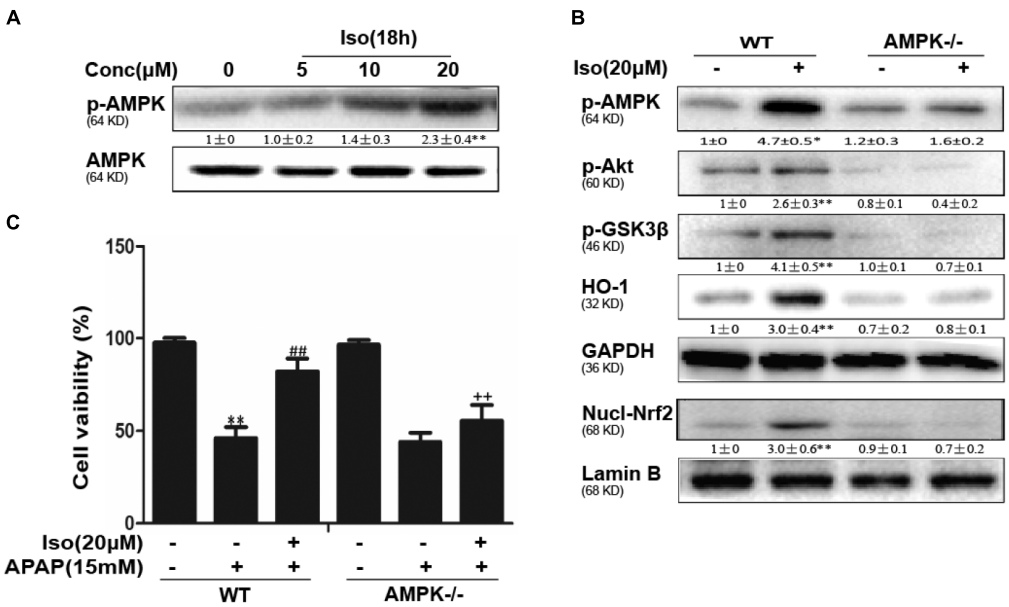

FIGURE 7 | Iso-induced AMPK phosphorylation is essential for AKT/GSK3β-mediated Nrf2 activity and cytoprotection in HepG2 cells. **(A)** HepG2 cells were cultured with Iso for 18 h. Western blot analysis was used to detect the expression of p-AMPK and AMPK. **(B)** HepG2 WT and AMPK$^{-/-}$ cells were incubated with or without Iso (20 μM) for 18 h; Western blot analysis was used to detect the phosphorylation of AMPK, Akt, GSK3β and Nrf2. **(C)** HepG2 WT and AMPK$^{-/-}$ cells were pretreated with 20 μM Iso for 1 h before APAP (15 mM) treatment for 24 h; cell viability was determined with an MTT assay. All the data presented as the average of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the WT control group; $^{\#\#}p < 0.01$ versus the WT APAP group; $^{++}p < 0.01$ versus the WT APAP plus Iso group.

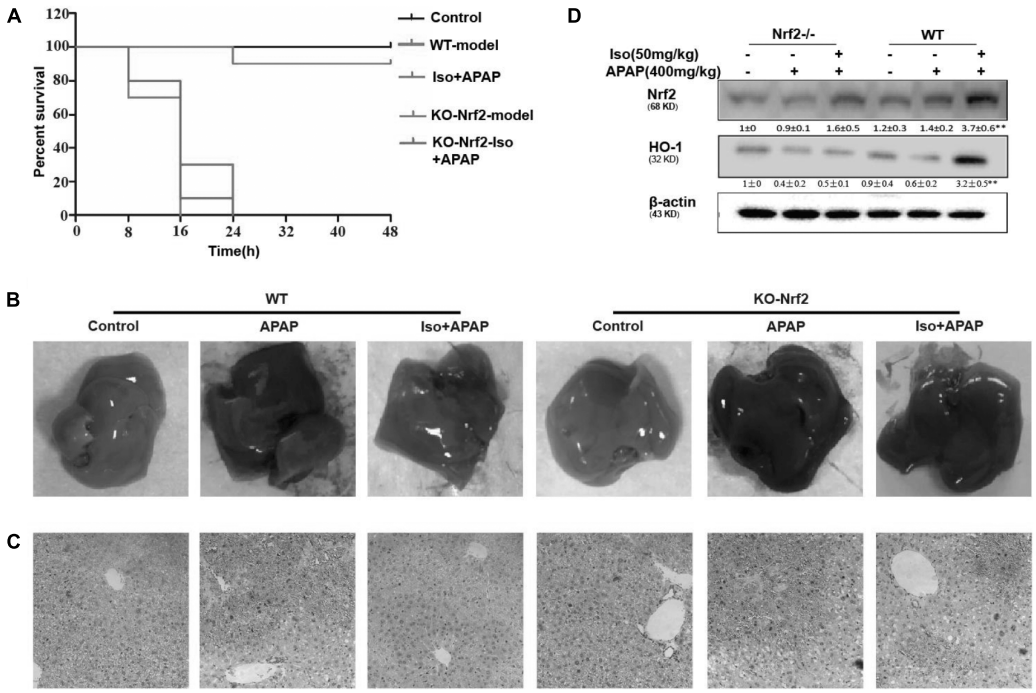

FIGURE 8 | Protective effects of the Iso-mediated regulation of Nrf2 against APAP-induced AILI in mice. WT and $Nrf2^{-/-}$ mice were intraperitoneally injected with Iso (50 mg/kg) twice at 12 h intervals. **(A)** Survival rate of WT and $Nrf2^{-/-}$ mice that received a lethal dose of APAP (900 mg/kg) 1 h after the last dose of Iso (50 mg/kg). The data represent the percentage of surviving mice at each time point. $n = 15$ in each group. **(B)** One hour after the last dose of Iso and 6 h after the APAP (400 mg/kg) challenge, livers ($n = 5$) from each experimental group were processed for gross examination. **(C)** Representative histological sections of the liver were stained with hematoxylin and eosin (H&E). **(D)** Effects of Iso on the protein expression of Nrf2 and HO-1. All data are presented as the means ± SEM ($n = 5$ in each group). $^{\#\#}p < 0.01$ versus the control group; $^{**}p < 0.01$ versus the APAP group.

Iso Enhanced Cell Viability Through an AMPK/AKT/Nrf2-Dependent Mechanism *in vitro*

Several mechanisms are involved in the regulation of Nrf2 activation, such as the phosphorylation of Akt. We further examined the effect of Iso on AKT phosphorylation and Akt-mediated GSK3β inhibitory phosphorylation. As shown in **Figures 6A,B**, Iso significantly increased AKT and GSK3β phosphorylation in a dose-dependent manner. AMPK acts upstream of AKT to increase the inhibitory phosphorylation of GSK-3β and could be activated by Iso in a dose-dependent manner (**Figure 7A**). Primary hepatocytes also have the same result (**Supplementary Figure 1B**). In addition, preincubation of cells with LY294002 (an AKT inhibitor) or HepG2 $AMPK^{-/-}$ cells prevented the Iso-induced Nrf2/ HO-1 activation and cytoprotection (**Figures 6C,D**, **7B**,**C**).

The Suppressive Effects of Iso on APAP-Induced Liver Injury Were Dependent on Nrf2

The dependency of the hepatoprotective role of Iso on Nrf2 was further assessed in WT and $Nrf2^{-/-}$ mice. First, as shown in **Figure 8A**, $Nrf2^{-/-}$ mice appeared to be more vulnerable to APAP-induced lethality than WT mice, as Nrf2 knockout reduced the survival rate from approximately 20% to approximately 0. Second, for WT mice, the final survival rates were 20 and 90% for the vehicle group and the Iso-treated group, respectively, while, for $Nrf2^{-/-}$ mice, the final survival rates were 0% in both the vehicle group and the Iso -treated group. We further examined Nrf2 and HO-1 expression and histopathological changes in the liver. As shown in **Figures 8B–D**, the inhibitory effects of Iso were abolished in $Nrf2^{-/-}$ mice.

DISCUSSION

Natural products have made enormous contributions to drug discovery, as they have many advantages over conventional chemical compound-based medications, such as fewer side effects, less long-term toxicity, and variable bioavailability and biological activity (Kim, 2018). In recent years, intensive studies have demonstrated the protective effects of natural products against APAP-induced hepatotoxicity due to their multiple mechanisms of action in inflammation, oxidant/antioxidant balance and damage responses (Ko et al., 2017; Lv et al., 2018). Iso (or homoorientin) is a chemical flavonoid-like compound that has been shown to exert anti-inflammatory effects and exhibit antioxidant potential (Ye et al., 2016). To our knowledge, no report has investigated the protective activity of Iso against APAP-induced hepatotoxicity.

The present study aimed to investigate the protective effects of Iso against APAP-induced hepatotoxicity and further explore the molecular mechanisms *in vivo* and *in vitro*.

APAP overdose-induced liver dysfunction is the most common cause of drug-induced liver injury (DILI) worldwide, which is characterized by lethality, high ALT and AST levels in serum and pathological changes in the liver (Brodsky et al., 2009). Using an APAP-induced mouse model, we found that APAP markedly increased mortality, serum ALT and AST levels and liver histopathology changes, as reported previously (Zhao et al., 2018). However, Iso treatment significantly prevented these elevations, suggesting that Iso protected liver tissues against the toxic effects of APAP. An abundance of evidence has shown that oxidative stress contributes to histopathological changes by inducing MDA and MPO formation and by decreasing in hepatic levels of SOD and GSH after APAP challenge (Wang L. et al., 2016; Xu et al., 2017). The results of our investigation show that Iso significantly reduced MDA and MPO levels and reversed GSH and SOD depletion, indicating that Iso treatment dramatically alleviated APAP-induced oxidative injury in mice. Oxidative stress-mediated JNK activation and mitochondrial dysfunction play central roles in APAP-induced hepatic injury (Saito et al., 2010); in these processes, JNK phosphorylation enhances the release of cytochrome c and AIF from the mitochondria and enhances Bax translocation to the mitochondria. Western blot analyses in liver tissue clearly demonstrated that Iso suppressed JNK phosphorylation, Bax mitochondrial translocation, and AIF and cytochrome c release. Together, the current results emphasize that Iso may serve as a hepatoprotective agent for inhibiting oxidative stress and mitochondrial dysfunction.

Iso has been reported to possess a notable hepatoprotective effect mediated by the regulation of the respiratory chain complexes and the activity of phase II detoxifying enzymes (Yuan et al., 2016). Nrf2, as a coordinator of multiple signaling pathways, plays a key role in maintaining cellular redox homeostasis and defensing against oxidative stress. Nrf2 activation is observed in hepatic stellate cells, Kupffer cells and parenchymal hepatocytes (Yeligar et al., 2010). We further investigated the critical involvement of Nrf2 in Iso-mediated protection against APAP-induced liver injury. Our data showed that Iso induced Nrf2 and HO-1 activation and translocation in liver tissue. We further determined the upstream processes involved in Nrf2 activation by Iso. Notably, AMPK, a sensor of cellular energy status, regulates cell survival and death in the presence of oxidative stress (Shirwany and Zou, 2014). Iso has been reported to activate AMPK in pancreatic cancer cells (Ye et al., 2016). In our study, Iso treatment significantly increased the phosphorylation of AMPK, AKT, and GSK-3β, which might contribute to Nrf2 activation and hepatoprotective effects *in vivo*. Previous studies have demonstrated that a suite of signaling pathways play important roles in the process of hepatotoxicity, including GSK3β/Nrf2 (Mobasher et al., 2013), PKCzeta-Akt-GSK3beta (Shieh et al., 2009) and AMPK/Akt/GSK3β (Wang L. et al., 2016) pathways. Such studies may provide potential targets for pharmacological candidates for the treatment of liver injury.

To further determine the functional role of Nrf2 in the hepatoprotective effects of Iso and to decipher the mechanisms of Iso-induced Nrf2 activation, we utilized $HepG_2$ cells challenged with APAP *in vitro*. As shown in **Figure 4**, Iso significantly suppressed APAP-induced cytotoxicity, apoptosis and ROS generation. In addition, Iso increased Nrf2 expression and nuclear translocation as well as HO-1 expression in a dose-dependent manner. Considering the key role of Nrf2 in HO-1 regulation and in hepatoprotective effects (Wang W. et al., 2016), we speculated that the upregulation of HO-1 expression and the suppression of cytotoxicity were dependent on Nrf2 activation. This hypothesis was verified by the finding that the Iso-induced cytoprotection and increased HO-1 expression that occurred in normal HepG2 cells were abolished in HepG2 $Nrf2^{-/-}$ cells. Together, these results suggest that Iso protects against cytotoxicity and that oxidative stress depends on Nrf2 activation. Given the results of our *in vivo* study, we further explored the effect of the AMPK/Akt/GSK3β pathway on Iso-induced Nrf2 activation *in vitro*. Our results demonstrated that Iso induced AMPK, Akt, GSK3β, and Nrf2 activation *in vitro*, and the Nrf2 activation and cytoprotective effect of Iso were abolished by an AKT inhibitor or by AMPK knockout (**Figures 6**, **7**). A large number of natural products process potent anti-APAP-induced hepatotoxicity, such as dioscin. The difference is that dioscin showed a remarkable protective effect against APAP-induced hepatotoxicity by adjusting mitochondrial function (Zhao et al., 2012), and Iso ameliorated APAP-induced hepatotoxicity by activating Nrf2 via the AMPK/Akt/GSK3β pathway.

Given all of these results, we used Nrf2-deficient mice to further elucidate whether the inhibition of APAP-induced hepatotoxicity by Iso involves Nrf2 activation. Unlike in WT mice, Iso could not alleviate the mortality or histopathological changes in the liver associated with APAP challenge in Nrf2-deficient mice. In summary, our findings demonstrated the hepatoprotective effect of Iso against APAP-induced oxidative damage and mitochondrial dysfunction, an effect that may be strongly dependent on Iso-mediated Nrf2 activation.

AUTHOR CONTRIBUTIONS

XF and HL conducted the experiments and wrote the paper. LW conducted the experiments. XD and XC contributed to the design and improvement of the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01334/full#supplementary-material

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Herbal Extracts and Natural Products in Alleviating Non-alcoholic Fatty Liver Disease via Activating Autophagy

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Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease world-wide, and currently therapeutic options for NAFLD are limited. Herbal medicine (HM) may offer an attractive alternative for the treatment of NAFLD. Recent years have witnessed a growing interest in the autophagy-inducing agents, and autophagy activation has been recognized as an efficient strategy in managing NAFLD and related complications. Pharmacological studies have demonstrated certain potential of HM extracts and natural products in inducing autophagy, which might contribute to the efficacy of HM in preventing and treating NAFLD. This review aims to summarize current understanding of mechanisms of HM extracts and natural products in preventing and treating NAFLD. Specially, we focused on mechanisms by which autophagy can target the main pathogenesis events associated with NAFLD, including hepatic steatosis, inflammation, oxidative stress, and apoptosis. It is hoped that this brief review can provide a general understanding of HM extracts and natural products in treating NAFLD, and raise awareness of potential clinical application of HM in general.

Keywords: autophagy, Chinese herbal medicine extracts, non-alcoholic fatty liver, steatosis, inflammation, oxidative stress, apoptosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease around the world, affecting one third of population in certain areas (Younossi et al., 2016). It is universally acknowledged that sedentary lifestyle, in conjunction with food abundance in industrialized countries are the main causes of NAFLD (Farrell et al., 2013). Clinically, NAFLD covers a broad spectrum of liver abnormalities, ranging from simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, to cirrhosis (Cobbina and Akhlaghi, 2017). The hallmark of NAFLD is characterized by excessive accumulation of fat deposits in the liver, resulting from causes other than alcohol abuse. While simple steatosis is considered pathologically benign, NASH often indicates liver injury that may progress into severe pathology. Although NAFLD is a major component of metabolic syndrome and chronic liver diseases, a satisfactory explanation of any pathological aspects of NAFLD is unavailable at the moment.

Herbal medicine (HM), an alternative approach in the treatment of NAFLD has drawn growing attention among practitioners. In China, HM accounts for the majority of treatments in traditional Chinese medicine (TCM); plant elements and extracts are nature products that by far the most common elements used clinically. Although being used initially as empirical prescription for

individuals, some of HM has been supported by clinical evidence in the treatment of NAFLD (Pan et al., 2013; Yu et al., 2015). The extracts of HM have also shown benefits in alleviating NAFLD (Xu et al., 2015); clinical trials have obtained evidence of natural product from HM, such as berberine (Yan et al., 2015), resveratrol (Chen et al., 2015), and curcumin (Panahi et al., 2017) in improving NAFLD parameters. Recently, studies have suggested that HM extracts could induce autophagy (Hu et al., 2016), which might offer an explanation of the efficacy of HM in NAFLD therapy.

Autophagy has been inferred in the pathogenesis of NAFLD and lipid dysregulation (Ueno and Komatsu, 2017). It is generally accepted that autophagy is activated during the early stage of NAFLD, in response to acute increase in lipid availability, thus attenuates lipid accumulation within the liver. However, hepatic autophagy is impaired upon sustained availability of lipids, such as long-lasting high fat dieting (Ueno and Komatsu, 2017). Autophagy is considered as one of the pathways in lipid breakdown (lipophagy), and is intimately associated with metabolism of lipid droplets (Kwanten et al., 2014). Autophagy can be cutely activated by a variety of means, such as caloric restriction, physical exercise, rapamycin, AMP-activated protein kinase (AMPK)-targeting agents, and hydrogen sulfide. Invariably, autophagy activation is associated with attenuation of NASH as well as improvement of various metabolic parameters (e.g., body weight, circulating glucose or triglyceride levels, and insulin sensitivity) (Lee et al., 2017). Moreover, autophagy activation with carbamazepine can reduce hepatic fibrosis in a model of α1-antitrypsin deficiency-associated liver disease (Puls et al., 2013). In contrast, inhibition of autophagy with a Beclin 1-interacting negative regulator results in accelerated lipid accumulation and pathogenesis of NAFLD (Tanaka et al., 2016).

The role of HM extracts in inducing autophagy and its implication in tumorigenesis has been investigated extensively (Wang et al., 2017; Zhang et al., 2018). Increasing attention has begun to focus on HM extracts and their effects on autophagy in NAFLD pathogenesis and related hepatic and metabolic complications. This review summarizes the current knowledge on the interrelationship between autophagy, autophagy inducing effect of HM extracts or natural products, and NAFLD.

AUTOPHAGY AND THE REGULATING MOLECULES

Autophagy is a highly conserved self-digestion process, bring dispensable or potentially dangerous cytoplasmic material, such as damaged organelles and misfolded or unfolded proteins, to lysosomes for degradation. To date, at least three autophagy processes have been described, namely microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Macroautophagy is by far the most extensively characterized autophagy process, whereas the macroautophagy and CMA processes are lesser understood. Thus, the term autophagy is generally referred to the macroautophagy process. Autophagy is fundamental in the preservation of organismal fitness, and is central to adaptation to stress, usually alleviating damage of cells exposed to infections or else nutritional, metabolic physical or chemical (Kim and Klionsky, 2000).

In eukaryotic cells, autophagy is initiated by the formation of autophagosomes and autolysosome that leads to lysosome-mediated degradation. The process of autophagosome formation involves three major steps: initiation, nucleation, and elongation (Klionsky and Emr, 2000). Once the autophagosome enclosed, it can fuse with lysosome in the cytoplasm and assemble into autolysosome. More than 30 autophagy-related genes (ATGs) are involved in autophagy process (Amaravadi et al., 2016). The initiation of autophagosome is controlled by the ULK1-Atg13-FIP200 complex (Chan, 2009; Chan et al., 2009). The nucleation step requires the Beclin-1-class III phosphatidylinositol 3 Kinase (PI3K) complex that includes Beclin-1, Vps34 (class III PI3K), Vps15, Atg14L/Barkor, and Ambra-1 (Mizushima and Komatsu, 2011). Two conjugation systems are involved in the elongation of autophagosomes. The Atg12-Atg5-Atg16 complex and the cleavage of light chain 3 (LC3)/Atg8 cascade, leading to the soluble form LC3-I, and later forms the autophagic double-membrane associated LC3-II protein that allowing the closure of the autophagic vacuole (Ohsumi, 2001). The appearance of LC3-II and the autophagic adaptors p62 are commonly used to monitor autophagy influx. Closed autophagosomes fuse with lysosomes to generate autolysosomes for degradation. A large number of factors/actors regulate the autophagosome-lysosome fusion. In the autophagosome-lysosome fusion process, soluble *N*-ethylmaleimide-sensitive factor attachment protein, cytoskeleton proteins, and small GTPases are involved (Yu et al., 2017).

Mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) exerts prominent autophagy-suppressing functions by catalyzing the inactivating phosphorylation of ATG13 and ULK1 (Noda and Inagaki, 2015). Such an inhibition can be relieved upon the inactivation of mTORC1 by AMPK, which is sensitive to cAMP accumulation (a consequence of ATP consumption) and also catalyzes the phosphorylation of ULK1 and Beclin 1 (Niso-Santano et al., 2015; Selleck et al., 2015).

AUTOPHAGY INDUCING HM EXTRACTS AND NATURAL PRODUCTS ON HEPATIC STEATOSIS

Hepatic lipid accumulation is the most notable feature of NAFLD. The liver is not a *de factor* organ for lipid storage. Under normal physiological conditions, the amount of fat that the liver contains is less than 5% of its weight. Thus, excessive lipid accumulation within the liver, known as ectopic lipid accumulation, is hallmark of hepatic steatosis, a typical characteristic of NAFLD. In the liver, triglyceride and cholesterol esters are the main constitutes of lipid droplet, and autophagy is closely associated with lipid droplets metabolism. LC3-positive structures are seen to co-localize with lipid droplet markers in liver tissue (Shibata et al., 2009) and in cell lines (Shibata et al., 2010; Sinha et al., 2012, 2014). Lipid droplets have also been shown to associated with lysosomes (Tuohetahuntila et al., 2017). Defective autophagy of lipid droplets in hepatocytes has recently been

identified as a possible pathophysiological mechanism of NAFLD (Tuohetahuntila et al., 2017; Zhang et al., 2017). Thus, activating autophagy might be a promising strategy to attenuate hepatic lipid accumulation.

Some HM extracts and natural products are considered to be effective in attenuating lipid accumulation via, at least partly, activating autophagy. Ginsenoside Rb2, one of the major ginsenosides in *Panax ginseng*, is able to prevent hepatic lipid accumulation in *db/db* mice, HepG2 cells and primary mouse hepatocytes (Huang et al., 2017). Rb2 could partly reverse the repression of autophagic pathways involving AMPK or silent information regulator 1 (SIRT1). Inhibition of AMPK or SIRT1 pathway thus blocked the beneficial effects of Rb2 (Huang et al., 2017), suggesting that the effect of Rb2 on alleviating hepatic steatosis may be achieved through autophagy induction.

Resveratrol, a natural polyphenol, has been reported to improve complications associated with NAFLD pathology. Administrate resveratrol (200 mg/kg bodyweight) to rats under high fat diet conditions significantly prevented hepatic steatosis and hepatocyte ballooning after 18-week treatment, along with the up-regulation of SIRT1 and autophagy markers LC3-II, Beclin 1, and P62 (Ding et al., 2017). Similarly, another study has also shown beneficial effects of resveratrol on hepatic steatosis, and demonstrated that the effect is achieved partially through inducing autophagy and the cAMP-PRKA-AMPK-SIRT1 signaling pathway (Zhang et al., 2015a). In methionine-choline-deficient diet (MCD)-induced NASH animals, 4-week resveratrol intervention significantly decrease lipid accumulation in the liver, along with the increase LC3-II levels and decrease P62 expressions (Ji et al., 2015). When the autophagy inhibitor, chloroquine (CQ) is added, the beneficial effects of resveratrol on AML12 cells are then abolished (Ji et al., 2015), suggesting that the effects of resveratrol are likely associated with autophagy activation.

Akebia saponin D (ASD), extracts from *Akebia quinata*, has been implicated in treating NAFLD. Administration of ASD in *ob/ob* mice results in a significantly decrease in hepatic steatosis and an accompanied increase in autophagic flux (e.g., increased expression of LC3-II and decreased P62 accumulation). In oleic acid stressed Buffalo rat liver cells, ASD also prevented excessive lipid droplets formation and increased autophagic flux, however, CQ or siRNA mediated ATG7 knockdown could abolish the effect (Gong et al., 2016), suggesting that ASD is an activator of autophagy and a possible candidate for treating NAFLD.

Bergamot polyphenol fraction (BPF), one of the dietary polyphenols, could strongly reduce hepatic steatosis and counteract the pathogenic increase of serum triglycerides, blood glucose and obesity in experimental rats (Parafati et al., 2015).

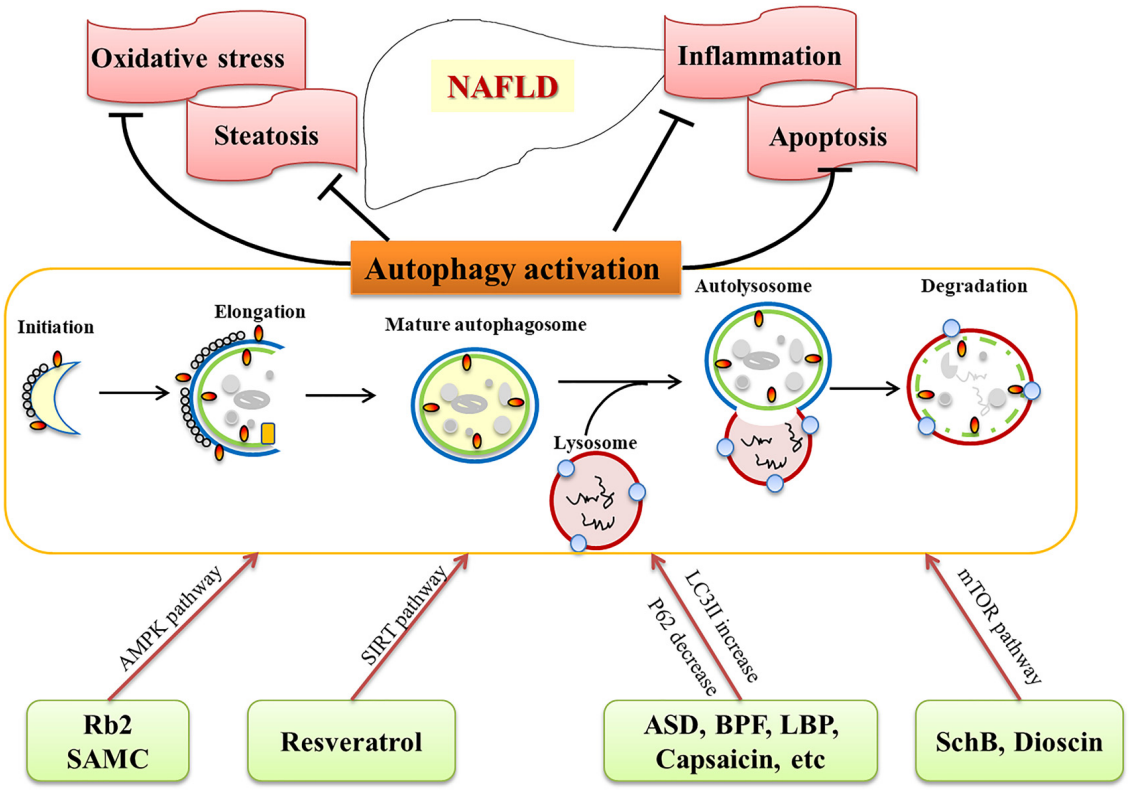

FIGURE 1 | Herbal medicine (HM) extracts on NAFLD via autophagy activation. HM extracts and natural products activate autophagy through different pathways (AMPK, SIRT, mTOR, etc.), and target related pathological processes (steatosis, oxidative stress, apoptosis, inflammation) of NAFLD.

TABLE 1 | Herbal medicine and natural products that exert an effect on autophagy.

Herb name/active components	Targeted pathways	Reference
Ginsenoside Rb2	Activate AMPK and SIRT1 in *db/db* mice, HepG2 cells, and primary mouse hepatocytes	Huang et al., 2017
Resveratrol	Up-regulate SIRT1 and autophagy markers LC3-II, Beclin 1, and P62 in diet induced NAFLD animals	Ji et al., 2015; Zhang et al., 2015a; Ding et al., 2017
Akebia saponin D	Increase expression of LC3-II and decrease P62 accumulation in *ob/ob* mice and primary rat hepatocytes	Gong et al., 2016
Bergamot polyphenol fraction	Increase LC3 and Beclin 1 and reduce SQSTM1/p62 in diet induced NAFLD rats	Parafati et al., 2015
Capsaicin	Activate TRPV1 in HepG2 cells	Li et al., 2013
Glycycoumarin	Block mitochondrial activation in palmitate stressed cells and NASH animals	Zhang E. et al., 2016
Lycium barbarum polysaccharides	Activate Atg5 and LC3-II and down-regulate p-mTOR and p62 in NASH animals	Xiao et al., 2014
S-allylmercaptocysteine	Suppressed LKB1/AMPK and PI3K/Akt pathways in NAFLD rats	Xiao et al., 2013b
Schisandrin B	Inhibit PI3K/Akt/mTOR signaling pathway in AML-12 and RAW 264.7 cells	Zhang et al., 2015b
Dioscin	Decrease p-mTOR/mTOR in NASH animals	Xu et al., 2017

Importantly, BPF can stimulate autophagy as suggested by increased levels of LC3 and Beclin 1, and concomitant reduction of SQSTM1/p62, suggesting autophagy stimulation (Parafati et al., 2015).

Capsaicin, an extract of *Capsicum annuum* and a common dietary supplement, has been shown to exert beneficial effects on NAFLD (Gong et al., 2016). Treatment of capsaicin in HepG2 cells results in activation of the transient receptor potential vanilloid 1 (TRPV1), which is accompanied with significantly increased the expression of autophagy-related proteins, such as LC3 II, Beclin 1, Atg5, and Atg7 (Li et al., 2013).

AUTOPHAGY INDUCING HM EXTRACTS AND NATURAL PRODUCTS ON OXIDATIVE STRESS

Oxidative stress describes an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage (Braud et al., 2017). Although mechanisms underlying NAFLD pathogenesis remain undefined, the widespread theories, such as "two hit hypothesis" and "multiple parallel hits" all centralized oxidative stress as a driving factor for NAFLD progression (James and Day, 1998; Tilg and Moschen, 2010). Since lipid oxidative mainly occurs in mitochondria, the excess ROS generated from oxidative stress conceivably would cause mitochondrial damage and subsequently dysfunction. As the formation of autophagosome is usually initiated from the membrane of mitochondria or endoplasmic reticulum (ER), the dysfunction of mitochondria presumably contribute to inhibition of autophagy. Supporting this assumption is a recent observation that mediating oxidative stress can efficiently stimulate autophagy (Tang et al., 2017).

Licorice is a popular herbal plant widely used in the treatment of various diseases including liver diseases (Jung et al., 2016). Glycycoumarin (GCM) is a representative of coumarin compounds isolated from licorice. It is reported that GCM could block mitochondrial activation in palmitate stressed cells (HepG2, AML-12, and L02) and MCD diet induced NASH mice, in line with the improved metabolic disorders, and the effects are partially associated with reactivation of the impaired autophagy (Zhang E. et al., 2016).

Lycium barbarum is one of the commonly used HM; *L. barbarum* polysaccharides (LBP) are the main components of *L. barbarum*, accounting for 20–50% of the total extracts. Studies showed that LBP could significantly inhibit oxidative stress in various animal models and cell lines (Zhu et al., 2015; Varoni et al., 2017). In high fat diet-induced NASH rat model, 4 weeks LBP treatment showed ameliorative effects on metabolic and inflammatory parameters of NASH (Xiao et al., 2014). In addition, certain autophagic markers, such as Atg5 and LC3II, were significantly up-regulated while certain autophagic negative regulators, such as p-mTOR and p62, became down-regulated (Xiao et al., 2014).

AUTOPHAGY INDUCING HM EXTRACTS AND NATURAL PRODUCTS ON APOPTOSIS

Accumulation of unfolded proteins in the ER triggers an adaptive response, known as the unfolded protein response (UPR), to restore ER homeostasis (Song et al., 2017). The UPR pathway is also required to maintain hepatic functions. However, prolonged UPR leads to ER stress and pro-apoptotic transcription factor activation (Schroder and Sutcliffe, 2010). Apoptosis is inferred in the pathogenesis of NAFLD, whereas handling apoptosis is considered to be beneficial in alleviating NAFLD and related complications.

Garlic-derived S-allylmercaptocysteine (SAMC) has been shown to ameliorate hepatic injury in a NAFLD rat model (Xiao et al., 2013a). Administration of SAMC during NAFLD development could protect the liver from chronic injury and reduced the number of apoptotic cells in these rats (Xiao et al., 2013b). SAMC treatment could also enhance the expression of key autophagic markers in the liver, with a concomitant decrease in the activities of LKB1/AMPK and PI3K/Akt pathways as well as a decrease in the activity of antiautophagic regulator mTOR (Xiao et al., 2013b).

However, some other herbal extracts activate autophagy and apoptosis simultaneously. Schisandrin B (Sch B) is an active dibenzocyclooctadiene isolated from *Schisandrae fructus*, with a wide array of pharmacological activities (Gao et al., 2016; Ran et al., 2018). Sch B has been shown to exhibit potent proapoptotic and proautophagic effects in AML-12 and RAW 264.7 cells (Zhang et al., 2015b). The inhibition of PI3K/protein kinase B (Akt)/mTOR signaling pathway is thought to associated with the proautophagic activities of Sch B (Zhang et al., 2015b).

AUTOPHAGY INDUCING HM EXTRACTS AND NATURAL PRODUCTS ON INFLAMMATION

Inflammation is the typical pathological feature of NASH. Inflammation is frequently triggered by various signals, including pro-inflammatory cytokines and chemokines, that are released from injured hepatocytes and activated Kupffer cells (Liu et al., 2016). Suppression of autophagy has been observed in Kupffer cells in the steatotic liver, and Kupffer cells with low autophagy activity are sensitized to endotoxin and subsequent inflammatory process (Fukada et al., 2012).

Dioscin is a saponin extracted and isolated from *Polygonatum Zanlanscianense* Pamp. Dioscin has been shown to markedly decrease serum ALT and AST levels in animals with liver injury, and rehabilitate inflammation via decreasing the expression levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) (Zhang W. et al., 2016). In addition, dioscin has been shown to suppress collagen synthesis (Liu et al., 2015). Further studies have shown that Dioscin significantly decrease the expression of p-mTOR/mTOR level and sequentially activate autophagy (Xu et al., 2017).

Resveratrol has also been shown to decrease inflammatory infiltration in the liver of MCD-induced NASH animals, and decreased serum levels of ALT, AST, IL-1β, IL-6, and TNF-α in these animals which were associated with autophagy (Ji et al., 2015). In addition, resveratrol is reported to restore liver injury via activating autophagy and suppressing NF-κB activation in experimental animals (Li et al., 2014), suggesting autophagy activation could serve as an anti-inflammatory strategy and have the potential to prevent NAFLD progression.

CONCLUSION AND PERSPECTIVES

HM extracts and natural products are effective in treating NAFLD and related complications (**Table 1**), although the mechanisms are still under exploration. Recently studies provide valuable information on its role of HM extracts and natural products via activating autophagy. As discussed above, autophagy inducing agents are confirmed to be beneficial in NAFLD treatment in animal models and cell lines. The commonly used HM extracts and natural products, such as resveratrol, Rb2, dioscin, LBPs, GCM, are potential candidates for preventing and treating NAFLD/NASH, and biochemical and histological experiments have demonstrated their ability to activate autophagy (**Figure 1**). Numerous HM extracts and natural products are indicated in activating autophagy, however, the results should be interpreted with caution, since multiple targets are involved, and most of the data are obtained from studies with animals or cells, clinical evidence are urgently needed to evaluate the therapeutic effects and safety. Nevertheless, HM and natural products provide a promising choice, and activation of autophagy might be an underlying mechanism for the efficacy of the compounds.

AUTHOR CONTRIBUTIONS

GJ proposed the topic and made the frame. LZ composed most of the context. GJ and ZY revised the manuscript.

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6-Bromoindirubin-3′-Oxime (6BIO) Suppresses the mTOR Pathway, Promotes Autophagy, and Exerts Anti-aging Effects in Rodent Liver

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Liver aging is associated with age-related histopathological and functional changes that significantly enhance the risk of numerous diseases or disorders developing in elderly populations. 6-Bromoindirubin-3′-oxime (6BIO), a potent inhibitor of glycogen synthase kinase-3 (GSK-3), has been implicated in various age-related diseases and processes, such as tumorigenesis, neurodegeneration, and diabetes. Recent studies have also revealed that 6BIO increases autophagy in yeast, mammalian cell lines, and dopaminergic neurons, which is one of the classical mechanisms strongly associated with liver aging. However, the impact or the mechanism of action of 6BIO in liver remains entirely unknown. Here, we find that 6BIO reduces oxidative stress, improves lipid metabolism, enhances autophagy, and significantly retards liver aging *via* modulating the GSK-3β pathway and mTOR pathway. Our findings suggest that 6BIO could be a potential agent to protect the liver in the field of anti-aging pharmacology.

Keywords: 6-bromoindirubin-3′-oxime, aging, autophagy, mammalian target of rapamycin, glycogen synthase kinase-3β

INTRODUCTION

Aging is broadly defined as the time-dependent functional decline of a living organism, usually accompanied by the age-related gradual accumulation of damaged biomolecules, which eventually results in the disruption of cellular homeodynamics. This deterioration is the primary risk factor for major human pathologies, including neurodegenerative diseases cancer and diabetes (Lopez-Otin et al., 2013). The development of pharmacological intervention in age-related functional decline and pathological changes has attracted much attention in the field of biology (Kennedy and Pennypacker, 2014; Vaiserman et al., 2016).

GSK-3 was first identified by its role in glycogen synthase phosphorylation (Embi et al., 1980) and later reported as a key protein that may function as an intermediate in inflammation and metabolism (Orellana et al., 2015). 6-Bromoindirubin-3′-oxime (6BIO) is a selective inhibitor of glycogen synthase kinase-3α/β (GSK-3α/β) derived from Tyrian purple indirubin (Zhao et al., 2017). It is reported as a promising novel agent for therapeutic

intervention in several age-related diseases, including diabetes, neurodegenerative disorders, leukemia, and cancer (Avrahami et al., 2013; Shen et al., 2015; Zhang et al., 2017; Li et al., 2018; Liu et al., 2018). It also suppresses the cellular senescence-related accumulation of damaged biomolecules and modulates cellular processes associated with aging, such as inflammation, oxidative stress, cellular viability, proliferation, and apoptosis (Nicolaou et al., 2012; Tsakiri et al., 2017; Zhang et al., 2017).

Recent studies have revealed that 6BIO is also a potent autophagy modulator. Accordingly, 6BIO reduces the oxidative load and upregulates autophagy-related protein Beclin1 in human diploid skin fibroblasts (Sklirou et al., 2017). Another study found that 6BIO induced autophagy in dopaminergic neurons of mice midbrain in order to clear toxic protein aggregates in a preclinical model of Parkinson disease, the second most common neurodegenerative disorder, and a type of age-related disease. This research also implies that 6BIO diminishes phosphorylation of ribosomal protein S6 kinase B1 (RPS6KB1) and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), which are downstream targets of the mammalian target of rapamycin (mTOR) (Suresh et al., 2017).

However, especially in *in vivo* models, relevant studies about 6BIO are mainly confined to the area of neurology, immunology, and oncology (Braig et al., 2013; Klamer et al., 2013; Suresh et al., 2017; Zhao et al., 2017). Whether 6BIO can suppress liver aging, and the mechanisms underlying its probable anti-aging effects, remains unclear. Considering that lipid metabolism is closely related to liver function and that autophagy, as well as inflammation and oxidative stress, is a vital factor in liver aging (Martinez-Cisuelo et al., 2016), we assume that 6BIO, which affects lipid metabolism and modulates autophagy, inflammation, and oxidative stress, may also play a pivotal role in liver aging *via* autophagic pathways.

Therefore, to determine the role of 6BIO in liver aging, we evaluated the effect of 6BIO on aging characteristics and age-related hepatic changes. We also investigated the possible molecular mechanisms involved in the geroprotective effect of 6BIO. Our results suggested that 6BIO treatment significantly ameliorates age-related changes, including reducing oxidative stress, improving lipid metabolism, and enhancing autophagy through the mTOR and GSK-3β pathways.

MATERIALS AND METHODS

Animals

Male young (2-month-old) and aged (18-month-old) mice were purchased from the Experimental Animal Center of the Chinese People's Liberation Army Fourth Military Medical University and were maintained on a 12:12 h light/dark cycle with lights on at 8:00 am. All mice had *ad libitum* access to water and food. The experiment was performed on four different groups, with eight mice per group: a young control group, an aged control group, a 6BIO treatment group, and a rapamycin treatment group. The rapamycin treatment was used as the positive control because rapamycin is reported to ameliorate liver aging by inducing mTOR-regulated autophagy (Ehninger et al., 2014). Young male mice were used in the young control group, and aged male mice were used in the other three groups. To test the anti-aging effect of 6BIO and rapamycin, the mice were injected with 6BIO (10 mg/kg) and rapamycin (4 mg/kg) in 10 ml/kg of saline intraperitoneally every day for 2 weeks (Siegmund et al., 2017; Suresh et al., 2017). The young control group and the aged control group received the same volume (10 ml/kg) of 0.9% NaCl. Food intake was measured daily. Body and fasting blood glucose measurements were recorded, and then the mice were sacrificed. Blood and liver samples were harvested after the injection period. Blood was drawn by cardiac puncture. All experimental protocols were approved by the Animal Experimentation Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University.

Biochemical Examinations and Enzyme-Linked Immunosorbent Assays (ELISAs)

Serum and hepatic levels of total cholesterol (TC) and triglyceride (TG) were determined using commercially available kits provided by Nanjing Jiancheng Institute of Biotechnology (Nanjing, China), according to the manufacturer's protocol. The extraction and analysis of liver lipids were conducted according to the procedure described by Kim et al. (2017)'s Materials and Procedure—extended for details, http://dx.doi.org/10.17504/protocols.io.iy7cfzn. Serum insulin and serum and hepatic IL-6 were measured using a mouse insulin ELISA kit (Alpine Immune Sciences, Hong Kong, China) and a mouse IL-6 ELISA kit (Biovendor, Czech Republic), respectively, following the manufacturer's instructions.

Assay of Antioxidant Markers

The liver specimens were homogenized in PBS (phosphate-buffered saline) to prepare a 10% liver homogenate, and centrifugated at 3,000 rpm for 10 min at 4°C. The supernatant was then collected for the assays. Hepatic malondialdehyde (MDA) activities, superoxide dismutase (SOD) levels, and glutathione (GSH) content were measured spectrophotometrically using commercially available kits supplied by the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). All assays were performed according to the manufacturer's instructions.

Western Blot Analysis

Western blot was performed using a standard protocol. Tissue specimens were homogenized and lysed with RIPA lysis buffer. The concentrations of total protein were determined using the BCA (bicinchoninic acid) method. Proteins were separated equally from each specimen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Thereafter, the membranes were blocked with 5% skim milk and incubated overnight with the appropriate antibodies at 4°C followed by horseradish peroxidase (HRP)-labeled secondary antibody for 1 h at room temperature. The primary antibodies included

GSK-3β rabbit mAb (1:1,000; Cell Signaling Technology Inc., the US. #12456), phospho-GSK-3β (Ser9) rabbit mAb (1:1,000; Cell Signaling Technology Inc. #5558), mTOR rabbit mAb (1:1,000; Cell Signaling Technology Inc. #2983), phospho-mTOR (Ser2448) rabbit mAb (1:1,000; Cell Signaling Technology Inc. #5536), AKT rabbit mAb (1:1,000; Cell Signaling Technology Inc. #4691), phospho-AKT (Ser473) rabbit mAb (1:1,000; Cell Signaling Technology Inc. #4060), P53 rabbit mAb (1:1,000; Proteintech #10442-1-AP), P16 rabbit mAb (1:1,000; abcam. ab51243), β-gal rabbit mAb (1:1,000; Cell Signaling Technology Inc. #27198), LC3I/II rabbit mAb (1:1,000; Cell Signaling Technology Inc. #3868), P62 rabbit mAb (1:1,000; Cell Signaling Technology Inc. #88588), beclin-1 rabbit mAb (1:1,000; Cell Signaling Technology Inc. #3495), and GAPDH rabbit mAb (1:5,000; abcam.ab8245). Finally, the immunoreacting bands were visualized using an enhanced chemiluminescence (ECL) method.

Oil Red O Staining

Frozen specimens were cut into thin sections about 10 μm thick and then stained with filtered Oil Red O (cat. no. 1.02419; EMD Millipore, Billerica, MA, USA) dissolved in 60% isopropanol for 15 min at room temperature. Next, the slides were incubated in hematoxylin to counterstain the nuclei and transferred to aqueous mounting medium. Images were captured under a light microscope (magnification ×200 and ×400).

Transmission Electron Microscopy

Liver samples were fixed in 3% glutaraldehyde for 24 h at 4°C, post-fixed in 1% osmium tetroxide in sodium phosphate buffer at room temperature, and cut into ultrathin sections (50–70 nm) on an ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate, and then viewed and photographed using a Hitachi H-7650 transmission electron microscope at 120 kV. Ultrastructural analysis was performed using ImageJ (NIH) analysis.

Statistical Analysis

Sample sizes, as described in the figure legends, were selected based on effect size and availability, according to the usual standard. Statistical analysis was performed using GraphPad Prism 7.0 software. Data were presented as mean ± standard deviation (SD). One-way ANOVA analysis was applied to assess the statistical significance, using Tukey's *post hoc* analysis when required. $p < 0.05$ was considered statistically significant.

RESULTS

6BIO Attenuated Hepatic Oxidative Stress and Inflammation

Oxidative stress is strongly believed to contribute to the aging and the pathogenesis of a considerable number of degenerative diseases (Yew et al., 2018). Therefore, we evaluated the effects of 6BIO on aged mouse liver in relation to antioxidant enzymes SOD, GSH, and MDA. As shown in **Figures 1A–C**, compared with the young mouse liver, in the aged mouse liver, the level of SOD and GSH was significantly ($p < 0.05$) reduced, while the level of MDA was significantly ($p < 0.05$) increased. The treatment with 6BIO or rapamycin was observed to bring the level of SOD close to that in the young control. 6BIO and rapamycin significantly ($p < 0.05$) increased the level of GSH in the old mouse liver. In addition, they reduced the level of MDA.

Aging is also accompanied by increased levels of pro-inflammatory cytokines such as IL-6; this appears to be linked to the immunosenescence process (da Cunha and Arruda, 2017).

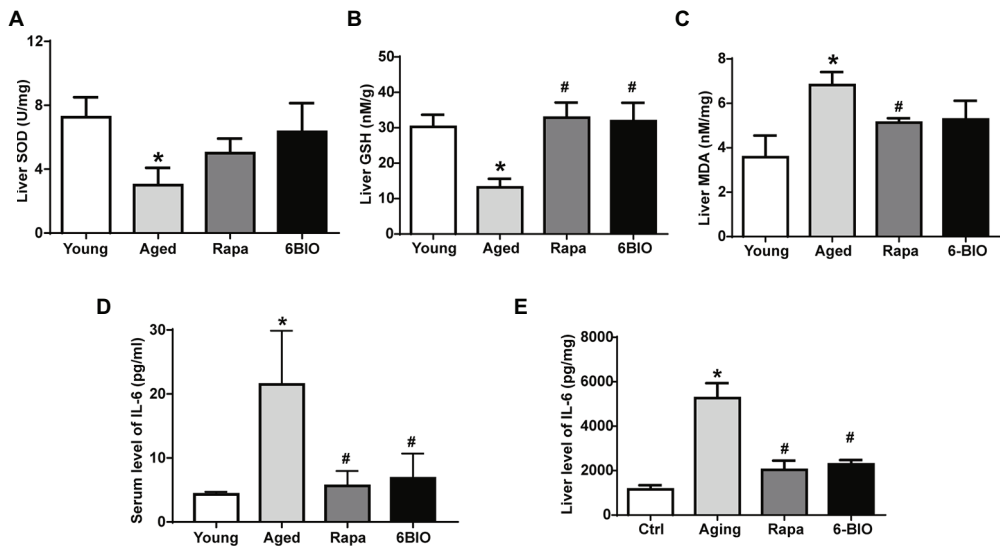

FIGURE 1 | 6BIO attenuated hepatic oxidative stress and inflammation. The levels of **(A)** SOD, **(B)** GSH, and **(C)** MDA in liver tissue were measured by using commercially available kits. The **(D)** serum and **(E)** hepatic levels of IL-6 were measured by ELISAs. The results are shown as the mean ± SD of eight animals per group. $^{*}p < 0.05$ compared with the young control group; $^{\#}p < 0.05$ compared with the aged control group; $^{\&}p < 0.05$ compared with the rapamycin treatment group.

As shown in **Figures 1D,E**, the mean values of both serum and hepatic IL-6 were significantly ($p < 0.05$) higher in the aged group than in the young control group. The 6BIO-treated group and rapamycin-treated group showed significantly ($p < 0.05$) lower IL-6 levels in serum and liver than the untreated aged group.

These results suggest that 6BIO and rapamycin treatment may attenuate oxidative stress and inflammation associated with aging in rodent liver.

2-Week Treatment With 6BIO, Compared With Rapamycin, Exerted a Strong Effect on Hepatic Lipid Metabolism but Only a Mild Effect on Glucose Metabolism

There was no significant difference in mean food intake between the aging mice, the rapamycin-treated mice, and the 6BIO-treated mice (**Figure 2A**). The changes in serum lipid levels are shown in **Figures 2B,C**. Analyses of serum lipid concentration showed significantly ($p < 0.05$) higher levels of serum triglycerides (TG) and TC in the aged group than in the young control group. 6BIO and rapamycin significantly ($p < 0.05$) reduced the average levels of both serum TG and serum TC in the aged mice. The serum lipid-reducing effect of 6BIO was even significantly ($p < 0.05$) stronger than that of rapamycin.

We next analyzed the levels of hepatic TG and TC content (**Figures 2D,E**). 6BIO and rapamycin significantly ($p < 0.05$) reduced the average levels of hepatic TG and TC in the old-aged mice, closer to the level observed in the young control group. To further investigate whether 6BIO was able to affect hepatic steatosis, we then performed Oil Red O staining to illustrate the hepatic lipid content (**Figure 2F**). Histological analysis of the liver specimens showed marked hepatic steatosis in the old-aged group. A remarkably decreased accumulation of lipid droplets in the aged liver was observed in both the 6BIO-treated group and the rapamycin-treated group.

We also investigated the effect of 6BIO on glucose metabolism in the mice. Accordingly, the 2-week treatment with rapamycin or 6-BIO did not significantly alter blood glucose levels (**Figure 2G**). Nevertheless, rapamycin significantly ($p < 0.05$) reduced the level of fasting insulin, thereby indicating an improvement in insulin sensitivity. In the 6BIO-treated group, however, no significant difference was observed in fasting insulin compared to the aged control group (**Figure 2H**).

Overall, 6BIO reduced serum lipid concentration and lipid accumulation in liver and ameliorated the hepatic steatosis. The treatment with 6BIO even showed a mildly stronger effect than the rapamycin treatment on lipid metabolism. Nevertheless, the 2-week 6BIO treatment resulted in little improvement in insulin sensitivity.

6BIO Regulated the Senescence Markers of Mice Livers

To verify the effects of 6BIO on hepatic senescence, Western blot was used to analyze the levels of the senescence markers p16, p53, and β-gal (β-galactosidase). Previous studies had reported the upregulation of p16 and β-gal and the downregulation of p53 to be associated with age-related dysfunction. These molecular markers of senescence could be promoted as well-established endpoints when investigating anti-aging interventions (Niedernhofer et al., 2017).

As shown in **Figures 3A–D**, compared with the young control group, in the aged mouse liver, the levels of p16 and β-gal were found to be significantly elevated, while the expression of p53 decreased markedly, in line with the findings of other studies (Roos et al., 2016). The expression of p53 in both the 6BIO-treated group and the rapamycin-treated group was higher than that in the untreated old group ($p < 0.05$), while the levels of p16 and β-gal were significantly elevated in both the 6BIO-treated group and rapamycin-treated group.

Overall, both 6BIO and rapamycin exhibited a marked anti-aging effect. 6BIO treatment even showed a slightly better anti-aging effect than the rapamycin treatment, which is deemed to be one of the most promising anti-aging drugs (Wang et al., 2017).

6BIO Activated Autophagy in Old-Aged Murine Livers

It has been widely reported that autophagy plays a crucial role in the progression of aging (Nakamura and Yoshimori, 2018). Hence, we investigated whether autophagy is involved in the anti-aging effect of 6BIO. As shown in **Figures 4A–D**, the expression level of autophagy-related proteins LC3, Beclin1, and p62 was measured by Western blot. 6BIO treatment, as with rapamycin treatment, significantly increased the LC3II:LC3I ratio and the level of Beclin1, along with markedly reducing the level of p62 in the aged mouse liver. In addition, under the transmission electron microscope, fewer autophagosomes were observed in the aged mouse liver than in the young mouse liver, whereas more autophagosomes were observed in the liver specimen of the 6BIO-treated group and the rapamycin-treated group (**Figures 4E,F**).

Taken together, these results indicate that 6BIO is a promising autophagy inducer, and accordingly, autophagy may play a pivotal role in the positive effect of 6BIO treatment.

6BIO Inhibited Both the mTOR and the GSK-3β Signaling Pathway

It has been reported that the mTOR signaling pathway plays a pivotal role in the process of aging (Baar et al., 2016), and that it is a critical mechanism in terms of regulating autophagic capacity (Wong et al., 2017). To confirm the mechanism of the 6BIO-induced anti-aging effect, we investigated the mTOR signaling pathway. According to Western blotting analysis, both the 6BIO treatment and the rapamycin treatment inhibited phosphorylation of the mTOR (**Figures 5A,B**). In both the 6BIO-treated group and the rapamycin-treated group, Akt phosphorylation was significantly higher than that in the aged untreated group (**Figures 5A,C**), which supports the hypothesis that due to the inhibition of mTOR, the reduction in its downstream molecule S6K attenuates the S6K-mediated negative feedback control, thereby resulting in Akt activation (Lashinger et al., 2011).

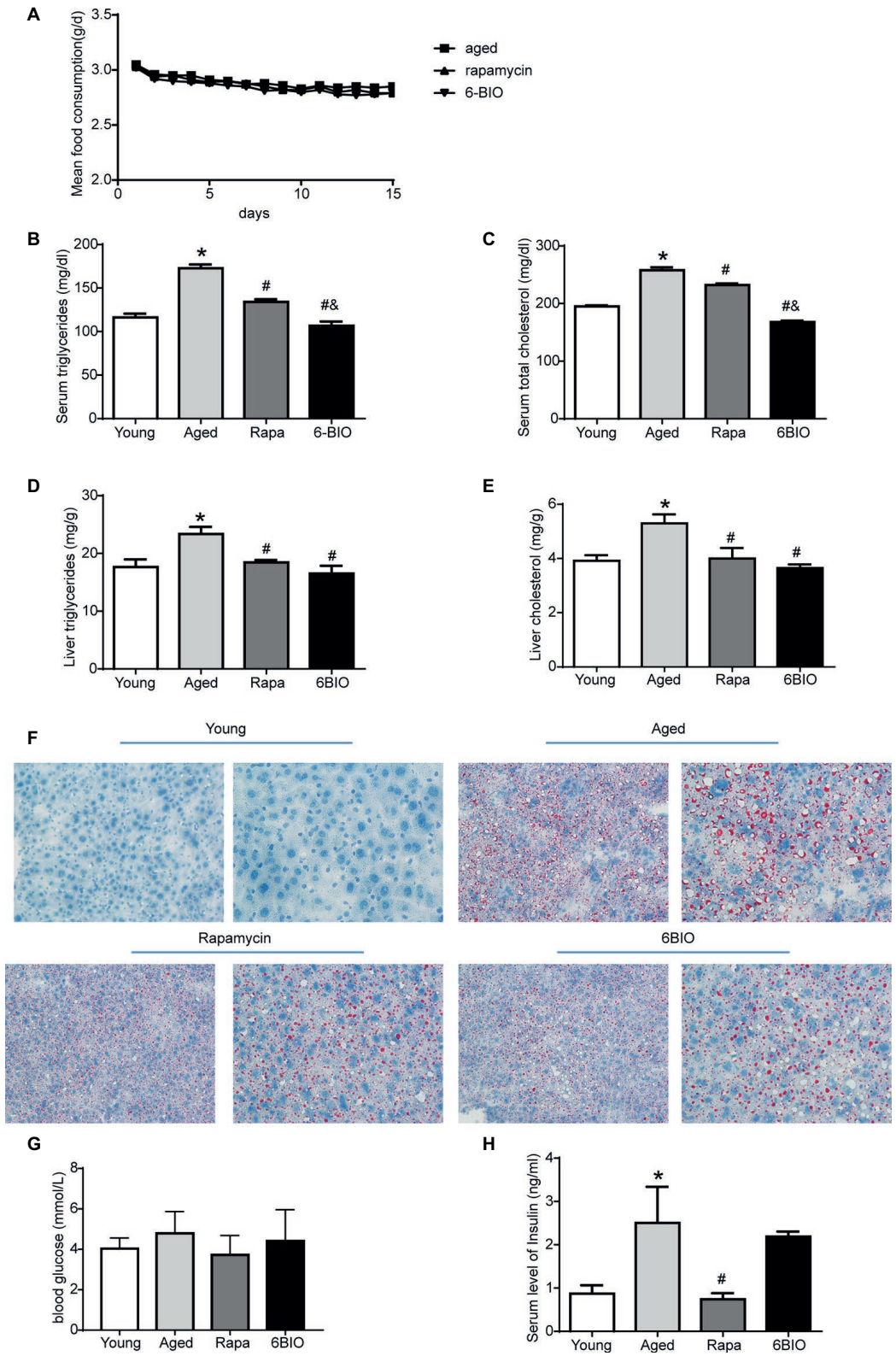

FIGURE 2 | Two-week treatment with 6BIO, compared with rapamycin, exerted a strong effect on hepatic lipid metabolism, but little effect on glucose metabolism. **(A)** Mean food consumption per mouse per day **(B)** Serum triglycerides. **(C)** Serum total cholesterol. **(D)** Liver triglycerides. **(E)** Liver cholesterol. **(F)** Liver steatosis assessed by Oil Red O staining. **(G)** Blood glucose. **(H)** Serum level of insulin. All values were expressed as mean ± SD of eight animals per group. $^{*}p < 0.05$ compared with the young control group; $^{\#}p < 0.05$ compared with the aged control group; $^{\&}p < 0.05$ compared with the rapamycin treatment group.

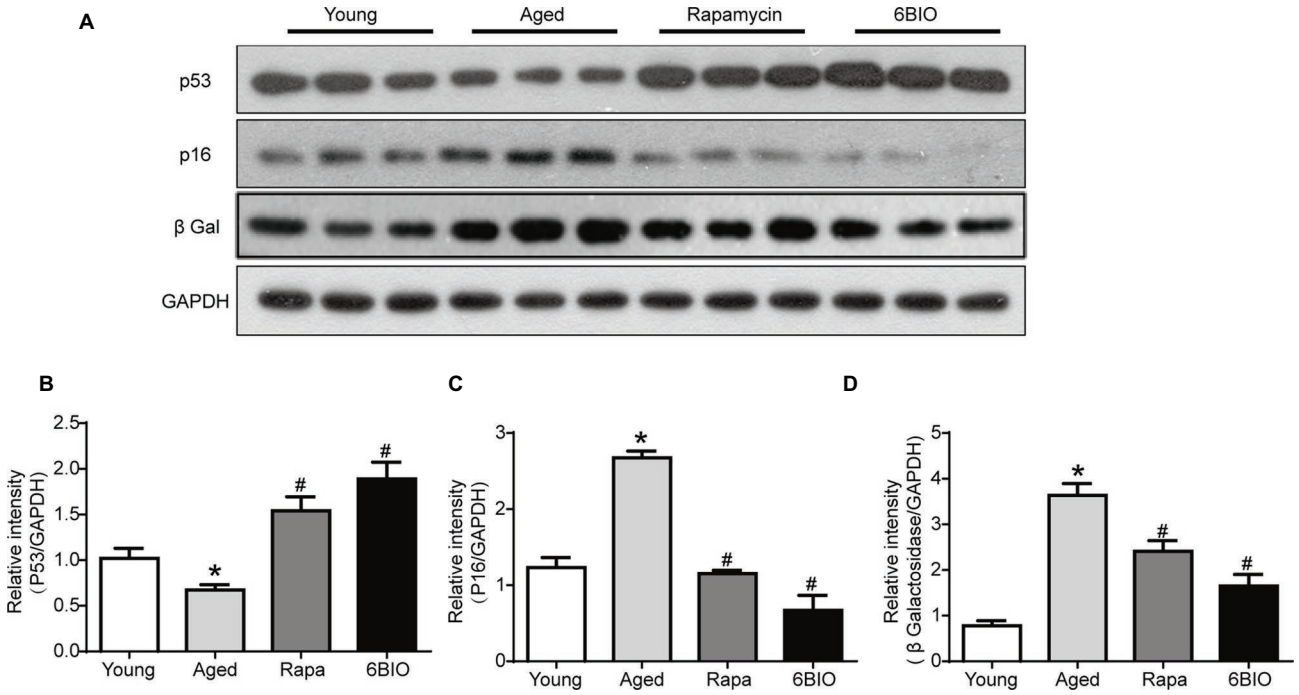

FIGURE 3 | 6BIO regulated the senescence markers of mouse livers. **(A)** Western blotting results revealed the changes in senescence markers. Protein expression levels of **(B)** p53, **(C)** p16, and **(D)** β-gal were evaluated by Western blot analysis. The results are shown as the mean ± SD of eight animals per group. $^{*}p < 0.05$ compared with the young control group; $^{\#}p < 0.05$ compared with the aged control group; $^{\&}p < 0.05$ compared with the rapamycin treatment group. GADPH was used as a loading control.

As 6BIO is a known potent inhibitor of GSK-3α/β, we then explored the expression of p-GSK-3β. The level of GSK-3β phosphorylation on serine-9 was reduced significantly in the 6BIO-treated group, while conversely, the rapamycin-treated group exhibited an increasing level of p-Gsk-3β with increasing upregulation of Akt phosphorylation compared with the aged group (**Figures 5A,C,D**). GSK-3β Serine-9 phosphorylation is part of a negative feedback loop and 6BIO-mediated inhibition of GSK-3B activity was previously shown to be associated with the downregulation of this inhibitory phosphorylation (Sklirou et al., 2017).

These results indicate that 6BIO inhibits both the mTOR and the GSK-3β signaling pathway, while rapamycin positively regulates GSK-3β signaling.

DISCUSSION

Anti-aging pharmacology helps prevent the vast majority of degenerative disorders and prolong lifespan by delaying aging rather than targeting all the age-related pathological manifestations one by one (Kennedy and Pennypacker, 2014). It has been reported that 6BIO, a potent inhibitor of GSK-3α/β, has made a promising impact on age-associated diseases such as cancer and neurodegenerative diseases (Beurel et al., 2015; McCubrey et al., 2016). By contrast, little research has focused on the effect, and mechanism of action, of this potent drug on models of natural aging, especially liver aging. In this study, we aimed to investigate the anti-aging effect, and molecular mechanism, of the novel anti-aging drug 6BIO on naturally aged mouse liver. Rapamycin, a well-known promising anti-aging drug that delays aging through mTOR-dependent autophagy (Zhou and Ye, 2018), was used as the positive control in the study. To our knowledge, this is the first study to demonstrate the effects of 6BIO treatment in models of natural aging.

Our results indicated that 6BIO ameliorates the decline of liver function with age, including lipid metabolism disorder, and attenuates hepatocyte senescence in aged mice, as revealed by alterations in the cellular senescence markers. Also, we investigated the possible molecular anti-aging mechanisms of action, and respective signaling pathways, of 6BIO, some of which relate to age-associated changes, too, such as the changes in canonical p53/p21 and p16/pRB signaling pathways, oxidative stress, inflammation, autophagy, the GSK-3β signaling pathway, and the mTOR signaling pathway.

Lipid and glucose metabolic disorders are common manifestations in aging models, thereby correlating closely with a decline in liver function. One characteristic of hepatic senescence is fat deposition (He et al., 2018). Our results showed an increase in fat deposition and disordered lipid metabolism during the aging process of the aged mice. In the 6BIO treatment group, the serum levels of TC and TG decreased, thereby indicating an amelioration of lipid metabolic disorders as a result of improved liver function. Moreover, 6BIO treatment rectifies the hepatic levels of TC and TG, and lipid droplet accumulation in liver. Overall, the 6BIO treatment even exerted

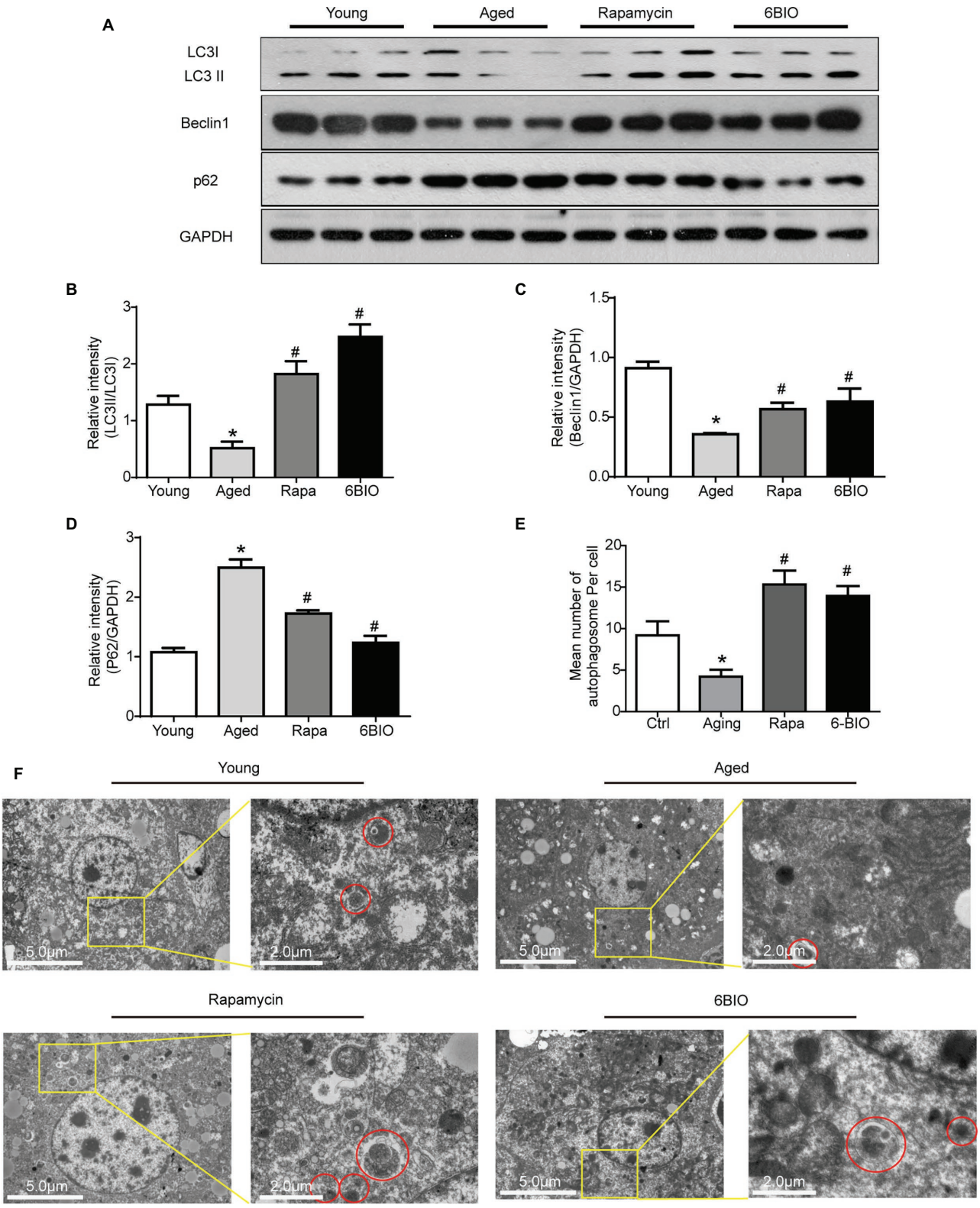

FIGURE 4 | 6BIO activated autophagy in aged murine livers. **(A)** Western blotting and the analysis of the Western blot results of autophagy-related proteins **(B)** LC3II/I, **(C)** beclin1, and **(D)** p62. **(E)** Mean number of autophagosome per cell under electron microscope (three hepatocytes were counted per group at 1.2k× magnification). **(F)** Observation of autophagosomes through an electron microscope. All values were expressed as mean ± SD of eight animals per group. $^{*}p < 0.05$ compared with the young control group; $^{\#}p < 0.05$ compared with the aged control group; $^{\&}p < 0.05$ compared with the rapamycin treatment group.

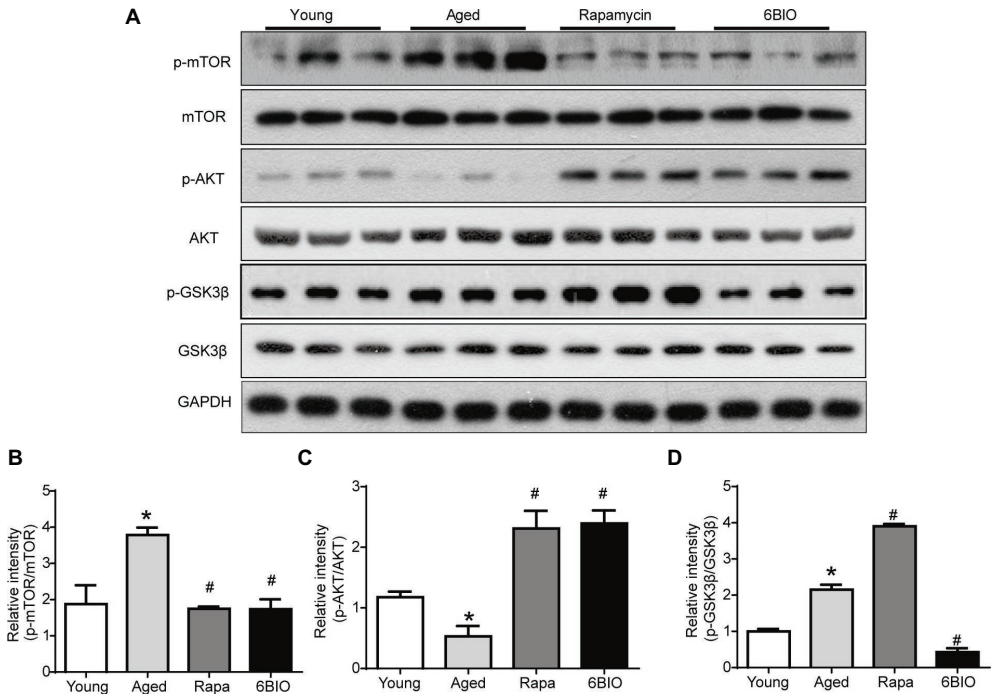

FIGURE 5 | 6BIO inhibited both the mTOR and the GSK-3β signaling pathway. **(A)** The phosphorylated mTOR, the total mTOR expression, the phosphorylated Akt, the total Akt expression, the phosphorylated GSK-3β, and the total GSK-3β expression were examined by Western blot. **(B)** p-mTOR to mTOR ratio. **(C)** p-Akt to Akt ratio. **(D)** p-GSK-3β to GSK-3β ratio. The results are shown as the mean ± SD of eight animals per group. $^{*}p < 0.05$ compared with the young control group; $^{\#}p < 0.05$ compared with the aged control group; $^{\&}p < 0.05$ compared with the rapamycin treatment group.

a slightly better effect on lipid metabolism—or, in other words, on lipid metabolism in liver—than the rapamycin treatment. Apart from lipid-related metabolic disorder, the higher serum level of insulin in the aged group suggested an impaired insulin sensitivity resulting from aging. Nevertheless, a 2-week treatment with 6BIO may be too short a duration to exert a significant improvement in insulin sensitivity.

We also examined cell senescence markers to better understand aging in hepatocytes. Molecular senescence markers such as p53, p16, and β-gal are well-established endpoints for quantifying the degree of cell aging (Niedernhofer et al., 2017). According to our findings and those of other studies, p53 was downregulated, and the level of p16 elevated, by the aging process in the aged mice. β-Gal is an enzyme whose protein expression levels in the aged mice were found to be higher than in the young control mice in our study, which is consistent with the findings of another study (Niedernhofer et al., 2017). 6BIO treatment significantly ameliorated the level of these senescence markers, indicating the attenuation of cell senescence. Moreover, p53 and p16 are not only the biomarkers of cell senescence but also controllable factors regulating the aging process (Yi et al., 2013). It is reported that p53 extends lifespan and maintains normal tissue homeostasis (Qian and Chen, 2013), the ablation of p16 cells in rodents promotes longevity and alleviates age-related dysfunction (Baker et al., 2016), the deficiency of p16 induces elevated p53, and the activity of β-gal is strongly associated with the expression of p16 (Niedernhofer et al., 2017). Hence, our finding that 6BIO activated p53, reduced p16, and diminished β-gal also suggests that an overexpression of p53, reduction in p16, and downregulation of β-gal could constitute a possible pathway for the anti-aging effect of 6BIO. Further research is needed to determine the specific interactions between 6BIO and these molecules.

Oxidative stress and inflammation are two of the most well-known canonical aging mechanisms (Park et al., 2014; Zhang et al., 2015). SOD functions as an antioxidant enzyme, while GSH is a most vital catalase and a nonenzymatic antioxidant. The end product of lipid peroxidation, MDA, is measured to reflect the level of hepatic oxidative damage. Similar to the findings of previous studies (Jimenez et al., 2018), the aged mice exhibited lower levels of SOD and GSH than the young mice, whereas a marked increase in MDA was observed in the aged group, indicating that liver oxidative stress increases with increasing age. Studies also report that the production of pro-inflammatory cytokines, such as IL-6, is linked to the aging process (Kim et al., 2016). Our results showed that the aging process was accompanied by a hepatic pro-inflammatory response, which is reflected by the upregulation of IL-6. In the 6BIO treatment group, a marked improvement in oxidative stress and inflammation in the aged livers was observed in our study. SOD and GSH activities were found to be significantly elevated, MDA levels decreased markedly, and the IL-6 expression was significantly upregulated when the aged mice were treated with 6BIO. Oxidative stress and inflammation were found to be similarly diminished in the rapamycin treatment group.

Autophagy is a vital degradation process that controls cellular and organismal homeostasis in mammals and plays a pivotal

role in liver aging. There is a strong correlation between autophagy and age-associated liver dysfunction (Czaja, 2010). In addition, recent studies have revealed that 6BIO is a novel autophagy inducer in human diploid skin fibroblasts and in dopaminergic neurons of mice midbrain. Nevertheless, so far, no research has proven whether this agent regulates autophagy in liver. In the present study, the decrease in LC3II:I ratio and beclin-1 level, the increase in p62 level, and the reduced number of autophagosomes observed under microscopy in the aged group demonstrated a decline in autophagy with aging. Our findings showed that 6-BIO remarkably induces autophagy. Our study revealed—for the first time—that 6BIO treatment induces autophagy in aging liver. 6BIO may delay aging and ameliorate age-related alterations through an autophagy pathway.

Meanwhile, taking into consideration that the mTOR pathway negatively controls autophagy, we also investigated whether 6-BIO affects the mTOR signaling pathway. Since 6BIO is known to be a potent GSK-3α/β inhibitor, it seemed logical to observe the GSK-3β pathway also. The level of Akt phosphorylation was observed because Akt is upstream of both the mTOR pathway and GSK-3β pathway. Previous studies have reported that inhibition of the mTOR pathway and GSK-3β pathway ameliorated age-related pathologic changes (Chen et al., 2018; Yuan et al., 2018). The results showed that 6BIO not only inhibits GSK-3β signaling, in concordance with the findings of previous studies (Ko et al., 2018), but also inhibits mTOR signaling, whereas rapamycin inhibits the mTOR pathway but activates GSK-3β signaling *via* feedback activation of Akt originating from S6K (Wu et al., 2018). Moreover, the phosphatidylinositol-3 kinase (PI3-K)/phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway, which positively regulates cell growth, is implicated in some anti-aging mechanisms (Haga et al., 2009). The inhibition of PI3K or PDK1 will lead to the suppression of Akt phosphorylation and downstream mTOR signaling. However, the increase in the p-Akt/Akt ratio in the 6BIO-treated group indicates that 6BIO inhibits the phosphorylation of mTOR and GSK-3β rather than affecting PDK1/Akt signaling. In previous studies, 6BIO was found to alleviate age-related diseases *via* the GSK-3β pathway. Our own results indicated that 6BIO may exert its anti-aging effect on liver *via* crosstalk with another novel pathway—the mTOR signaling pathway. It is reported that, additionally, GSK-3β inhibition may induce autophagy in human pancreatic cancer cells and in prostate cancer cells (Marchand et al., 2015; Sun et al., 2016). Overall, our results support the model illustrated in **Figure 6**. 6BIO treatment might induce autophagy to exert an anti-aging effect *via* the inhibition of both mTOR and GSK-3β signaling.

Currently, 6BIO has been tested in certain preclinical disease models, such as in Parkinson disease models, intracerebral hemorrhage models, and leukemia models, and such research has provided promising evidence of efficacy (Shen et al., 2015; Suresh et al., 2017; Zhao et al., 2017). Our study also points to the clinical potential of therapeutic 6BIO in liver. However, to date, no clinical trial concerning 6BIO has been conducted. In fact, some matters still require attention before being applied to a clinical setting. A recent study indicated that GSK-3β inhibitor suppresses B- and T-cell development in stem cells, which signifies it should be tested carefully, particularly in conjunction with stem cell transplantation and adoptive T-cell immunotherapy (Shen et al., 2015).

In summary, according to this study, 6BIO delays liver aging and ameliorates age-associated alterations, including lipid metabolism, hepatic oxidative stress, and hepatic inflammation. In particular, in terms of balancing lipid metabolism, 6BIO seems to be even slightly more effective than rapamycin. Besides, the levels of the senescence markers also indicated that the 6BIO-treated group fared better, although not significantly, than the rapamycin-treated group. Overall, despite the fact that rapamycin appears to have a more positive effect than 6BIO on insulin resistance, the 6BIO treatment showed a remarkable anti-aging effect, functioning even slightly better than the rapamycin treatment, which is deemed to be one of the most promising anti-aging drugs (Wang et al., 2017). Accordingly, we suggest that besides inhibiting the GSK-3β pathway, 6BIO may exert its anti-aging

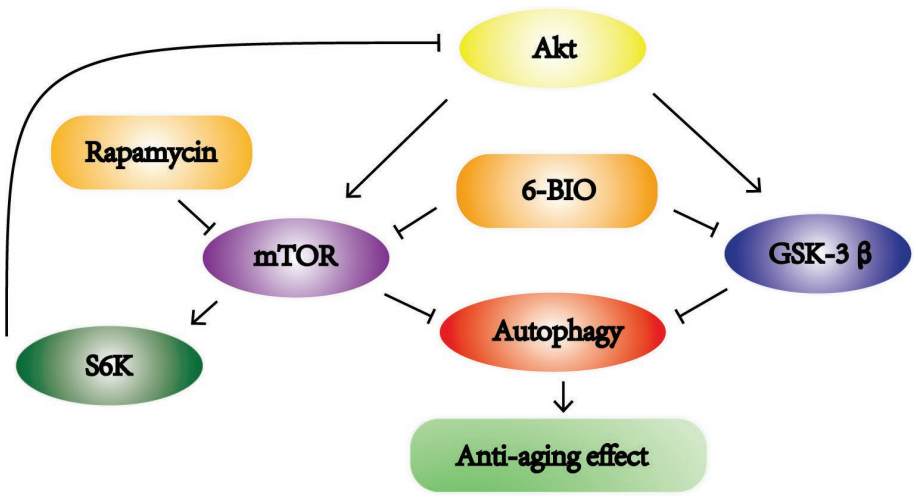

FIGURE 6 | Schematic illustration of this study. 6BIO treatment induced autophagy to exert an anti-aging effect by inhibiting mTOR and GSK-3β signaling.

effect in liver by inducing autophagy through mTOR inhibition. FOXO transcription factors are important mediators of autophagy-related gene expression and aging (Martins et al., 2016). It will be interesting to analyze how these factors are regulated in liver upon 6BIO-treatment. Cytoprotective effects of 6BIO were shown to depend on Nrf2, an inducer of antioxidant genes which is compromised during aging (Sklirou et al., 2017; Tsakiri et al., 2017; Zhou et al., 2018). Understanding the exact nature of the underlying mechanisms by which 6BIO delays or reverses aging downstream to mTOR/GSK-3B requires further investigations possibly in these directions.

AUTHOR CONTRIBUTIONS

DG designed the research, performed the experiments, analyzed the data, and drafted the manuscript. YZho and YM supervised the research, critically revised the manuscript for important intellectual content, and obtained funding. YS, WL, QL, YZha, CP, and BC were also involved in performing the experiments. All authors read and approved the final manuscript.

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Salvianolic Acid B Inhibits Activation of Human Primary Hepatic Stellate Cells Through Downregulation of the Myocyte Enhancer Factor 2 Signaling Pathway

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Various isoforms of myocyte enhancer factor 2 (MEF2) have been shown to play a role in the activation of rat hepatic stellate cells (HSCs) in culture. The signals that regulate MEF2 in HSCs are unknown. In addition, whether MEF2s regulate the activation of human HSCs (H-HSCs) is unclear. Here, we studied the expression and function of MEF2s in H-HSCs. Our data showed that the levels of MEF2A, C, and D proteins were high in liver tissues from patients with cirrhosis and increased during culture-induced activation of primary H-HSCs. Exposure of H-HSCs to transforming growth factor beta 1 (TGF-β1) led to a significant increase in MEF2A and C protein levels and enhanced MEF2 activity. Interestingly, TGF-β1 did not further enhance MEF2D levels. Furthermore, TGF-β1 activated p38 mitogen-activated protein kinase (MAPK) and led to increased phosphorylation of MEF2C at its p38 recognition site. Inhibition of p38 MAPK inhibited both TGF-β1- and culture-induced activation of MEF2. The activity of collagen I reporter in H-HSCs was significantly reduced when MEF2A and MEF2C were blocked with overexpression of dominant negative MEF2 mutants. Salvianolic-acid B (SA-B), a water-soluble element of *Salvia miltiorrhiza* known to have anti-fibrosis effects, attenuated both basal and TGF-β1-induced increased levels of MEF2A and C mRNA and protein. In addition, SA-B inhibited MEF2 activity, which correlated with reduced expression of the HSC activation markers, α-smooth muscle actin (α-SMA), and collagen I. Administration of SA-B reduced MEF2A *in vivo*, which was accompanied by reduced levels of α-SMA in a model of dimethylnitrosamine-induced rat liver fibrosis. We concluded that the MEF2 transcription factor was stimulated by TGF-β1 in H-HSCs. Antagonizing TGF-β1-induced activation of the MEF2 signaling pathway may account in part for the anti-fibrosis effects of SA-B.

Keywords: myocyte enhancer factor 2, hepatic stellate cell, TGFβ1, salvianolic acid B, liver fibrosis

INTRODUCTION

Hepatic stellate cells (HSCs) are the main cells responsible for liver fibrosis. Activation of HSCs, which involves the transition of quiescent cells into highly proliferative, fibrogenic, and contractile myofibroblasts, represents the final common pathway of the hepatic response to liver injury induced by various factors. Transforming growth factor beta1 (TGF-β1) activates HSCs and is considered the most potent cytokine to perpetuate the fibrogenic response in the liver (Friedman, 2008; Tsuchida and Friedman, 2017). The pro-fibrogenic effects of TGF-β1 are mediated through several key downstream effectors. These primarily include p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1 and 2 (ERK1, 2), and Smad signaling pathways (Zhang, 2018).

Interestingly, many studies suggest that HSCs isolated from rats and humans, and myofibroblasts, their activated counterparts, in particular, express a number of neuronal and muscle cell markers, such as neurotrophins, neurotrophin receptors, brain-derived nerve growth factor, MyoD, and α-smooth muscle actin (α-SMA) (Trim et al., 2000; Geerts, 2001; Vincent et al., 2001; Cassiman et al., 2002). This suggests that factors involved in the regulation of neuronal and muscle cells may also play a role in HSCs. Indeed, our previous studies have shown that the transcription factor, myocyte enhancer factor 2 (MEF2), represents a class of proteins known to play key roles in neurons and muscle cells and participates in the activation of rat HSCs in culture (Wang et al., 2004). Four mammalian isoforms of MEF2, A–D, have been identified. The MADS and MEF2 domains present at the N terminus are highly homologous among MEF2s and are responsible for mediating dimerization among them and DNA binding. The C-terminus regions of MEF2 proteins are required for transcriptional activation (Black and Olson, 1998). Many important signaling pathways converge on MEF2. These include p38 MAPK, Smads, and calcium/calmodulin/calcineurin pathways (Mao and Wiedmann, 1999; Quinn et al., 2001; Olson, 2004; Wales et al., 2014). The p38 MAPK signaling pathway has been shown to regulate MEF2 transcriptional activity in various cell types and to promote neuronal survival and differentiation (Phiel et al., 2001; Illi et al., 2005; Ju et al., 2005; Ramachandran et al., 2008). However, whether and how MEF2 is regulated by TGF-β1 during liver fibrosis is not entirely clear.

Great efforts have been made to understand the molecular mechanisms that underlie HSC activation during liver fibrosis. Currently, much of our knowledge of how stellate cells behave during liver injury has been gained through studies using animal model systems (Tsuchida and Friedman, 2017). However, whether and to what degree the mechanisms identified in animal systems operate in (H-HSCs) is not entirely clear and requires validation in order to establish their relevance to human disease. Therefore, we studied the regulation of MEF2 in H-HSCs. Our data revealed high levels of MEF2 expression in human cirrhotic liver tissues and freshly isolated H-HSCs. Expression of MEF2 was activated by TGF-β1 *via* p38 MAPK.

Salvianolic acid B (SA-B) is an effective water-soluble component of *Salvia miltiorrhiza*. Previous research indicates that SA-B inhibits the synthesis of collagen I (Col I) in LX-2 cells independently of TGF-β1 stimulation, and the anti-fibrotic effect of SA-B is due to direct inhibition of p38 signaling and inhibition of the cross-talk between Smad and ERK signaling (Lv and Xu, 2012). In another study, we found that SA-B lowers portal pressure in rats with dimethylnitrosamine (DMN)-induced cirrhosis *in vivo*. In addition, SA-B attenuates endothelin-1 (ET-1)-induced HSC contraction by inhibiting the activation of RhoA and Rho-associated coiled coil-forming protein kinase (ROCK) II and downstream MYPT1 phosphorylation at Thr^{696} *in vitro* (Xu et al., 2012). The present study indicates that SA-B inhibits MEF2 activity in H-HSCs and attenuates its levels in a liver fibrosis model in the rat.

Abbreviations: DMN, dimethylnitrosamine; ERK, extracellular signal-regulated kinase 1; GFAP, glial fibrillary acidic protein; H-HSCs, human hepatic stellate cells; p38 MAPK, p38 mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; SA-B, salvianolic acid B; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor β1.

MATERIALS AND METHODS

Materials

Human liver specimens were obtained from patients who had undergone liver resection or transplantation for liver carcinoma or end-stage chronic liver diseases, following the guidelines and with the approval of the institution. The study was approved by the Ethics Committee of Shuguang Hospital. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Salvianolic acid B (SA-B), an effective water-soluble element of Radix *Salvia miltiorrhiza*, was purified and provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Polyclonal antibodies for MEF2A and monoclonal antibodies for p-38, p-ERK1/2, and ERK1 were purchased from Santa Cruz Biotechnology (TX, United States); polyclonal antibodies for MEF2C and p-p38, from Cell Signaling; polyclonal antibodies for collagen I, from Calbiochem (CA, United States); monoclonal antibody for MEF2D, from BD Biosciences (MD, United States); and monoclonal antibodies for β-actin and α-SMA from Sigma-Aldrich (St Louis, MO, United States). Inhibitors of ERK (PD98059) and p38 MAPK (SB203580) were purchased from Calbiochem (CA, United States).

Isolation and Culture of HSCs

The HSCs were isolated as described previously (Zhang and Xu, 2007). Briefly, H-HSCs from ~50 g of liver tissue and HSCs from adult male Sprague–Dawley rats (~400 g) were isolated with pronase (Roche, Germany)/collagenase (Serva, Germany) treatment, followed by density gradient centrifugation using Nycodenz (Axis-shield, Norway) solution to isolate the buoyant and lipid rich stellate cells. Isolated HSCs were cultured in medium 199 (Gibco) containing 10% fetal calf serum. When the cultures reached confluence, they were trypsinized and passaged at a ratio of 1:3.

Immunofluorescent Staining

The H-HSCs were plated on glass coverslips (Fisher Scientific) in 12-well plates and cultured with 10% fetal calf serum in medium 199. After fixation in methanol/acetone (1:1), cells were washed twice with phosphate-buffered saline and incubated with blocking solution for 1 h. Primary antibodies (Neo Markers) and fluorescence labeled anti-rabbit IgG or anti-mouse IgG were incubated with the cells for 1 h respectively. Cells were mounted on glass slides using the ProLong Antifade kit (p-7481, Molecular Probes, Eugene, OR, USA), viewed, and photographed with a Leica TCS-Sp2 (UV) confocal laser scanning microscope (Leica, Germany).

RNA Preparation and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was prepared from H-HSCs using the TRIzol total RNA preparation kit, following the manufacturer's instructions (Gibco). The mRNA expression was measured using SYBR Green Real-time PCR Master Mix (TOYOBO), and the ViiA 7 Real-Time PCR System (ABI, Carlsbad, CA, United States). The MEF2A primers (sequence 5′ to 3′) were as follows, forward: TGC GAC AGC CCA GAC CCT G; reverse: GAG GTG GCA GAC CAG GTG CG; and MEF2C primers (sequence 5′ to 3′), forward: CCA GTA TGC CAG CAC CGA C; reverse: CGT CTC CAC GAT GTC TGA G. The housekeeping gene β-actin was used as a reference gene for normalization.

Cellular Protein Extracts and Western Blot

Cytoplasmic and nuclear extracts were prepared from H-HSCs (NUC-101, Sigma) and protein concentrations of the supernatant were measured using a protein concentration kit (DC Protein Assay; Bio-Rad) according to the manufacturer's instructions. All western blot analyses were repeated at least three times. In general, data from a representative experiment are shown.

Luciferase Reporter Gene Assay

The MEF2 and collagen α1 (I) luciferase reporter assays were performed as previously described (Gong et al., 2003). The H-HSCs were transfected with either an MEF2 luciferase reporter gene (MEF2 binding site underlined, wild-type: TCGACGGGCTATTTTTAGGGCC; mt: 5-TC-GACGGGC GATTTTTCGGGCCG-3 (Shin et al., 1999), the italics indicate mutated sites) or a collagen I (Rippe et al., 1997) promoter construct, along with a plasmid encoding β-galactosidase (β-Gal) (CMV-β-Gal) by the Lipofectamine 2000 transfection method (Invitrogen) unless stated otherwise. Cellular extracts were assayed for both luciferase and β-Gal activities. Relative fold change in luciferase activity was calculated based on the efficiency of transfection (β-Gal value).

Induction of Liver Fibrosis in Rats

Male Sprague-Dawley rats (150–160 g) were injected with DMN (10 mg/kg body weight) intraperitoneally for three consecutive days each week. A peritoneal injection of normal saline (N.S). was administered to the normal control group. The SA-B treated group was treated with SA-B (12.5 mg/kg) intragastrically for 3 weeks (Xu et al., 2012). The animal experiments were approved by the Committee on the Care and Use of Live Animals for Teaching and Research of the Shanghai University of Traditional Chinese Medicine.

Histology and Immunohistochemistry

Portions of liver tissue (approximately $1 \times 0.8 \times 0.3$ cm^3) were fixed by immersion in 8% buffered methanol and then processed and paraffin-embedded. After deparaffinization, sections of 4-μm thickness were stained with hematoxylin-eosin (H&E) and sirius red. In preparation for immunostaining, paraffin sections of the liver samples were rehydrated and washed for 5 min, three times in Tris-buffered saline. The tissue sections were blocked for 60 min at 37°C. The specimens were then incubated with the primary antibody overnight. The sections were washed in phosphate-buffered saline three times for 10 min and incubated with the secondary antibody for 45 min. The slides were mounted with glycerol.

Statistical Analysis

Comparisons between groups were performed using the Student's *t*-test or one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

RESULTS

Isolation and Phenotypic Characterization of H-HSCs

We successfully isolated primary H-HSCs. The isolated primary H-HSCs were plated onto uncoated plastic dishes, trypsinized after 7 days, and sub-cultured. Phenotypically, the freshly isolated H-HSCs were round and lipid rich, smaller than hepatocytes and bigger than Kupffer cells. Primary H-HSCs on day 4 in culture had extended pseudopods and assumed a stellar shape. By day 7 in primary culture, the lipid storage in cells was significantly decreased. Passage 1 H-HSCs was proliferated at a faster rate, and these cells developed a fibroblastic morphology and prominent contractile filaments, similar to those observed in rat HSCs (**Figure 1A**). Immunocytochemical studies have shown that isolated H-HSCs express desmin, a specific marker for HSCs (Wilhelm et al., 2016), throughout the period of culture. In contrast, levels of α-SMA and glial fibrillary acidic protein (GFAP), markers of intermediate filament proteins for activated HSCs (Lim et al., 2008), increased progressively, suggesting that H-HSCs become activated during culture. The high levels of α-SMA and GFAP were maintained following subculture (**Figure 1A**).

Expression of MEF2s in Cultured Primary H-HSCs and Freshly Isolated HSCs From Normal and Cirrhotic Human Livers

Examination of MEF2s in cultured primary H-HSCs revealed that levels of MEF2A, C, and D were significantly increased

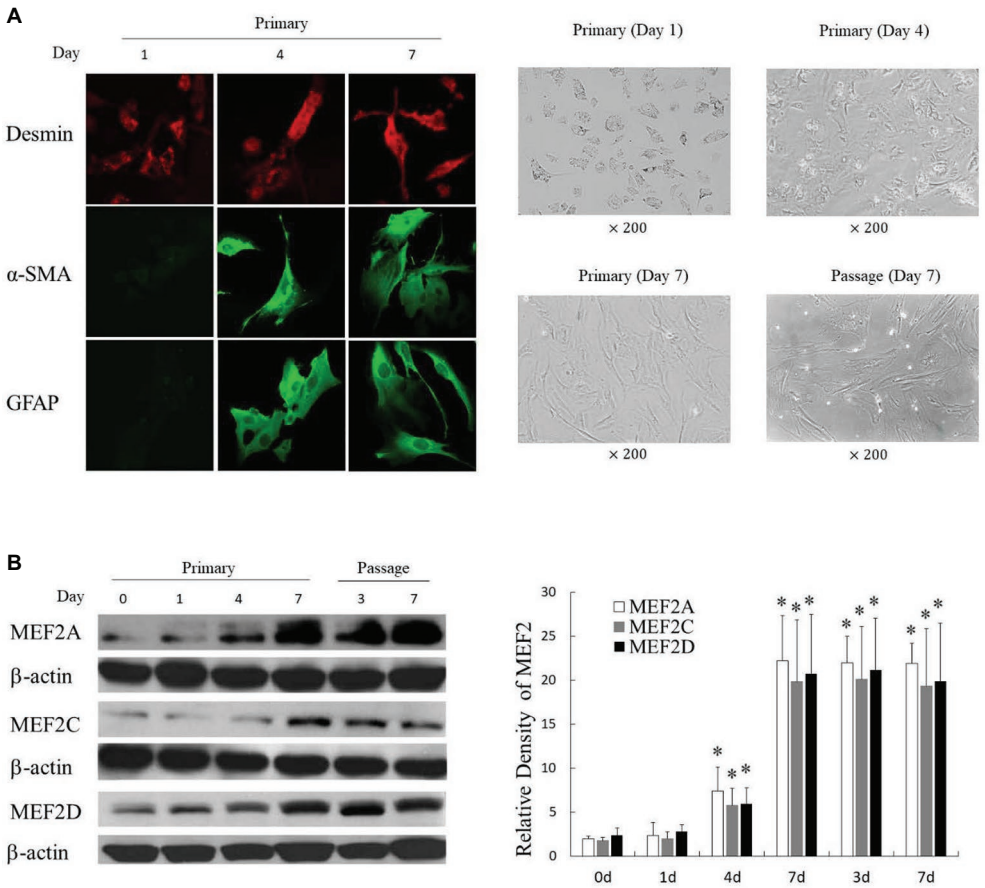

FIGURE 1 | Induction of MEF2 expression during culture-induced activation of primary H-HSCs. **(A)** Expression of activation markers in cultured H-HSCs. The left panel was determined by immunofluorescence. The right panel shows the morphology of H-HSCs under a phase contrast microscope. 'Day' indicates the number of days in culture. **(B)** Expression of the MEF2 protein in cultured H-HSCs. The levels of MEF2A, MEF2C, and MEF2D proteins were determined by western blot using isoform-specific antibodies. The right panel shows densitometry values of MEF2 proteins relative to β-actin. Each value represents the mean ± SEM ($n = 3$, $^*p < 0.05$ vs. controls).

during culture, as H-HSCs became more activated (**Figure 1B**). The levels of MEF2 proteins were maintained at a high level following subculture. The kinetics of the increase in MEF2 observed in cultured H-HSCs suggests that high levels of MEF2 proteins are associated with human liver fibrosis. We compared the levels of MEF2 proteins in freshly isolated H-HSCs from normal and cirrhotic human livers. In comparison to the low levels of MEF2A, C, and D proteins in H-HSCs from normal patients, the levels of MEF2 proteins were much higher (**Figure 2**) in H-HSCs isolated from cirrhotic livers, indicating that increased levels of MEF2s correlate well with the development of liver cirrhosis in humans.

Regulation of MEF2 Expression by TGF-β1 in H-HSCs

As a potent profibrogenic cytokine of HSCs, TGF-β1 is a key regulator of liver fibrosis (Friedman, 2008). To determine whether TGF-β1 induces MEF2 expression in H-HSCs, we cultured the H-HSCs as described in **Figure 1** and determined the expression of MEF2 proteins following the addition of TGF-β1. Exposure of H-HSCs to TGF-β1 after 7 days in culture led to a robust increase in the levels of both MEF2A and MEF2C (**Figure 3A**). In contrast, MEF2D levels remained unchanged following TGF-β1 treatment. To correlate the increases in MEF2A and MEF2C protein expression, we determined their mRNA levels in primary H-HSCs cultured for 7 days, by RT-qPCR. Our data showed that TGF-β1 increased the mRNA levels of MEF2A and C (**Figure 3B**). We then examined the potential of MEF2 to activate gene transcription in H-HSCs using a sensitive MEF2-dependent reporter gene assay. The H-HSCs cultured for 7 days were transiently transfected with a luciferase reporter plasmid containing two copies of the MEF2 binding sites in the 5′-regulatory region of the luciferase gene. The same luciferase reporter with mutated MEF2 sites was used as the control. The cells were cotransfected with a plasmid for β–Gal as an internal control for transfection efficiency. Cultured H-HSCs showed high levels of MEF2-dependent luciferase activity (a nearly eight-fold increase in wild-type MEF2 reporter activity vs. mutant MEF2

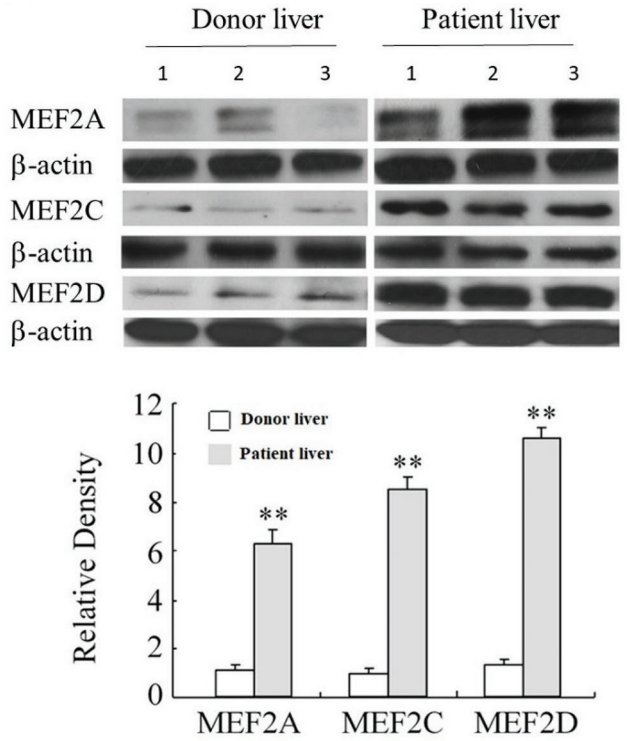

FIGURE 2 | Expression of MEF2 in normal and cirrhotic human livers. The levels of MEF2A, MEF2C, and MEF2D proteins were determined by western blot. Numbers 1 to 3 indicate the samples from individual human. The bottom panel shows densitometry values of MEF2 proteins relative to β-actin. Each value represents the mean ± SEM (n = 3, $^{**}p < 0.01$ vs. controls.).

reporter control) (**Figure 3C**, left graph). Interestingly, TGF-β1 further enhanced MEF2 activity (**Figure 3C**).

Regulation of MEF2 and Human HSC Activation by TGFβ1-p38 MAPK

The p38 MAPK is downstream of the TGF-β1 signal and has been shown to directly phosphorylate and stimulate MEF2s in neurons (Mao et al., 1999; Liu et al., 2003). We tested the role of p38 MAPK in TGF-β1-induced regulation of MEF2s in HSCs. Western blot results showed that TGF-β1 causes a progressive increase in the levels of phosphorylated p38 (p-p38), indicating activation of p-38 (**Figure 4A**). This increase in p38 activity correlated well with enhanced phosphorylation of MEF2C at its p38 recognition site (**Figure 4B**). To confirm whether p38 regulates MEF2s in response to TGF-β1 in HSCs, we inhibited p38 MAPK with a specific inhibitor SB203580. Inhibition of p38 MAPK significantly attenuated the TGF-β1-induced increase in MEF2 levels and activity (**Figure 4C**). Consistently, inhibition of p38 MAPK also downregulated the TGF-β1-induced activation of the collagen I promoter and greatly attenuated the TGF-β1-induced increase in the levels of collagen I and α-SMA (**Figures 4D**,**E**).

To show that p38-mediated regulation of MEF2s is involved in collagen and α-SMA expression, we tested the effects of dominant negative MEF2A, which carries mutations at its p38 phosphorylation sites and does not respond to p38 MAPK signaling in H-HSCs. Overexpression of this MEF2A mutant significantly inhibited the activity of the collagen I promoter (**Figure 4F**). Similarly, overexpression of MEF2C R24L, which inhibits endogenous MEF2s *via* a different mechanism, also reduced collagen promoter activity.

Effects of Antifibrogenic Agent, SA-B, on MEF2

The proliferation and activation of rat HSCs are effectively inhibited by SA-B (Lv et al., 2010). We confirmed that treating H-HSCs with SA-B reduces the TGF-β1-induced increase of α-SMA and collagen I production. Similarly, SA-B also reduced collagen reporter activity in H-HSCs at day 5 in culture (**Figure 5A**).

To verify if SA-B regulates HSC activation *via* downregulation of MEF2, we treated H-HSCs with SA-B and determined its effect on MEF2 proteins. The SA-B evidently caused a decline in the levels of MEF2A and C protein and mRNA. Consistently, SA-B also reduced MEF2-dependent luciferase reporter activity (**Figure 5B**). We tested whether the effects of TGF-β1 on MEF2 were inhibited by SA-B in H-HSCs. The SA-B inhibited the TGF-β1-induced increase in MEF2 mRNA and protein levels and attenuated TGF-β1-mediated activation of MEF2 activity (**Figure 5C**). These results suggest that SA-B is capable of inhibiting TGF-β-induced activation of MEF2 at multiple levels in H-HSCs.

Effects of SA-B on MEF2A and α-SMA in the Rat Liver, Following DMN-Induced Fibrosis

Our results above showed that HSCs from patients with cirrhosis express higher levels of MEF2 proteins. In addition, SA-B is capable of inhibiting the expression of MEF2 stimulated by TGF-β1 in H-HSCs. To correlate MEF2 levels and fibrogenic response *in vivo*, we examined the expression of MEF2A and α-SMA in the rat liver using a well-established model of DMN-induced fibrosis. Injection of DMN induced liver fibrosis and cirrhosis in the rat (**Figure 6A**). Compared with the normal rat liver control, the expressions of MEF2A and α-SMA were significantly induced after treatment with DMN for 4 weeks (**Figure 6B**). The levels of MEF2A and α-SMA were evidently reduced by SA-B, which correlates well with evidence of reduced Sirius red staining.

DISCUSSION

Most previous studies on the complex mechanisms involved in the development of liver fibrosis in humans have been conducted primarily using HSCs isolated from animals (Jiao et al., 2016; Vilaseca et al., 2017; Du et al., 2018). However, whether the molecular mechanisms identified in animal cells can be directly translated to human HSCs, particularly, primary human HSCs, is unclear. We addressed this question by testing the role and regulation of the MEF2s, which are transcription factors that have been identified to play a role

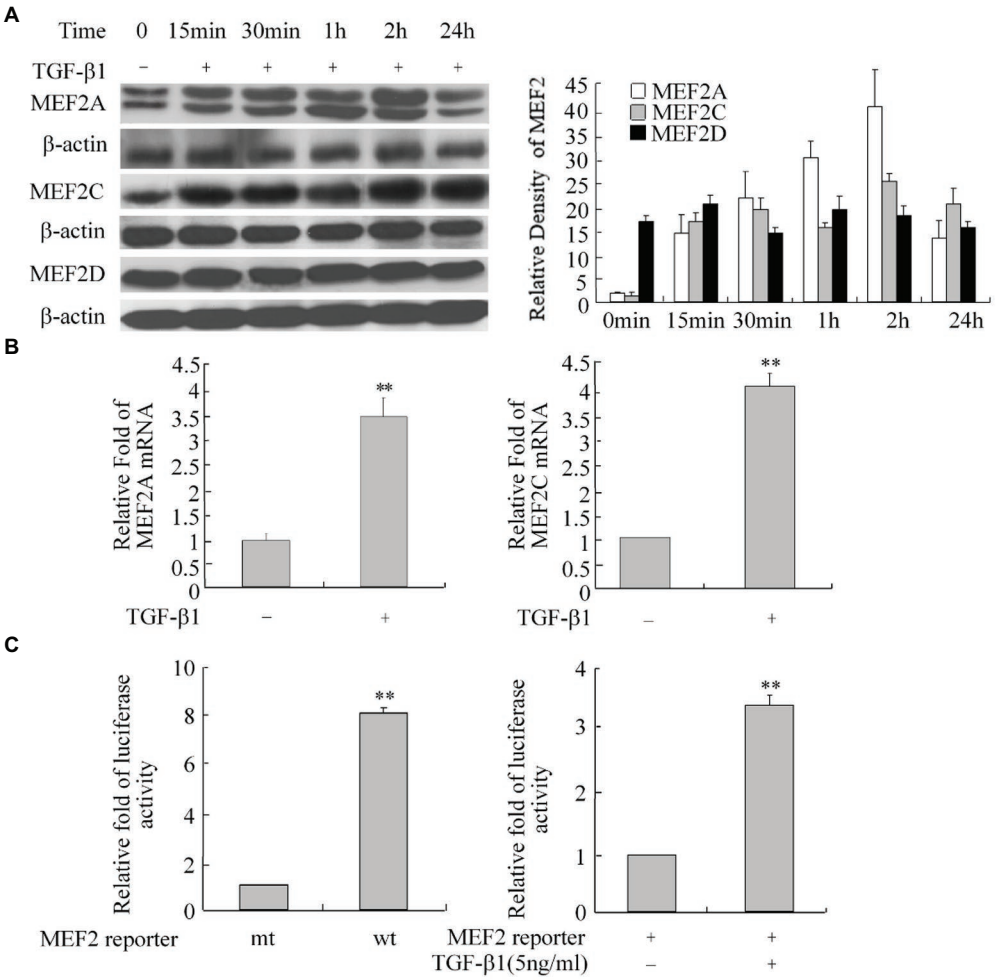

FIGURE 3 | Increase in MEF2 expression during TGF-β1-induced activation of H-HSCs. **(A)** Regulation of MEF2 protein expression by TGF-β1. The levels of MEF2 proteins and β-actin at different time points following TGF-β1 (10 ng/ml) treatment were determined by western blot on day 7 in primary H-HSCs. The right graph represents quantification ($n = 5$). **(B)** TGF-β1-induced increase of MEF2 mRNA expression. Primary H-HSCs were treated with TGF-β1 for 2 h at day 7 in culture. MEF2 mRNAs was determined by RT-qPCR ($n = 3$, $^{**}p < 0.01$ vs. without TGF-β1-induction). **(C)** Increase in MEF2-dependent gene transactivation activity after TGF-β1 treatment. Left panel, primary H-HSCs on day 5 in culture were transfected with a vector for the MEF2 luciferase reporter for 48 h. Right panel, primary H-HSCs on day 5 in culture were transfected with a vector for the MEF2 luciferase reporter for 46 h and treated with TGF-β1 (5 ng/ml) during the last 2 h. (wt, wild-type MEF2 luciferase reporter; mt, reporter with mutated MEF2 binding sites; $n = 3$). Luciferase activity was determined ($n = 3$, $^{**}p < 0.01$, TGF-β1 vs. untreated control).

in rat HSCs (Wang et al., 2004). Our current studies showed that MEF2s function as downstream effectors of TGF-β1 signals to regulate human HSC activation and the fibrogenic response. We identified p38 MAPK as the major mediator activated by TGF-β1 to regulate MEF2 in H-HSCs during liver fibrosis. The MEF2 proteins show higher levels in the HSCs isolated from cirrhotic patients than those from control patients. Moreover, SA-B, a herbal extract with known clinical antifibrogenic effects in liver fibrosis (Liu et al., 2002a), attenuated TGF-β1-induced activation of MEF2s and MEF2-dependent collagen I gene expression in H-HSCs and in a liver fibrosis model in the rat. Given that the process of liver fibrosis is required for the development of cirrhosis, our results show that MEF2 participates in the development of human liver fibrosis and may contribute to the pathogenesis of cirrhosis.

Although transcriptional regulation of MEF2 in rat HSC activation has been demonstrated (Wang et al., 2004), the manner in which this class of factors is regulated by profibrogenic cytokines has not been established prior to our study. Our studies using human primary HSCs revealed differential regulation of various isoforms of MEF2s and extended findings in rat HSCs. We showed that although plating H-HSC MEF2A led to activation of H-HSCs and an increase in the protein levels of MEF2A, C, and D, TGF-β1 treatment of H-HSCs for 7 days enhanced only the expression of MEF2A and C but not MEF2D. The reason for this difference in MEF2 isoform response is presently unknown. As MEF2 proteins form either

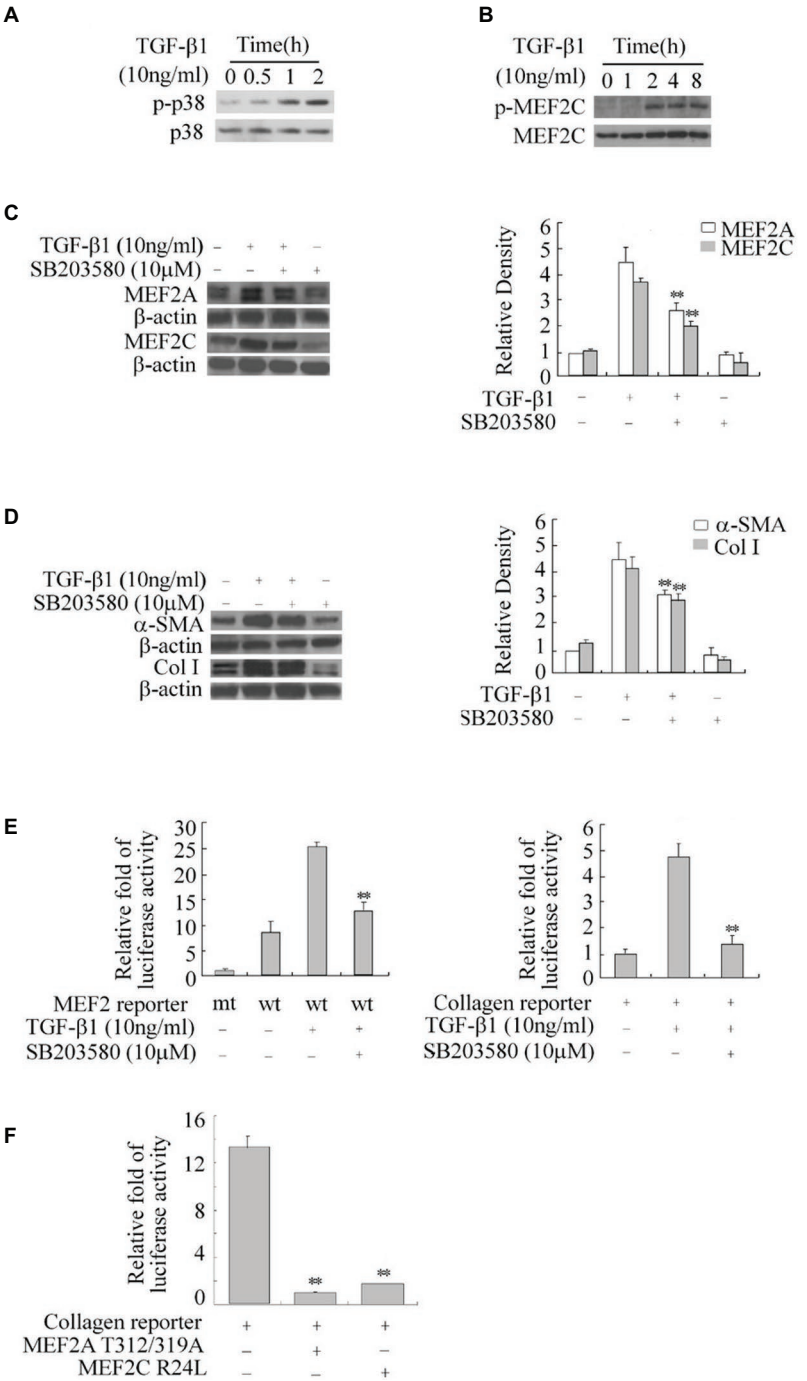

FIGURE 4 | Regulation of the expression of MEF2, α-SMA, and collagen I by TGF-β1-p38 MAPK in HSCs. **(A)** Activation of p38 by TGF-β1. H-HSCs were treated with TGF-β1 for different lengths of time. Levels of phosphorylated p38 (p-p38) and total p38 were determined by western blot. **(B)** TGF-β1-induced phosphorylation of MEF2C. Levels of p-MEF2C and total MEF2C were determined by western blot. **(C)** Inhibition of TGFβ1-induced regulation of MEF2 by the p38 inhibitor. Primary H-HSCs were pretreated with SB203580 (p38 inhibitor) or vehicle for 24 h and treated with TGFβ1 during the last 2 h. MEF2A and MEF2C were determined by western blot. **(D)** Inhibition of TGFβ1 induced-α-SMA and collagen I expression by p38 inhibitor. The assays were performed as described in **(C)** ($n = 3$, $^{**}p < 0.01$ TGF-β1-treated samples with SB203580 vs. without SB203580). **(E)** Inhibition of TGFβ1 induced MEF2 transactivation activity and collagen I reporter in H-HSCs. H-HSCs on day 5 in culture were transfected with mutated (mt) or wild type (wt) MEF2 reporter overnight, and then treated with TGFβ1 for 2 h with or without SB203580 pretreatment over 24 h. The luciferase assay was performed ($n = 3$; $^{**}p < 0.01$ wild-type reporter induced by TGF-β1 with SB203580 treatment vs. without treatment). **(F)** Inhibition of collagen I reporter by blocking MEF2. The collagen I reporter gene assay was determined at day 7 in primary culture, 48 h after transfection with the co-expression of dominant negative MEF2 as indicated ($n = 3$, $^{**}p < 0.01$ vs. controls.).

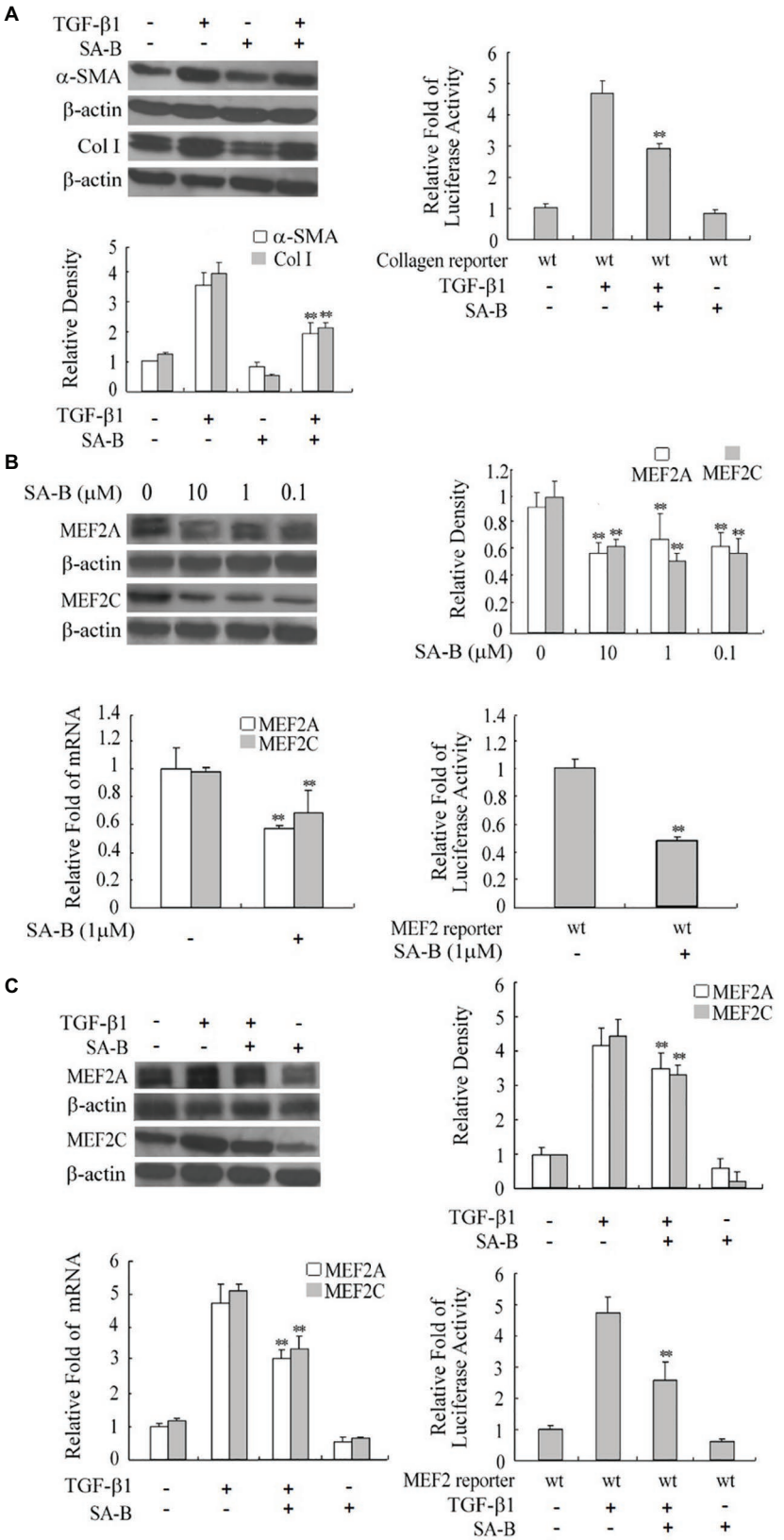

FIGURE 5 | Continued

FIGURE 5 | Reduction of the expression of MEF2s and markers of H-HSC activation by SA-B. **(A)** Expression of Col I and α-SMA in H-HSC treated with SA-B or TGF-β1. Day 5 H-HSCs were treated with SA-B (1 μM) for 48 h, and then treated with TGF-β1 (10 ng/ml) for 2 h ($n = 3$; $^{**}p < 0.01$; Col I and α-SMA induced by TGF-β1 pretreated with SA-B vs. non-treated). The collagen luciferase reporter gene assay was determined. Relative fold change in luciferase activity is shown ($n = 3$, $^{**}p < 0.01$, wild-type reporter induced by TGF-β1 with SA-B treatment vs. without SA-B treatment). **(B)** Expression of MEF2 in H-HSC treated with SA-B. Day 5 H-HSCs were treated with SA-B at different concentrations for 48 h. Protein level was analyzed by western blot (top left panel). Densitometry values of MEF2 relative to β-actin are shown (top right panel) ($n = 3$; $^{**}p < 0.01$ vs. non-treated with SA-B). The MEF2 mRNA expression was determined by RT-qPCR following SA-B (1 μM) treatment as described above (bottom left panel) ($n = 3$, $^{**}p < 0.01$, with vs. without SA-B). The MEF2-dependent luciferase reporter gene assay was determined (bottom right panel: $n = 3$; $^{**}p < 0.01$, wild-type reporter treated with SA-B vs. without SA-B). **(C)** Expression and activity of MEF2 treated with SA-B or TGF-β1. Day 5 H-HSCs were treated with SA-B (1 μM) for 48 h and then treated with TGF-β1 (10 ng/ml) for 2 h. Protein level of MEF2 was determined by western blot (top left panel) ($n = 3$; $^{**}p < 0.01$, TGF-β1-treated samples with SA-B vs. without SA-B). MEF2 mRNA expression and reporter activity assay was determined (bottom panel) ($n = 3$; $^{**}p < 0.01$, TGF-β1 treated samples pretreated with SA-B vs. without SA-B).

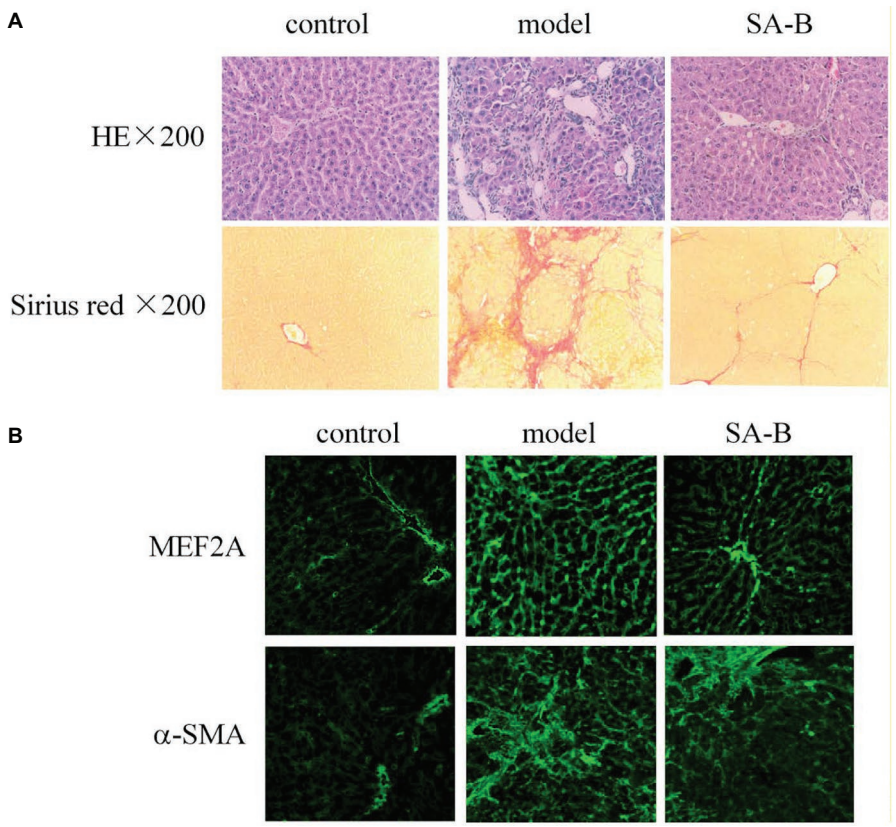

FIGURE 6 | Inhibition of MEF2 and α-SMA expression by SA-B in the rat liver following DMN-induced fibrosis. **(A)** DMN-induced rat liver fibrosis. Sections were stained with H&E and Sirius red. **(B)** MEF2 and α-SMA expression in normal control, DMN-induced fibrosis, and SA-B-treated livers. Proteins were determined by immunofluorescence.

homodimers or heterodimers to regulate gene expression (Tang et al., 2005), it is not clear whether MEF2D (not induced by TGF-β1) may contribute to the overall activity of MEF2s in cells.

Our data defined TGF-β1 as a stimulus of MEF2 expression during the activation of H-HSCs. Several pathways have been shown to mediate the TGF-β1 signal in various cell types (Friedman, 2008). Previous studies have shown that p38 MAPK directly phosphorylates and activates MEF2s in response to survival signals in neurons (Mao et al., 1999). Our current findings suggest that p38 MAPK plays a major role in mediating TGF-β1-induced activation of MEF2s. It is interesting to note that although inhibition of p38 MAPK clearly attenuated TGF-β1-induced increase in the MEF2 protein, this inhibition was incomplete. Whereas p38 MAPK is known to directly interact with MEF2 proteins, TGF-β1 also affects MEF2 function by increasing the levels of MEF2 mRNAs. These data are consistent with the possibility that other downstream mediators of the TGF-β1 signal may also participate in regulating MEF2s. As MEF2s are known to interact with the Smad family of proteins in muscle cells (Quinn et al., 2001), it would be interesting to test whether mediators other than p38, such as Smad or repressors of the TGF-β1 signal, may participate in the regulation of MEF2s, particularly at the level of transcription (Gong et al., 2003).

Previous studies have described the antifibrogenic effects of SA-B in rat HSCs. SA-B suppressed HSC proliferation concentration dependently, inhibited soluble type I collagen secretion, and decreased the matrix collagen deposition. SA-B at 1 and 10 μmol/L decreased the cell active TGF-β1 secretion by 63.3% and 15.6% of the control (Liu et al., 2002b). The mechanisms by which SA-B exerts its anti-fibrosis effects are not entirely clear. Our data suggest that SA-B is a potent antifibrogenic agent against the culture-induced activation of primary human HSCs. In addition, SA-B directly antagonizes the TGF-β1-induced fibrogenic response in human HSCs. Moreover, SA-B not only reduces the increase in MEF2 mRNA levels but also attenuates the accumulation of MEF2 proteins. Our studies suggest that antagonizing TGF-β1-induced activation of MEF2 activity at both protein and RNA levels may account in part for the anti-fibrosis effects of SA-B.

CONCLUSION

The MEF2 transcription factor is stimulated by TGF-β1 in H-HSCs. Antagonizing TGF-β1-induced activation of the MEF2 signaling pathway may account in part for the anti-fibrosis effects of SA-B.

AUTHOR CONTRIBUTIONS

WZ and JP performed the experiments and wrote the manuscript. YZ assisted with the animal experiments. GC assisted with histomorphological experiments. LX designed the experiments and interpreted data.

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REVIEW
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Lipophagy and Alcohol-Induced Fatty Liver

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This review describes the influence of ethanol consumption on hepatic lipophagy, a selective form of autophagy during which fat-storing organelles known as lipid droplets (LDs) are degraded in lysosomes. During classical autophagy, also known as macroautophagy, all forms of macromolecules and organelles are sequestered in autophagosomes, which, with their cargo, fuse with lysosomes, forming autolysosomes in which the cargo is degraded. It is well established that excessive drinking accelerates intrahepatic lipid biosynthesis, enhances uptake of fatty acids by the liver from the plasma and impairs hepatic secretion of lipoproteins. All the latter contribute to alcohol-induced fatty liver (steatosis). Here, our principal focus is on lipid catabolism, specifically the impact of excessive ethanol consumption on lipophagy, which significantly influences the pathogenesis alcohol-induced steatosis. We review findings, which demonstrate that chronic ethanol consumption retards lipophagy, thereby exacerbating steatosis. This is important for two reasons: (1) Unlike adipose tissue, the liver is considered a fat-burning, not a fat-storing organ. Thus, under normal conditions, lipophagy in hepatocytes actively prevents lipid droplet accumulation, thereby maintaining lipostasis; (2) Chronic alcohol consumption subverts this fat-burning function by slowing lipophagy while accelerating lipogenesis, both contributing to fatty liver. Steatosis was formerly regarded as a benign consequence of heavy drinking. It is now recognized as the "first hit" in the spectrum of alcohol-induced pathologies that, with continued drinking, progresses to more advanced liver disease, liver failure, and/or liver cancer. Complete lipid droplet breakdown requires that LDs be digested to release their high-energy cargo, consisting principally of cholesteryl esters and triacylglycerols (triglycerides). These subsequently undergo lipolysis, yielding free fatty acids that are oxidized in mitochondria to generate energy. Our review will describe recent findings on the role of lipophagy in LD catabolism, how continuous heavy alcohol consumption affects this process, and the putative mechanism(s) by which this occurs.

Keywords: alcohol, autophagy, ethanol, lipolysis, lipophagy, liver, steatosis

ALCOHOL ABUSE CAUSES LIVER INJURY

Heavy drinking is a major cause of liver disease worldwide (O'Shea et al., 2010). Current trends indicate that, in the United States, between 2007 and 2014, alcohol-induced liver disease (AILD) became the second most frequent cause of cirrhosis and hepatocellular carcinoma (HCC)-related mortality after non-alcoholic fatty liver disease (NAFLD) (Kim et al., 2019). Fatty liver (steatosis) is the earliest response to excessive drinking in 90% or more of alcohol abusers (O'Shea et al., 2010; You and Arteel, 2019). Steatosis is characterized by excessive deposition of fat, seen microscopically as intracellular lipid droplets (LDs). These are hydrophobic islands of stored fat, each surrounded by a phospholipid monolayer, which, itself, harbors specific proteins that maintain LD integrity. Under the microscope, LDs can be seen suspended alone in the soluble cytoplasm (cytosol) or in physical contact with other membranous organelles, including the endoplasmic reticulum (ER), from which LDs are believed to originate (Ploegh, 2007; Fei et al., 2009; Choudhary et al., 2015). LDs also interact with mitochondria, lysosomes, peroxisomes, and membranes of the Golgi apparatus (Barbosa et al., 2015). Each of the latter organelles has a functional interaction with LDs, the extent of which appears to depend on the cell's lipid content (Krahmer et al., 2018).

In this review, the terms "autophagy" and "lipophagy" will, be used interchangeably or together. The two are closely-linked, as LDs are taken up and degraded with other cellular constituents, by "bulk autophagy." During "true lipophagy," LDs are selectively taken up and degraded. In addition, the classical autophagy/lipophagy pathway, mentioned in the Abstract has some non-canonical variations to be described herein.

AILD IS LINKED TO ETHANOL OXIDATION

The liver sustains the greatest injury after excessive drinking because it is the principal site of ethanol oxidation (metabolism) (Lieber and DeCarli, 1991; Osna et al., 2017), as depicted in **Figure 1**. The bulk of hepatic ethanol oxidation is catalyzed by alcohol dehydrogenase (ADH) and cytochrome P450 2E1(CYP2E1). Catalase, an enzyme, which inhabits peroxisomes and is abundant in liver, has an accessory function in hepatic ethanol oxidation, but a more prominent role in brain ethanol metabolism (Zimatkin et al., 2006). ADH, the primary oxidative enzyme, is located in the liver cytosol (soluble cytoplasm). ADH has a high catalytic efficiency (K_{cat}) and a high affinity ($K_{m\ ethanol}$ = 1–2 mM) for ethanol as a substrate. CYP2E1 is predominantly associated with the membranes of the smooth ER. It has a lower affinity for ethanol ($K_{m\ ethanol}$ = 10–20 mM) and a lower K_{cat} than ADH. All three enzymes oxidize ethanol to generate acetaldehyde, which is toxic because it is highly reactive and covalently binds to proteins, lipids and nucleic acids (Kenney, 1982; Donohue et al., 1983; Brooks and Zakhari, 2014). In hepatocytes, acetaldehyde's toxicity is minimized because it is rapidly oxidized to acetate by the mitochondrial aldehyde dehydrogenase2 (ALDH2). Both ADH-catalyzed ethanol oxidation to acetaldehyde and ALDH2-catalyzed acetaldehyde oxidation to acetate utilize nicotinamide adenine dinucleotide (NAD^+) as a hydrogen and electron acceptor. Together, both reactions generate excess quantities of NADH, which lowers the intracellular NAD^+/NADH ratio, also known as the cellular redox potential. The latter change in redox initiates significant metabolic shifts toward reductive synthesis by accelerating the synthesis and slowing the oxidation of fatty acids, thereby exacerbating steatosis. In addition, ethanol oxidation stimulates fatty acid biosynthesis by enhancing *de novo* synthesis of lipogenic enzymes, the syntheses of which are governed by activation of three transcription factors: the sterol regulatory element binding protein-1c (SREBP-1c), the carbohydrate response element binding protein (ChREBP) and early growth response-1 (Egr-1). We and others have described the induction and regulatory features of these factors in other articles and reviews (You et al., 2002; Liangpunsakul et al., 2013; Thomes et al., 2013b; Thomes and Donohue, 2017; You and Arteel, 2019).

CYP2E1, the other major ethanol metabolizing enzyme is unique in two ways: (1) The enzyme is induced by ethanol, which elevates CYP2E1's intracellular content (Lieber, 1970, 2004; Roberts et al., 1995), thereby accelerating the overall rate of ethanol oxidation; (2) CYP2E1 possesses a unique catalytic cycle, coupled with a broad substrate specificity. The latter properties allow the enzyme to produce, not only higher levels of acetaldehyde but also greater quantities of other reactive oxygen species (ROS), including hydroxyethyl radicals ($\bullet CH_3CH_2OH$), hydroxyl radicals ($\bullet OH$), and superoxide anions (O_2^-). Superoxide can undergo secondary reactions with nitric oxide to form peroxynitrite ($OONO^-$), which can covalently bind to tyrosine residues on proteins (Osna et al., 2004). All the aforementioned reactive species are unstable, but they heighten oxidant stress in the hepatocyte to enhance hepatocellular damage (Wu et al., 2012; Yang et al., 2012) (also see Takahashi). It is now clear that development of alcohol-induced fatty liver is the "first hit" that propagates injury, as described in the next section.

METABOLIC SOURCES OF ALCOHOL-INDUCED HEPATIC LIPIDS AND THEIR HEPATOTOXICITY

Alcohol-induced fatty liver was reported in humans (Buck, 1948) decades before the metabolic pathways affected by heavy drinking were revealed and well before it was discovered that certain fatty acids are hepatotoxic (Savary et al., 2012). Hepatic fatty acid (and lipid droplet) accumulation after alcohol abuse arises from: (1) accelerated hepatic lipogenesis (You et al., 2002; You and Crabb, 2004); (2) enhanced fatty acid import into the liver from the plasma (Wei et al., 2013); (3) defective secretion of lipoproteins (e.g., very low density lipoproteins VLDLs) from the liver into the plasma, resulting in their hepatic retention (Kharbanda et al., 2009); (4) reduced fatty

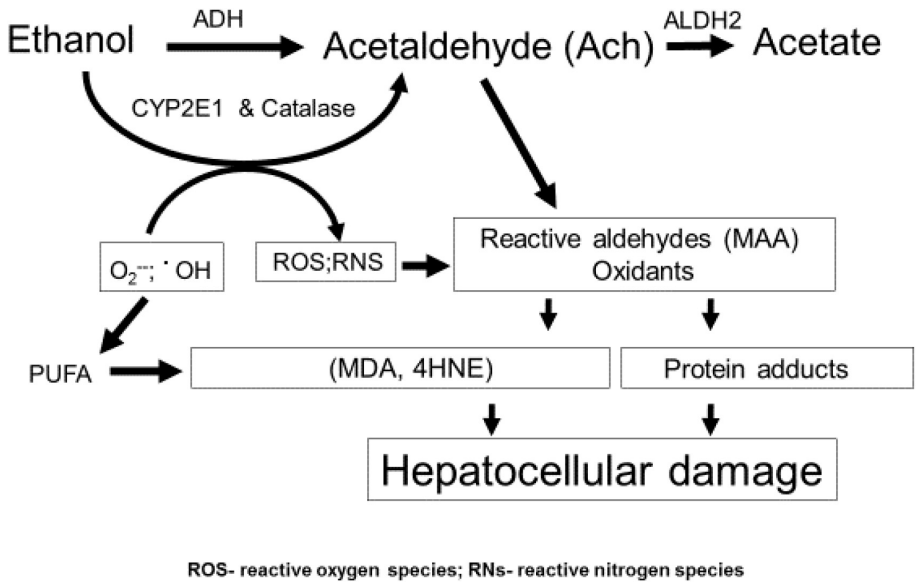

FIGURE 1 | Ethanol metabolism and hepatic oxidant stress. Ethanol is oxidized principally in liver hepatocytes by ADH, CYP2E1, and catalase to acetaldehyde (Ach). Ach is a highly reactive intermediate that, itself, covalently binds to protein or can undergo secondary reactions to form MAA (see text). CYP2E1 is induced by ethanol and produces radicals, including superoxide and hydroxyl radicals, which by themselves are reactive and can undergo secondary reactions with PUFA, producing ROS and RNS as defined in this figure and in the text. The latter reactive molecules can also form adducts with proteins.

acid oxidation (FAO) by mitochondria (Fischer et al., 2003); and now, (5) decelerated lipophagy (Rasineni et al., 2017; Schulze et al., 2017a).

Steatosis from heavy drinking elevates intrahepatic levels of saturated and polyunsaturated fatty acids (PUFA). The latter are particularly reactive, because their double bonds, allow them to combine with hydroxyl radicals (●OH) derived from ethanol oxidation and/or mitochondrial respiration, to form lipid peroxides. These undergo subsequent modifications, including the iron-driven Fenton reaction and peroxide fragmentation to produce highly reactive lipid aldehydes, including 4-hyroxynonenal (4-HNE) and malondialdehyde (MDA), each of which covalently binds to proteins (Houglum et al., 1990). Acetaldehyde, generated from ethanol oxidation can also react with MDA to form a larger, highly reactive hybrid molecule, malodialdehyde-acetaldehyde (MAA) that binds to proteins, forming MAA adducts (Tuma et al., 1996, 2001; **Figure 1**) Proteins that bear such adducts exhibit altered biological function as well as proinflammatory and profibrogenic properties that can exacerbate liver injury beyond simple steatosis (Kharbanda et al., 2001, 2002; Tuma, 2002). It is also noteworthy that earlier *in vitro* studies revealed that substoichiometric concentrations of acetaldehyde (Ach) alone can form adducts with a specific lysine residue on the alpha subunit of tubulin to disrupt its polymerization into functional microtubules (Smith et al., 1989) These findings suggest that metabolically-derived Ach has similar properties *in vivo*, which may explain how chronic ethanol consumption disrupts vesicle trafficking in hepatocytes during autophagy. **Figure 2** depicts such a scenario.

LD CATABOLISM

Until 2009, it was held that LDs are degraded by lipases in the cytosol and by the lysosomal acid lipase (LAL), but do not undergo macroautophagy. That concept was discarded after Singh et al. (2009) demonstrated the macroautophagic degradation of LDs. These investigators also added the term, "lipophagy" to the list of autophagic organelle degradation pathways. That list includes mitophagy (mitochondria) (Lemasters, 2005), pexophagy (peroxisomes) (Katarzyna and Suresh, 2016), ERphagy or reticulophagy (endoplasmic reticulum), (Schuck et al., 2014) ribophagy (ribosomes) (An and Harper, 2018) nucleophagy (nuclei) (Mochida et al., 2015), lysophagy (lysosomes) (Hung et al., 2013), and in photosynthetic cells, chlorophagy (chloroplasts) (Ishida et al., 2008; Wada et al., 2009).

Schulze et al. (2017b) reviewed the multiple pathways by which LDs are degraded in liver cells. These are depicted in **Figure 3**. Macrolipophagy is the canonical vesicular pathway during which LDs (or portions thereof) are selectively sequestered in autophagosomes, which are then trafficked by microtubules to fuse with lysosomes, forming autolysosomes in which the LDs_is/are degraded. During microlipophagy, LDs directly interact with (or are engulfed by) lysosomes in an endocytosis-like manner, for direct lipolysis of the LD contents. Chaperone-mediated autophagy (CMA), carries out the selective lysosomal degradation of specific proteins that reside on the LD membrane. Two such proteins are the perilipins 2 and 3 (PLIN2 and PLIN3) (Kaushik and Cuervo, 2016) These proteins are targeted by CMA to the lysosome because each carries,

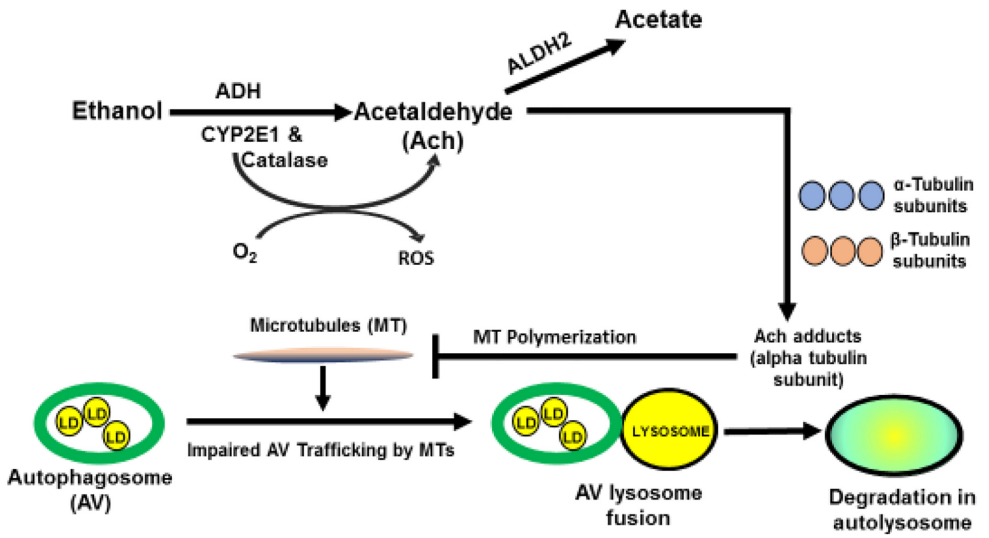

FIGURE 2 | Postulated mechanism by which acetaldehyde impairs microtubule function. Acetaldehyde (Ach) is generated from ethanol oxidation. Due to its high reactivity, it can, form adducts with the α-subunit of tubulin, to inhibit microtubule (MT) polymerization. Such inhibition impairs MT-dependent processes, including the movement of LD-bearing autophagosomes to lysosomes for degradation of LDs.

in its primary sequence, a specific pentapeptide (lys-phe-glu-arg-gln or KFERQ), which is recognized by and binds to the cytosolic form of the 70 kDa heat shock constitutive protein, (Hsc70) a chaperone, that directs each PLIN to the lysosomal membrane. There, the lysosome-associated membrane protein 2A (LAMP2A) facilitates each protein's internalization and degradation. The removal and degradation by CMA of PLINS from the LD membrane enhances recruitment of cytosolic lipases, including the cytoplasmic adipose triglyceride lipase (ATGL) to initiate lipolysis of the LD contents (Kory et al., 2015). In the context of this review, it is noteworthy that, prior to its degradation by CMA, PLIN 2 is phosphorylated by the energy-sensing adenosine monophosphate-activated kinase (AMPK) (Kaushik and Cuervo, 2016). The kinase is activated by nutrient deficit but inactivated by chronic alcohol consumption (You and Crabb, 2004; You et al., 2004). The reader should note that **Figure 3** identifies this and other specific steps in the lipophagic pathways illustrated, that are impaired by alcohol abuse.

PLIN2 is also ubiquitylated and degraded by the ubiquitin-proteasome system (UPS) (Xu et al., 2005). Ubiquitylation is a post-translational modification, during which polymers of ubiquitin, an 8.5 kDa polypeptide, are covalently attached to protein substrates at their free NH_2-termini or at the epsilon-amino groups of their internal lysine residues. The polyubiquitin chain is a signal for degradation of the protein substrate by the 26S proteasome (Ciechanover et al., 1980; Ciechanover, 1994; Bercovich et al., 1997; Ciechanover and Schwartz, 2004). Evidence indicates that the UPS regulates the content of PLIN2 in the cytosol by degrading its cytosolic form, but after it is localized in the LD membrane, PLIN2 evades UPS-catalyzed proteolysis (Takahashi et al., 2016) but is subject to degradation by CMA (Kaushik and Cuervo, 2016).

LD SIZE INFLUENCES ITS MODE OF CATABOLISM

In liver cells LD size varies widely from about 60 nanometers to more than 20,000 nanometers (20 microns) in diameter. Larger LDs are readily seen in liver histology sections from ethanol-fed rodents (Donohue et al., 2007a) and in liver biopsies of people with alcohol use disorders (AUDs) (Torruellas et al., 2014). Evidence now indicates that LD size influences which lipolytic pathway initiates LD breakdown. A recent investigation by Schott et al. (2017) (AASLD abstract; MS under review) revealed that autophagosomes and multivesicular bodies (MVB)/late endosomes, selectively sequester smaller-sized LDs, while larger LDs more frequently interact with the cytosolic adipocyte triglyceride lipase, (ATGL) the rate-limiting enzyme in triglyceride lipolysis. Thus, ATGL appears to preferentially lipolyze the contents of larger LDs, reducing them to sizes that allow their terminal sequestration into autophagosomes. These findings imply that the breakdown of very large LDs, such as those seen in livers with alcohol-induced macrovesicular steatosis proceeds by a sequential lipolysis-to-lipophagy mechanism.

The physio-biochemical causes of alcohol-induced LD enlargement can be traced partially to disruption of LD breakdown. Listenberger et al. (2018) reported that ethanol-induced LD enlargement is, in part, the result of decreased lipolysis, which coincides with a significant reduction in the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio in LD membranes of ethanol-fed animals. LDs that lack sufficient PC to shield internal lipid stores exhibit a tendency to fuse with each other, thereby creating enlarged LDs that can occupy the entire hepatocyte, pushing the nucleus to the periphery (Guo et al., 2008; Krahmer et al., 2011;

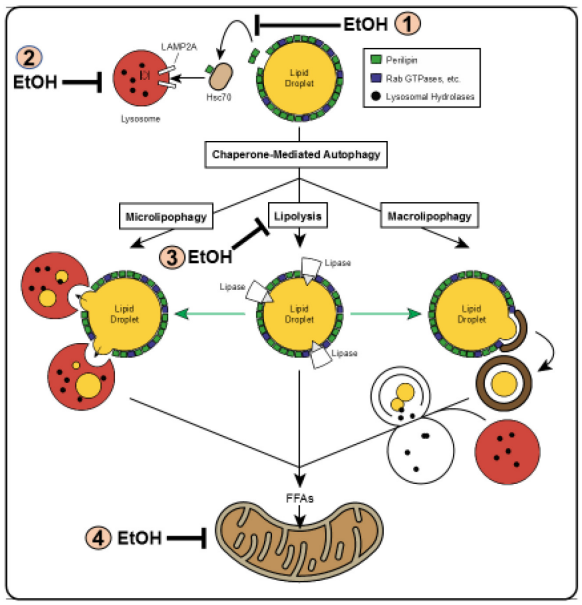

FIGURE 3 | Schematic of lipophagy pathways. Shown are the three major lipophagic pathways: macrolipophagy **(right)**, microlipophagy **(left)** and chaperone-mediated autophagy of lipid droplet proteins **(top center).** Details of each pathway are described in the text. Direct lipolysis by cytosolic lipases of a lipid droplet is shown in the **center**. Figure reproduced from Figure 1 by Schulze et al. (2017b) with permission. Note: The figure shows steps in the pathways that are inhibited by ethanol consumption. These are denoted by the numbered symbols indicated by (EtOH.) In **(1)** ethanol consumption blocks CMA by inhibiting AMPK; in **(2)** ethanol consumption prevents lysosome biogenesis by inhibiting TFEB; in **(3)** ethanol consumption impairs lipolysis by inhibiting ATGL and HSL; in **(4)** ethanol consumption blocks mitochondrial fatty acid oxidation by causing mitochondrial damage and inactivating PPAR- alpha. Details of each are described in the text.

You and Arteel, 2019). Furthermore, the reduction in LD surface PC:PE ratio on these enlarged LDs is associated with higher contents of PLIN2 and PLIN3 on the LD membrane. LD-associated PLINs confer structural integrity to the LD because they potently inhibit lipase activity (Brasaemle et al., 2009; Hall et al., 2010). The latter findings have been substantiated *in vitro* by studies conducted with liposomes, which model LDs, and were synthesized with PC:PE ratios identical to those detected in hepatic LDs of ethanol-fed rats. These analyses revealed that ethanol-induced changes in the LD phospholipid composition, contribute directly to quantitative changes in proteins that associate with the LD surface. Therefore, reducing the ratio of PC to PE increases PLIN2 and PLIN3 binding to the LD membrane (Listenberger et al., 2018). Comparable results were obtained when NIH 3T3 and AML12 cells were exposed to choline-deficient media, which also decreases the PC:PE ratio of LDs (Listenberger et al., 2018). Their data showed increased association of PLINs, and other specific proteins, with LDs. Taken together, these studies indicate that ethanol-induced phospholipid alterations on the LD surface (PC:PE ratio) directly impacts LD size and number by altering the LD surface proteome and inhibiting lipolytic pathways, including lipophagy.

AUTOPHAGY IS REGULATED BY ETHANOL OXIDATION

Active autophagy is essential for cell survival because it yields energy while it recycles substrates required for macromolecular synthesis. Whether ethanol exposure stimulates or slows autophagy *in vivo* or *in vitro* depends on the duration of ethanol consumption/exposure, the amount of ethanol administered, and the manner in which it is administered to animals or cells (Thomes et al., 2013a, 2015) Acute ethanol consumption, also called binge drinking, occurs when a large amount of alcohol is imbibed in a single bolus or at multiple times during a short (usually two to 12 h) time period (Zakhari and Li, 2007). Acute ethanol administration to naïve animals, usually performed by gastric intubation (gavage), accelerates hepatic autophagy (Ding et al., 2010; Thomes et al., 2015). In contrast, feeding the ethanol liquid diet (Lieber and DeCarli, 1986) to rodents for several weeks, retards hepatic autophagy, as judged grossly by liver enlargement (hepatomegaly), which reflects lipid and protein accumulation (Baraona et al., 1975; Donohue et al., 1989) and biochemically by the hepatic accumulation of two autophagy marker proteins: one is p62/SQSTM (sequestosome), an adaptor protein and lysosome substrate. The other is the lipidated form II of microtubule-associated protein 1 light chain 3 (LC3II), a classical marker of autophagosome content (Rubinsztein et al., 2009; Thomes et al., 2015; Rasineni et al., 2017).

Autophagy in Cultured Cells

We measured autophagy in ethanol-exposed recombinant VL-17A$^{ADH+/CYP2E1+}$ cells that metabolize ethanol (Donohue et al., 2006). Our studies revealed that 24 h or longer exposure of cells to 50 mM ethanol inhibits autophagy. This is due, in part, to the ethanol-induced depletion of lysosomes, which decrease by 50% while autophagosome numbers (seen microscopically as GFP-LC3II-positive puncta) nearly double in the same time period (Thomes et al., 2013a) The ethanol-induced rise in autophagosomes does not occur in parental Hep G2 cells, which express neither ADH nor CYP2E1. Similarly, autophagy proceeds normally when ethanol-treated VL-17A cells are co-treated with 4-methylpyrazole (4-MP) which blocks ethanol oxidation. LC3II levels also remain unchanged when reactive metabolites generated by ethanol oxidation are scavenged, by co-treating VL-17A cells with glutathione ethyl ester (Thomes et al., 2013a). The latter findings clearly indicate that blocking acetaldehyde/ROS formation or enhancing the removal of these metabolites avoids the disruptive effects of ethanol oxidation on autophagy in ethanol-metabolizing VL-17A cells.

Autophagy *in vivo*

More recent studies by Guo et al. (2015) showed that autophagy either slows down or proceeds normally in response to the level of acetaldehyde flux generated during ethanol metabolism. They report that ethanol-fed mice carrying the ALDH2 transgene exhibit significantly lower levels of blood and liver acetaldehyde than identically-treated control mice, carrying the friendly virus B

(FVB) viral vector, without the ALDH2 transgene. These findings suggest that ALDH2 transgenic mice clear acetaldehyde more rapidly than FVB control mice. Livers from ethanol-fed ALDH2 transgenic mice exhibit normal (control) levels of autophagy markers and a threefold reduction in hepatic triglycerides, compared with ethanol-fed FVB mice.

In vitro studies by these investigators revealed that direct ethanol (100 mM) or acetaldehyde (100 or 500 μM) exposure to ethanol-metabolizing VA-13$^{ADH+/CYP2E1-}$ cells, suppressed intracellular autophagy, while exposure to each compound caused increased cellular expression of interleukin-6 (IL-6), a pro-inflammatory marker (Osna et al., 2017). Both the latter responses were alleviated when ethanol or acetaldehyde-treated VA-13 cells were co-treated with the autophagy activator rapamycin or with the ALDH2 activator Alda-1. Their findings suggest that autophagy/lipophagy is suppressed by metabolically-derived acetaldehyde. However, accelerated acetaldehyde clearance by higher intrahepatic levels of ALDH2 or by Alda-1-activated ALDH2, prevents ethanol/acetaldehyde-elicited autophagy retardation and liver cell injury. The striking reduction in liver triglycerides in ethanol-fed ALDH2 transgenic animals, suggests that the reduction in the amount of metabolically-generated acetaldehyde allows lipophagy and subsequent lipolysis to proceed normally.

Other Metabolic Considerations

The study just described prompts the question of whether enhanced acetate formation due to accelerated acetaldehyde oxidation contributes to autophagy activation. This appears unlikely, as we reported that direct exposure of VL-17A cells to 10 mM acetate causes no change in LC3II content. In contrast, when we expose VL-17A cells to 100 μM acetaldehyde, LC3II content rises, indicating autophagy retardation (Thomes et al., 2013a). Acetate is metabolically converted to acetyl CoA, a substrate for acetyltransferases, which catalyze the attachment of acetyl groups to lysine side chains on proteins (Drazic et al., 2016). There are reports that acetylation of transcription factor EB influences its activity as the principal regulator of autophagy and lysosome biogenesis (discussed later in this review). However, published findings report mixed results. Some declare that acetylation of TFEB reduces its activity (Bao et al., 2016), while others assert that this modification activates the transcription factor (Li et al., 2017a,b). For now, our data indicate that excess acetate, by itself, does not influence autophagy, but studies designed to critically examine the incorporation of acetate derived directly from ethanol oxidation into acetyl CoA and subsequently into acetylated proteins, will likely reveal a more definitive answer to this question.

CHRONIC ETHANOL CONSUMPTION/EXPOSURE DISRUPTS LIPOPHAGY

There is solid evidence that, similar to affecting bulk autophagy, chronic ethanol consumption retards the lipophagic clearance of LDs. An *in vitro* study by McVicker et al. (2012) used alcohol-metabolizing WIFB cells to demonstrate that ethanol oxidation is strongly associated with impaired fat clearance and accumulation of LDs and of the lipid droplet protein PLIN2 (a.k.a. adipocyte differentiation related protein; ADRP in this paper). Their findings led them to suggest that ethanol metabolism retards lipophagy. Related to this report are several others that similarly, indicate that ethanol consumption by rodents not only disrupts the structural integrity of liver lysosomes (Donohue et al., 1994, 2007a; Kharbanda et al., 1995, 1997), but also retards lysosome biogenesis *in vivo* (Kharbanda et al., 1996; Chao et al., 2018b) and in cultured cells (Thomes et al., 2013a).

Ex vivo Studies Show Lipophagy Impairment by Chronic Ethanol Administration

Rasineni et al. (2017) reported that the clearance of LDs (as judged by triglyceride disappearance), in hepatocytes from ethanol-fed rats was slower than that in cells from pair-fed control animals. The latter finding was associated with lower intracellular levels of the active (phosphorylated) form of dynamin-2 (Dyn-2), a GTPase that catalyzes the scission of autolysosomes to sustain autophagic lysosome regeneration, a process that essentially "recycles" lysosomes from pre-existing autolysosomes (Chen and Yu, 2017). They also reported that hepatocytes from ethanol-fed rats contained lower levels than controls of the phosphorylated form of Src kinase, which phosphorylates, and activates Dyn-2. Consequently, hepatocytes of ethanol-fed rats contained 42% fewer lysosomes and 40–66% higher levels of the autophagy marker/substrate proteins LC3II and p62 than cells from pair-fed control animals. Their data indicate an ethanol-elicited retardation of LD degradation that likely results, in part, from faulty lysosome regeneration, adding to the previously-reported ethanol-induced decline in *de novo* lysosome biogenesis in livers of ethanol-fed rats (Kharbanda et al., 1996). Similar findings were recently reported in mice (Chao et al., 2018a,b), using the chronic ethanol feeding, followed by ethanol binge (NIAAA) feeding model of ethanol administration (Bertola et al., 2013a,b; Gao et al., 2017). The latter feeding regimen to rodents reportedly recapitulates the drinking patterns, as well as the degree of liver injury in humans with AUDs.

Related work by Schulze et al. (2017a) found that chronic ethanol consumption impairs Rab7, a small GTPase that facilitates lysosome recruitment to the LD. They report that hepatocytes from ethanol-fed rats exhibit lysosome clustering, suggesting a partial blockage of lysosome mobility and dispersion. They also detected a 50% decline in Rab7 activity in fasted (lipophagy-activated) hepatocytes from ethanol-fed rats, compared with identically-treated hepatocytes from control rats. Together, their findings indicate that ethanol exposure negatively affects GTPases that have accessory roles in lysosome function. These findings further support the notion that ethanol consumption significantly disrupts the degradative (lysosomal) phase of lipophagy. Their reported findings of ethanol-induced disruption in lysosome mobility

also indicate an ethanol-elicited decline in trafficking of these organelles by microtubules. As mentioned earlier, acetaldehyde, the primary oxidation product of ethanol oxidation, blocks polymerization of microtubule subunits into active molecular motors by forming adducts with the alpha tubulin subunit (Smith et al., 1989; **Figure 2**).

Chronic Ethanol Consumption Retards Triglyceride Breakdown (Lipolysis) and Fatty Acid Oxidation

Lipophagic degradation of LDs, yields free triglycerides and cholesteryl esters. These are hydrolyzed by lipases and esterases, respectively, generating free cholesterol, and high-energy fatty acids. Each fatty acid is activated by conjugation to coenzyme A (CoA), forming a fatty-acyl CoA, which is transported into the mitochondrion. There, it undergoes a stepwise series of beta (β) oxidations and cleavages, producing multiple two-carbon units of acetyl-CoA, which enter the Krebs cycle to generate ATP. Thus, the oxidative breakdown of one mole of palmitate, a C16 fatty acid, yields eight moles of acetyl CoA, to generate 96 moles of ATP. This is four-times more ATP than that yielded by the oxidation of one mole of glucose, a C6 monosaccharide (Devlin, 1992).

Ethanol consumption impedes triglyceride (TG) breakdown, as demonstrated by Schott, et al. who reported that β-adrenergic activation of the cytosolic ATGL and phosphorylation of the hormone-sensitive lipase (HSL) are lower in hepatocytes from ethanol-fed rats than in cells from pair-fed controls (Schott et al., 2017). These findings are closely linked to a reduction in LD breakdown in these cells.

Furthermore, chronic ethanol consumption impairs the mitochondrial oxidation of fatty acids released from hydrolyzed TGs by causing mitochondrial depolarization (Zhong et al., 2014) and by dysregulating the function of the peroxisome proliferator activated receptor alpha/retinoid X receptor (PPAR-α/RX receptor) a transcriptional regulator that governs expression of enzymes that catalyze FAO (Fischer et al., 2003). Evidence suggests that such disruption by alcohol administration is caused, in part, by direct binding of metabolically-generated acetaldehyde to PPAR-α, thereby diminishing its ability to bind target DNA sequences used to transcribe mRNAs that encode FAO enzymes (Galli et al., 2001).

Chronic Ethanol Administration Lowers the Nuclear Content of Transcription Factor EB (TFEB)

Transcriptional regulators of autophagy/lipophagy belong to the microphthalmia-associated/TFE subfamily of basic/helix-loop-helix/leucine zipper transcription factors. These include transcription factors EB and E3 (TFEB and TFE3) in mammals. TFEB is the major transcription factor which activates genes that encode proteins involved in autophagy, lysosome biogenesis and mitochondrial biogenesis (Settembre et al., 2011, 2012; Settembre and Medina, 2015). It was also demonstrated that TFEB promotes lipophagy (Settembre et al., 2013).

Our laboratory examined the levels of intranuclear (transcriptionally active) and cytosolic (inactive) TFEB in livers of C57Bl/6 mice subjected to acute and chronic ethanol administration. Interestingly, our analyses revealed that, compared with vehicle-gavaged control mice, TFEB nuclear content was elevated in livers of acutely-treated, ethanol-gavaged mice. These findings are consistent with similar studies conducted previously (Ding et al., 2010) and indicate that sudden hepatic oxidant stress in naïve animals enhances autophagy in liver, which was confirmed by changes in autophagy markers, LC3II and P62. Additionally, it is noteworthy that proteasome activity was unaffected in livers of acutely ethanol-gavaged mice. We obtained contrasting results, after subjecting mice to chronic ethanol feeding for 5–9 week. Liver nuclei of ethanol-fed mice exhibited lower nuclear TFEB content than pair-fed control mice Proteasome activity in livers of ethanol-fed animals was also significantly lower than pair-fed control mice (Thomes et al., 2015).

Recently, we examined the recovery from alcohol-induced steatosis in 6 week ethanol-fed Wistar rats, some of which were withdrawn from ethanol and fed control diet for 7 days. **Figures 4A,B** show that nuclear TFEB content in livers of ethanol-fed rats was three-fold lower than in pair-fed control rats, while the level of cytosolic TFEB in ethanol-fed rats was higher (**Figure 4C**), despite the fact that the level of mRNA that encodes TFEB was twofold lower in ethanol-fed rats than controls (data not shown); see Thomes et al. (2019). **Figures 4A,B** also demonstrate that nuclear TFEB levels returned to normal after 7 days of refeeding the control diet. Furthermore, **Figure 4D** illustrates a similar pattern of decline and restoration of proteasome activity in ethanol-fed rats, respectively, before and after 7 days of refeeding the control diet.

The question that arises from these studies is what mechanism(s) is/are responsible for the decline in nuclear TFEB content in chronically ethanol-fed rats and mice? **Figure 5** provides a likely scenario: The entry of TFEB into the nuclear compartment is blocked by its phosphorylation, catalyzed principally by the mechanistic target of rapamycin (mTOR), the "master kinase" that suppresses autophagy (Kim and Guan, 2015). Unphosphorylated TFEB in the cytosol can freely enter the nucleus to enhance coordinated lysosomal expression and regulation (CLEAR) gene transcription (Settembre and Medina, 2015), while its inactive, phosphorylated form remains in the cytosol, along with unphosphorylated TFEB produced by *de novo* synthesis.

Sha et al. (2017) demonstrated that the steady-state intracellular content of TFEB is principally regulated in the cytosol by the UPS. They report that a chaperone-dependent E3 ubiquitin ligase, STIP1 homology and U-Box containing protein 1 (STUB1) modulates TFEB content by preferentially ubiquitylating its phosphorylated form as a signal for degradation by the 26S proteasome. Thus, if either STUB1 or the 26S proteasome is inactivated, undegraded, phosphorylated TFEB accumulates in the cytosol thereby preventing autophagy gene activation by blocking the nuclear entry of unphosphoryated (active) TFEB. The latter scenario is possible because excess inactive TFEB monomers form

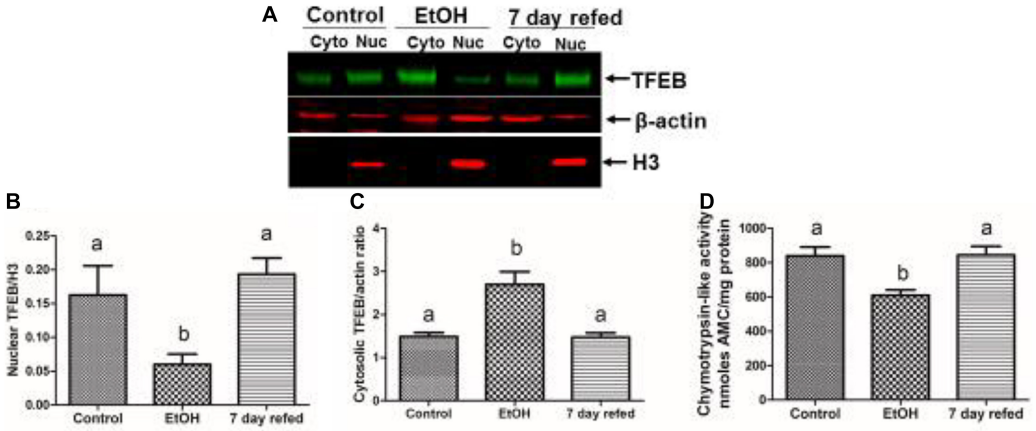

FIGURE 4 | Effect of chronic ethanol administration on the nuclear and cytosolic levels of TFEB in rats subjected chronic ethanol feeding and withdrawal. **(A)** Western blots of nuclear and cytosolic TFEB in livers of rats fed control diet ethanol diet and rats fed ethanol diet and then withdrawn from ethanol and fed control diet for 7 days. **(B)**–mean levels of nuclear TFEB, **(C)** mean levels of cytosolic TFEB, and **(D)** mean hepatic proteasome activities in the three groups of animals, as indicated (figure reproduced from Figure 4 by Thomes et al., 2019 with permission).

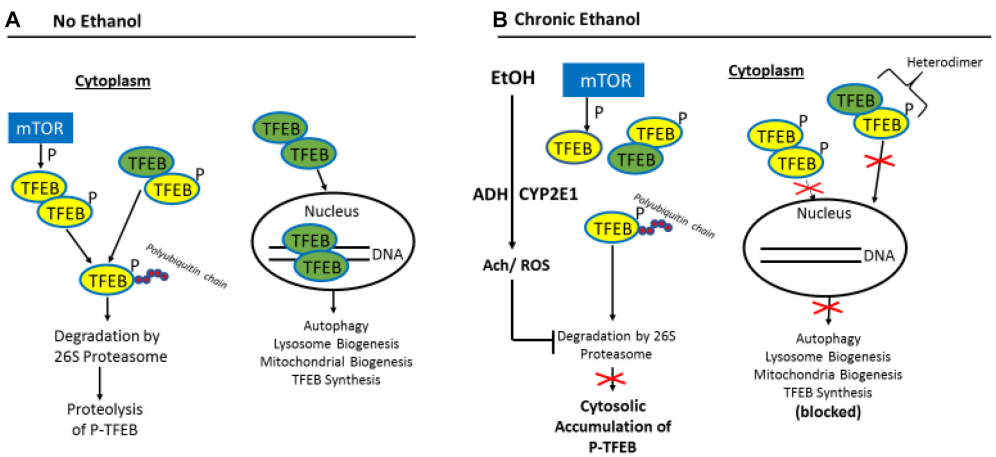

FIGURE 5 | Proposed model for the impairment of TFEB nuclear content by ethanol oxidation. **(A)** In the absence of ethanol, phosphorylated (inactive) TFEB (p-TFEB; yellow circles) is selectively ubiquitylated and degraded by the ubiquitin-proteasome system (UPS) in the cytosol, allowing translocation of active TFEB (green circles) into the nucleus to enhance transcription of CLEAR genes. **(B)** Chronic ethanol oxidation inhibits proteasome activity, which causes p-TFEP accumulation in the cytosol, resulting in heterodimer formation between pTFEB and active TFEB, thus preventing nuclear translocation of active TFEB and transcription of CLEAR genes (figure adapted from Figure 9 by Sha et al., 2017 with permission).

heterodimers with unphosphorylated TFEB monomers (Sha et al., 2017), thereby blocking the nuclear entry of active TFEB to initiate autophagy. Because chronic ethanol consumption/oxidation generates oxidants that inhibit liver proteasome activity (**Figures 4C**, **5**; Donohue et al., 1998, 2007b; Osna et al., 2004; Donohue, 2005; Thomes et al., 2015) such inhibition likely influences the ethanol-induced depletion of nuclear TFEB and the accumulation of cytosolic TFEB. This same scenario also partially explains why nuclear TFEB is either unaffected (or even rises) after acute ethanol treatment, as hepatic proteasome activity is unchanged by ethanol gavage (Thomes et al., 2012). Additionally other factors prevail under acute and chronic conditions, as we described before (Thomes et al., 2015) to explain these contrasting results.

ACTIONS AND AGENTS THAT ALLEVIATE ETHANOL-INDUCED AND/OR DIET-INDUCED STEATOSIS

Actions

Withdrawal of ethanol from ethanol-fed rats (Thomes et al., 2019) and humans rather rapidly and almost completely resolves their steatosis (Kirchgesner and Danse, 2014; Thiele et al., 2018) to suggest that the absence of ethanol oxidation allows resumption of normal lipophagy after ethanol withdrawal.

Agents

The following are dietary and other natural or synthetic agents that are reported to enhance autophagy. None have been

rigorously tested in humans with AUD, but some have undergone testing in ethanol-fed animals.

Dietary Components

Caffeine is a well-known component of coffee (Gonzalez de Mejia and Ramirez-Mares, 2014). Caffeine is believed to block mTOR signaling thereby stimulating lipophagy and FAO in mice (Sinha et al., 2014). Two meta-analyses in humans with NAFLD revealed that caffeine consumption alone does not affect liver fibrosis that arises from NAFLD. Interestingly, others report that regular coffee consumption provides protection against NAFLD-associated fibrosis (Marventano et al., 2016; Shen et al., 2016). Most of the latter findings indicate that, in addition to caffeine, other non-caffeine components in coffee are hepatoprotective (Chen et al., 2014).

Zinc is an essential metal that has a hepatoprotective effect when it is supplemented in the diets of human AUD patients and ethanol-fed rodents (McClain et al., 2017). Dietary supplematation with zinc reportedly reactivates PPAR-α, to stimulate β-oxidation of fatty acids, thereby attenuating ethanol-induced steatosis (Kang et al., 2009). Zinc, is also critical for lysosome acidification and biogenesis in mammary tissue (Rivera et al., 2018) to suggest that, by itself, zinc stimulates autophagy/lipophagy. The latter effect of zinc must be confirmed in liver.

Plant-Derived Agents

Corosolic acid is a compound extracted from the leaves of the banaba tree (*Langertroemia speciosa* L). The chemical protects the liver from alcoholic-induced liver injury, in part by subduing apoptosis and restoring hepatic autophagy after activating the AMP-activated protein kinase (AMP kinase). The latter enzyme suppresses mTORC1 activity, which allows autophagy activation (Guo et al., 2016).

Li et al. (2019) tested the efficacy of quercitin, a flavonoid found in fruits, vegetables, red wine, and herbal medications, on autophagy in livers of 15 week ethanol-fed C57Bl/6 mice. They report that quercitin, administered by gavage, effectively reversed the ethanol-induced blockade of TFEB nuclear localization, restoring both lysosome function and autophagic flux to normal.

Salvianolic acid A is a is a water-soluble phenolic carboxylic acid extracted from *Salvia miltiorrhiza*. It has been tested in ethanol-fed rats and is reported to ablate alcohol-induced liver injury by reducing alcohol-induced steatosis by enhancing autophagosome-lysosome fusion after restoring lysosomal cathepsin activities (Shi et al., 2018).

Repurposed Compounds

Lin et al. tested the effects of the autophagy inducers carbamazepine (an anti-seizure medication) and rapamycin (an immunosuppressant that blocks mTORC1). They also tested the lysosome inhibitor chloroquine, the antimalarial drug, which causes a rise in intralysosomal pH, on alcohol and diet-induced steatosis and injury (NAFLD). Their analyses revealed that treatment with the autophagy inducers attenuated fatty liver and injury in both models of AFLD and NAFLD. In contrast, chloroquine treatment exacerbated steatosis in these animals, but its effects were reversed by co-treatment with carbamezapine (Lin et al., 2013).

Nanoformulated Compounds

A comprehensive study by Wang et al. (2018) used a nanotechnology-enabled high throughput screen to test 15,000 compounds to identify those that activate TFEB. Three of these, digoxin, a cardiac glycoside used to treat atrial fibrillation, ikarugamycin, a natural antibacterial/anti-protozoan agent, used to treat infections, and alexidine dihydrochloride, an inhibitor of protein tyrosine phosphatase localized to the mitochondrion, activated TFEB, each by a distinct calcium-dependent mechanism. When the latter compounds were each formulated into nanoparticles and used to treat animals, each of them conferred hepatoprotection from diet-induced steatosis in mice and extended the lifespan of the roundworm *Caenorhabditis elegans* (*C elegans*). Whether these latter compounds would be effective agents to treat alcohol-induced fatty liver remains to be tested.

SUMMARY AND FUTURE DIRECTIONS

It is evident that autophagy is critical for maintaining normal liver function and it has a crucial role in ablating fatty liver disease that arises from excessive drinking. Although autophagy activity in the alcohol-induced fatty liver is affected by the duration of ethanol consumption/exposure, the amount of ethanol administered, and the way in which it is administered, there is solid evidence that chronic ethanol consumption retards the lipophagic/autophagic clearance of LDs that accumulate during the pathogenesis of alcohol-induced fatty liver. The reasons for retarded removal of LDs are threefold: (1) Ethanol oxidation disrupts the autophagic/lipophagic machinery (e.g., lysosomes) that degrade LDs; (2) alcohol-metabolism changes the phospholipid composition of the LD membrane, which, in turn, alters its content of resident proteins, conferring greater membrane resistance to lipophagy and lipolysis; and (3) ethanol metabolism thwarts the mitochondrial oxidation of fatty acids extracted from LDs by inhibiting PPAR-α and disrupting mitochondrial function.

While it is clearly evident that chronic alcohol consumption disrupts lipophagy, which contributes to alcohol-induced steatosis, the specific ethanol metabolite(s) that cause(s) such disruption(s) have/has yet be unequivocally identified. Strong circumstantial evidence, cited here, implicates metabolically-derived acetaldehyde as the offending agent. The challenges to confirming acetaldehyde as the sole autophagy disruptor are: (1) its volatility (bp = 21°C); (2) the high sensitivity required for its detection *in vivo* in its free and adducted forms. (3) detecting acetaldehyde or MAA adducts on specific autophagy/lipophagy-related proteins; and (4) defining whether such modifications influence the biological functions of these proteins.

Both acute and chronic alcohol consumption increase ROS production, which leads to oxidant stress. Our previous findings indicate that blocking acetaldehyde/ROS formation or enhancing their removal, avoids the disruptive effects of ethanol

oxidation on autophagy in ethanol-metabolizing cells. Studies have clearly shown that ethanol oxidation significantly disrupts the degradative (lysosomal) phase of autophagy/lipophagy.

Abstinence from alcohol along with proper pharmacological (Johnson, 2010) and behavioral therapies (Hagedorn et al., 2016) can effectively minimize recidivism and reverse steatosis in people with AUDs. Our animal studies, revealed that ethanol withdrawal from ethanol-fed rats almost completely resolves their steatosis after 7 days (Thomes et al., 2019). These findings also suggest that cessation of ethanol consumption restores lipophagy rates to normal, thereby accelerating the removal of excess LDs.

Finally, evidence, mostly from animal studies, but some human studies, suggest that caffeine/coffee, resveratrol, corosolic acid, zinc, carbamazepine, and rapamycin, individually activate autophagy/lipophagy and may also be used to prevent and treat alcohol-induced fatty liver. The clinical utility of these aforementioned compounds and of those tested recently (Wang et al., 2018) appear to have therapeutic promise but those that were most recently discovered to activate TFEB (Wang et al., 2018) must be tested for safety and efficacy in human trials.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing or the research in this review.

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REVIEW
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Relevance of Autophagy Induction by Gastrointestinal Hormones: Focus on the Incretin-Based Drug Target and Glucagon

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The biology of autophagy in health and disease conditions has been intensively analyzed for decades. Several potential interventions can induce autophagy in preclinical research; however, none of these interventions are ready for translation to clinical practice yet. The topic of the current review is the molecular regulation of autophagy by glucagon, glucagon-like peptide (GLP)-1 and the GLP-1-degrading enzyme dipeptidyl peptidase-4 (DPP-4). Glucagon is a well-known polypeptide that induces autophagy. In contrast, GLP-1 has been shown to inhibit glucagon secretion; GLP-1 also has been related to the induction of autophagy. DPP-4 inhibitors can induce autophagy in a GLP-1–dependent manner, but other diverse effects could be relevant. Here, we analyze the distinct molecular regulation of autophagy by glucagon, GLP-1, and DPP-4 inhibitors. Additionally, the potential contribution to autophagy by glucagon and GLP-1 after bariatric surgery is discussed.

Keywords: autophagy, incretin, GLP-1, DPP-4, glucagon

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INTRODUCTION

Recent advances with incretin-based drugs have opened new avenues in the management of diabetes. In the clinic, we can prescribe two types of incretin-based drugs: glucagon-like peptide 1 receptor agonists (GLP-1RAs) and dipeptidyl peptidase-4 (DPP-4) inhibitors. GLP-1 is produced from intestinal L-cells by proteolytical processing from proglucagon (ProG) and immediately degraded by DPP-4; its half-life is approximately 2 min. GLP-1RAs have been developed to avoid DPP-4-mediated cleavage of GLP-1 by introducing a mutation in the amino acid residue that DPP-4 targets. Exenatide (Exendin-4), a 39-amino-acid polypeptide isolated from the venom of the Gila monster lizard with 50% homology to human GLP-1, has been used in the clinic. Alternatively, to extend the half-life of endogenous GLP-1, DPP-4 inhibitors are prescribed. Recent clinical trials (Marso et al., 2016, 2017; Rosenstock et al., 2019) investigating the safety and efficacy of incretin-based drugs have provided diverse interpretations and provocative intellectual curiosities regarding the biology of incretin hormones and incretin-based drugs, specifically focusing on pleiotropic effects.

Autophagy, the cellular mechanism that promotes cell survival during nutrient depletion, may also be relevant under basal or nutrient excess conditions. This cellular process is specified by the formation of autophagosomes, by which cytosolic components are captured and fused with lysosomes to promote the degradation and/or recycling of its contents. The autophagic

process consists of four stages: initiation, nucleation, elongation, and fusion/degradation (Codogno et al., 2011). During nutrient depletion, autophagy can provide essential components for energy production and biosynthesis. However, it also acts in a similar manner by recycling damaged organelles, unnecessary proteins, and foreign substances for the quality maintenance of these intracellular components (Ueno and Komatsu, 2017). In circumstances of nutrient excess, autophagy plays important roles in eliminating unfolded proteins and toxic aggregates and facilitating endoplasmic reticulum (ER) homeostasis. The detailed mechanisms and biology of autophagy are summarized in subsequent sections of this issue.

Autophagy defects in certain diseases have been the subject of extensive research. In addition, liver autophagy defects have been shown to occur with several metabolic diseases, such as obesity, steatosis, and type 2 diabetes (Ueno and Komatsu, 2017). Early work in liver research indicates a link in the regulation between gastrointestinal hormones and liver autophagy (Ueno and Komatsu, 2017). Interestingly, incretin hormones and DPP-4 inhibitors have been associated with the amelioration of steatosis (Rowlands et al., 2018; Zheng et al., 2018). These drugs have been shown to induce autophagy in various cell types (Murase et al., 2015; Rowlands et al., 2018; Zheng et al., 2018).

In this review, we investigated the potential involvement of autophagy induction by GLP-1 and incretin-based dugs. In addition, we focused on glucagon, a known polypeptide that regulates glucose levels and a classic molecule that induces autophagy.

GLUCAGON AND GLP-1 SYNTHESIS FROM PROGLUCAGON

GLP-1 is produced from proteolytic cleavage of the precursor polypeptide pProG (Muller et al., 2017). The *pProG* gene (*Gcg*) is expressed in a specific population of enteroendocrine cells (L-cells) in the intestinal mucosa, islet cells in the pancreas, and some neurons within the nucleus of the solitary tract (NTS) (Han et al., 1986; Jin et al., 1988). Regulation of the *Gcg* transcription process is not completely known and distinct pattern of mRNA expression has been reported in intestinal endocrine cells and in pancreatic islet α-cells (Jin, 2008; Yi et al., 2008; Chiang et al., 2012; Muller et al., 2017). In addition to such unique transcriptional control in each cell type, posttranslational processing of prohormone plays an important role in the major cell types producing ProG peptides. In addition to glucagon and GLP-1, glucagon-like peptide-2 (GLP-2), oxyntomodulin, glicentin, glicentin-related pancreatic polypeptide (GRPP), and major proglucagon fragment (MPGF) are synthesized from ProG; however, the specific biological function of some of these fragments has not been identified (**Figure 1**). Such posttranslational regulation of these ProG peptides in their respective cell types relies on tissue-specific posttranslational modification by prohormone convertases (PCs). In intestinal L-cells and neurons of the NTS, a predominance of PC1/3 expression, GLP-1, oxyntomodulin, and GLP-2 are seen as physiologically relevant (Tucker et al., 1996; Larsen et al., 1997; Vrang et al., 2007); in pancreatic α-cells, high PC2 levels are responsible for the predominant glucagon synthesis (**Figure 1**)

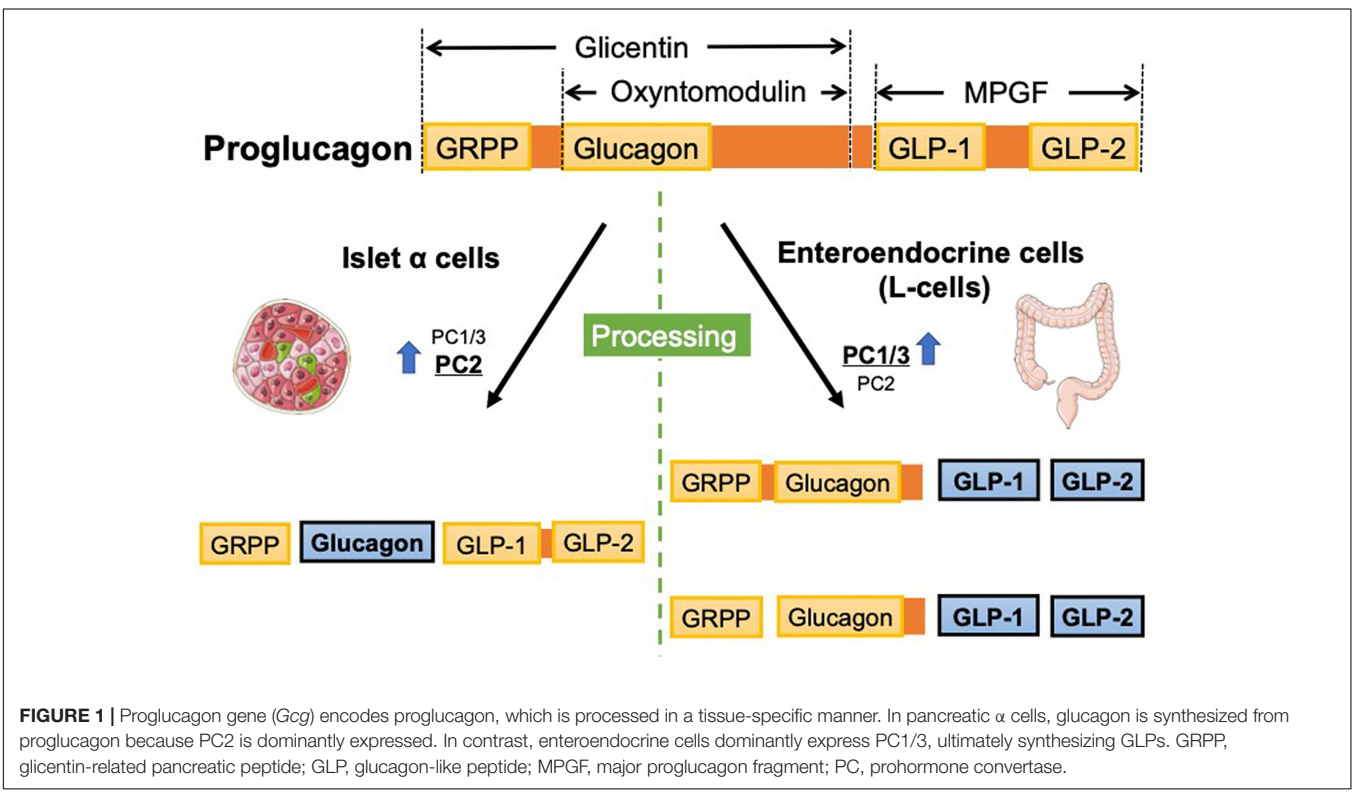

FIGURE 1 | Proglucagon gene (*Gcg*) encodes proglucagon, which is processed in a tissue-specific manner. In pancreatic α cells, glucagon is synthesized from proglucagon because PC2 is dominantly expressed. In contrast, enteroendocrine cells dominantly express PC1/3, ultimately synthesizing GLPs. GRPP, glicentin-related pancreatic peptide; GLP, glucagon-like peptide; MPGF, major proglucagon fragment; PC, prohormone convertase.

(Holst et al., 1994). PC2 is also expressed in the brain but does not colocalize with *Gcg*. Additionally, PC1/3 is expressed in α-cells but at lower levels than PC2, and the ratio of GLP-1 to glucagon expressed in islet cells has been shown to be increased during the progression of diabetes (O'Malley et al., 2014). *Gcg* expression and ProG levels are relatively lower in the proximal gut and higher in the distal part, with the highest expression in the colon (Bryant and Bloom, 1979).

GLUCAGON AND AUTOPHAGY

The association between autophagy and glucagon was reported approximately 50 years ago. In 1955, Christian de Duve reported on acid phosphatase-positive sac-like particles in rat liver cytoplasm (De Duve et al., 1955). Electron microscopy analysis revealed that such particles are surrounded by a lipoprotein membrane (Novikoff et al., 1956) and later showed acid hydrolases in these organelles, which were subsequently named lysosomes.

Secreted glucagon is recognized by the glucagon receptor (a G protein-coupled receptor) on the hepatocyte, subsequently adenyl cyclase-mediated productions of the second messenger cAMP was stimulated. Rise in the intracellular level of cAMP activates protein kinase A (PKA) and inhibits salt-inducible kinases (SIK). PKA phosphorylates Ser133 of cyclic AMP-responsive element-binding protein (CREB) and SIK dephosphorylates Ser171 of CREB-regulated transcription co-activator (CRTC). Ser133-phosphorylated CREB together with CRTC upregulates CREB target genes such as the gluconeogenesis-related genes PGC1α, nuclear receptor subfamily 4 group A member 1 (NR4A1) and TFEB which regulates gene expressions of autophagy proteins (Ueno and Komatsu, 2017).

In 1962, seminal work by Ashford and Porter (1962) found that glucagon administration increased the autophagy in liver. Subsequently, the role of glucagon in hepatocyte autophagy induction was confirmed *in vivo* by studies in rats (Arstila and Trump, 1968; Guder et al., 1970; Deter, 1971). Such effects of glucagon on the autophagy are likely tissue specific manner (Mortimore and Poso, 1987). Glucagon could induce autophagy by increasing the size and number of autophagic vacuoles (Guder et al., 1970; Deter, 1971; Shelburne et al., 1973); in addition, glucagon enhanced the fragility of hepatic lysosomes both mechanically and osmotically and altered sedimentation properties (Deter and De Duve, 1967). Such effects of glucagon on the hepatic lysosome appeared 30 min after intraperitoneal administration of glucagon, peaked for 15–30 min, and disappeared after approximately 4 h (Deter and De Duve, 1967). The number of hepatic lysosomes increased under conditions associated with an increase in endogenous glucagon levels, such as starvation (Guder et al., 1970), hypoglycemia induced by phlorizin (Becker and CornwallJr., 1971), or type 1 diabetes (Amherdt et al., 1974). Supporting these findings, a significant correlation between the parameters of hepatic lysosomal volume density and plasma glucagon was observed in rats with type 1 diabetes induced by streptozotocin, and insulin intervention in these rats led to suppression of glucose and glucagon levels (Amherdt et al., 1974). In addition, pancreatic transplantation normalized liver autophagy levels in rats with streptozotocin-induced diabetes by restoring insulin and glucagon levels (Brekke et al., 1983). Glucagon is relevant to glucagon-mediated glycogenolysis; glycogen granules are selectively enveloped by autophagosomes for catabolism into glucose. This special type of autophagy is termed glycophagy.

GLP-1-RELATED AUTOPHAGY

GLP-1RA has been shown to suppress glucagon levels (Mentis et al., 2011). Even though tissue diversity effects of glucagon on the autophagy induction, the liver is the established target organ for glucagon-induced autophagy; therefore, from this point of view, GLP-1 signaling could be relevant to inhibiting autophagy induction in liver. Recently, however, GLP-1 has also been implicated in the induction of autophagy in the liver (He et al., 2016) and in β cells (Zummo et al., 2017; Arden, 2018) as well.

GLP-1 can protect β cells from insults induced by chronic exposure to excess nutrients via induction of autophagosomal-lysosomal fusion (Zummo et al., 2017; Arden, 2018). Exendin-4, an agonistic polypeptide for human GLP-1R derived from the venom of the Gila monster lizard, has also been shown to enhance lysosomal function in β-cells, improve autophagosome clearance and protect against islet injury in a rat model of tacrolimus-induced diabetes (Lim et al., 2016). Indeed, in this study, β cells from rats administered Exendin-4 showed a reduction in the number of autophagosomes (Lim et al., 2016). Therefore, in certain environments, contrary to the hypothesis of the antiglucagon and antiautophagic signaling effects of GLP-1, GLP-1 receptor signaling could be relevant to the accelerated effects on autophagosomal-lysosomal fusion and the positive mediation of autophagic flux. However, the role of GLP-1 in β-cell autophagy is complex and likely dependent on stress conditions. In a rat model fed with high levels of fructose, GLP-1 analog intervention induced notable inhibition of β cell autophagy and enhanced β cell mass and function (Maiztegui et al., 2017). The detailed molecular regulation of the autophagy system in β cells via GLP-1 receptor signaling requires further investigation.

The liver is the potential target organ for GLP-1-induced autophagy (He et al., 2016). Among the organs, the level of GLP-1 in the liver is highest because of transport through the hepatic portal vein from the gut. Intervention of GLP-1 or its analogs could ameliorate several aspects of liver injury (D'Alessio et al., 2004; Cantini et al., 2016) and influence hepatic gluconeogenesis, glycogen synthesis, and glycolysis (**Figure 2**). For the effects of GLP-1, its receptor GLP-1R is essential; the presence of GLP-1R is controversial in hepatocytes and the focus of intense discussion. Protein expression of GLP-1R has been reported in transformed human hepatocyte cell lines, Hep-G2, HuH7, and primary human hepatocytes (Gupta et al., 2010). Even though there is confirmation of GLP-1R expression on hepatocytes, research has suggested that some effects of GLP-1 are indeed GLP-1R-independent events (Bullock et al., 1996;

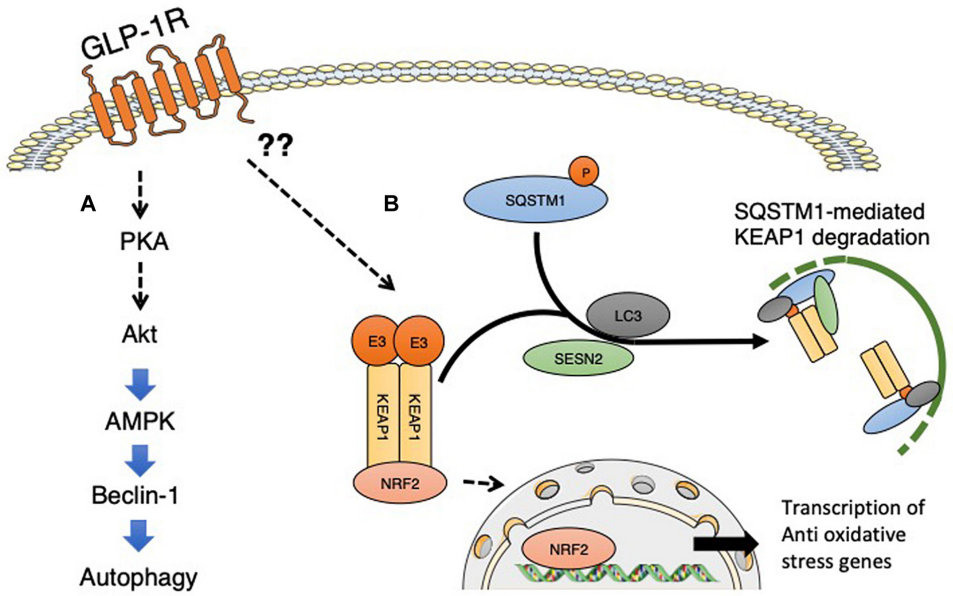

FIGURE 2 | GLP-1 receptor signaling and induction of autophagy. **(A)** GLP-1 receptor signaling activated PKA signaling, and subsequent activation of Akt and AMPK induced the proautophagic molecule Beclin-1. **(B)** GLP-1 receptor signaling is associated with NFR2 activation and the subsequent induction of oxidative stress-responsive genes. Even though detailed mechanisms have not yet been elucidated, GLP-1 signaling could potentially induce the interaction between SQSTM1 and KEAP1, resulting in the suppression of KEAP1-driven ubiquitination of NRF2; phosphorylated SQSTM1 and KEAP1 complexes are selectively degraded by autophagy. The involvement of sestrin2 associated with GLP-1 receptor signaling has not yet been analyzed. SESN2, sestrin2.

Flock et al., 2007; Aviv et al., 2009; Tomas et al., 2010). An alternative possible explanation could be based on the GLP-1 degradation products, such as GLP-1 9-36, GLP-1 28-36, or GLP-1 32-36. Studies have indicated that both GLP-1 28–36 and GLP-1 32–36 are cell-penetrating peptides that do not require a GLP-1R (Elahi et al., 2014). GLP-1(32–36) amide, a novel pentapeptide cleavage product of GLP-1, modulates whole-body glucose metabolism in dogs (Elahi et al., 2014). GLP1-derived nonapeptide GLP1(28–36) amide preserves pancreatic β cells from glucolipotoxicity (Liu et al., 2012) and activates PKA and Wnt signaling [reviewed in (Jin and Weng, 2016)]. The beneficial effects of GLP-1 fragments were reported to include kidney protective effects in *db/db* mice with diabetes (Moellmann et al., 2018). Whether such GLP-1 derived fragments are relevant for the GLP-1-induced liver autophagy induction is not known and required further investigation.

A series of experiments provided evidence that a GLP-1 agonist ameliorated hepatic steatosis and metabolic defects. Such antisteatotic and metabolic effects of GLP-1 on the liver could involve AMPK activation and suppression of the mTOR pathway. Liraglutide has been shown to halt the progression of steatosis and is associated with the induction of autophagy via activation of AMPK and suppression of mTOR pathways (He et al., 2016). By activating both macro- and chaperone-mediated autophagy, GLP-1 could protect hepatocytes from fatty acid-related apoptosis by suppressing a dysfunctional ER stress response. GLP-1 therapies have been shown to relieve the burden on the ER, reduce ER stress, and decrease subsequent hepatocyte apoptosis (Sharma et al., 2011).

The alternative explanation of autophagy induction by GLP-1 could be associated with suppression of oxidative stress. GLP-1 agonists have been shown to reduce oxidative stress in diverse preclinical studies. When the endogenous antioxidant system cannot remove free radicals appropriately, oxidative stress, the accumulation of such free radicals, accelerates a variety of disease conditions, such as diabetes and its complications, cancer, and neuronal disorders. Cellular defense systems for combatting reactive oxygen species (ROS) rely on the presence of antioxidants that scavenge ROS or induce genes involved in cytoprotection to neutralize ROS (Kaspar et al., 2009; Ma, 2013). The nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor is a master regulator of redox balance and is responsible for the transcription of various antioxidant and detoxification genes by binding to antioxidant response elements (AREs) (Kaspar et al., 2009; Ma, 2013). GLP-1 agonists have been shown to induce Nrf2 in β cells (Oh and Jun, 2017).

When cells are exposed to oxidative stress, SQSTM1, known as the ubiquitin-binding protein p62 and an autophagosome cargo protein, is phosphorylated at Ser349. Phosphorylated form of SQSTM1 physically interacted with KEAP1, an adaptor of the ubiquitin ligase complex for Nrf2, with high affinity (Ueno and Komatsu, 2017). The interaction between SQSTM1 and KEAP1 results in the suppression of KEAP1-driven ubiquitination of Nrf2; phosphorylated SQSTM1 and KEAP1 complexes are selectively degraded by autophagy (Ueno and Komatsu, 2017). Thereafter, Nrf2 is stabilized, translocates into the nucleus, and induces the expression of various essential cytoprotective genes, such as NAD(P)H dehydrogenase quinone 1, glutathione

S-transferase, glutamate-cysteine ligase catalytic subunit and heme oxygenase 1 (Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010; Taguchi et al., 2012; Ichimura et al., 2013). Sestrin 2, also known as an intracellular leucine sensor that negatively regulates mTORC1 signaling, binds with the SQSTM1 and KEAP1 complexes and functions as a scaffold protein for the SQSTM1-mediated autophagy of KEAP1 (Bae et al., 2013). Sestrin 2 is also induced under conditions of stress (Yang et al., 2014); Nrf2 activation might be regulated by selective autophagy under metabolic stress. Therefore, GLP-1-induced Nfr2 activation could be relevant to GLP-1-induced autophagy, but further study is needed. The association between GLP-1 and sestrin 2 has yet to be confirmed.

DPP-4 INHIBITORS-INDUCED AUTOPHAGY

DPP-4, a member of the serine peptidase/prolyl oligopeptidase gene family, was first found as a T cell differentiation antigen (CD26) and also as cell surface aminopeptidase. DPP-4 displays numerous biological functions, such as protease activity, interaction with adenosine deaminase and the extracellular matrix proteins, co-receptor activity mediating viral entry, and regulation of intracellular signals (Kameoka et al., 1993; Kahne et al., 1999; Lambeir et al., 2003; Lopez-Otin and Matrisian, 2007; Lu et al., 2013). Furthermore, the complexities of the biological functions of DPP-4 are indeed multiplying with diverse bioactive substrates of DPP-4, thus emphasizing the elegant role of DPP-4 in the biochemical tuning of multiple cell type and tissues. DPP-4 inhibitors exhibited multiple organ protective potential (Kroller-Schon et al., 2012; Itou et al., 2013; Kanasaki, 2016; Zhuge et al., 2016; Avogaro and Fadini, 2018) and also influenced cancer biology (Abrahami et al., 2018; Ye et al., 2018; Enz et al., 2019; Hollande et al., 2019; Yang et al., 2019).

Some preclinical studies have shown a potential link between DPP-4 inhibition and autophagy induction. In leptin-deficient *ob/ob* mice, sitagliptin at 50 mg/kg daily for 4 weeks ameliorated weight gain, metabolic disorders, and steatosis in the liver as well as insulin sensitivity. In this study, sitagliptin increased AMPK phosphorylation and decreased mTOR phosphorylation associated with the restoration of ATG5 and Beclin 1 messenger RNA expression that was suppressed in *ob/ob* mice. In addition, the relative level of LC3-II/LC3-I was significantly diminished in *ob/ob* mice and was restored to the basal level by sitagliptin (Zheng et al., 2018). Another report showed that autophagic responses were significantly diminished in OLETF rats after experimental myocardial infarction associated with a deficiency in AMPK/ULK-1 activation, Akt/mTOR/S6 signaling, and increased Beclin-1-Bcl-2 interaction, which are key molecular events for suppressing autophagy. Intervention with the DPP-4 inhibitor vildagliptin inhibited the Beclin-1-Bcl-2 interaction and enhanced both LC3-II protein and autophagosomes in the non-infarcted region in OLETF rats without normalization of either AMPK/ULK-1 or mTOR/S6 signaling. Such effects of vildagliptin on heart autophagy are associated with an 80% survival rate in OLETF rats; chloroquine, an autophagy inhibitor, diminished these beneficial effects of vildagliptin (Murase et al., 2015).

Reports have indicated that DPP-4 inhibitors could be associated with the induction of autophagy; however, the underlying mechanisms by which DPP-4 inhibition is related to autophagy induction are not absolutely clear. It may involve an increase in levels of GLP-1 by DPP-4 inhibitor treatment. DPP-4 inhibitor enhanced insulin secretion and induction of autophagy signals in the islets of high-fat-fed mice; such effects of DPP-4 inhibition on autophagy signaling were completely abolished by GLP-1R antagonist exendin 9-39 coadministration (Liu et al., 2016).

DPP-4 AND EXTRACELLULAR MATRIX INTERACTION: RELEVANCE TO AUTOPHAGY SUPPRESSION

Other than GLP-1 induction, the pleiotropic effects of a DPP-4 inhibitor may be relevant to the mechanisms of autophagy induction by DPP-4 inhibition.

The interaction with the extracellular matrix is an important determinant of cell fate. Integrins are glycoproteins that play vital roles in cell–cell or cell–matrix interactions through αβ heterodimers. Eighteen α and eight β subunits of integrins are known, and each of them displays diverse ligand binding and signaling properties (Pozzi and Zent, 2011). Integrin subunits consist of an extracellular domain that is important for their ligand binding properties and contains a transmembrane domain and a short cytoplasmic tail, which could interact with diverse cytosolic and transmembrane proteins by consisting a focal adhesion complex (with the exception of β4) (Pozzi and Zent, 2003). Integrins display physical interaction with several extracellular matrix (ECM) glycoproteins (such as collagens, fibronectins, and laminins) and cellular receptors (Plow et al., 2000; Hynes, 2002). Integrins are essential molecules in actin cytoskeleton remodeling and in regulating cell signals that regulate biological and cellular functions (Park et al., 2015). Integrins display intracellular signaling through ligand binding ("outside-in" signaling) (Ratnikov et al., 2005). Alternatively, integrins can alter their high- to low-affinity conformations, facilitating specific ligand binding ("inside-out" signaling) (Luo et al., 2007). The activation status of integrin relies on the cell type. In most cells that adhere to the basement membrane, integrins are activated; in contrast, integrins are inactive in circulating platelets or leukocytes until they are induced by platelet aggregation or stimulated by an inflammatory response. Integrins contain neither a catalytic site nor kinase activity but play a role as a bridge between the ECM and actin cytoskeleton. Such interaction between the ECM and the actin cytoskeleton through integrins allows integrins to maintain cytoskeletal organization, cell motility and intracellular-signaling pathways such as cell survival, cell shape, cell proliferation, and angiogenesis (Arnaout et al., 2007; Luo et al., 2007).

Indeed, DPP-4 is the molecule that interacts with the key integrin, integrin β1 (**Figure 3**), which can form a heterodimer with at least 11 α-subunits. Integrin β1 has the biological function

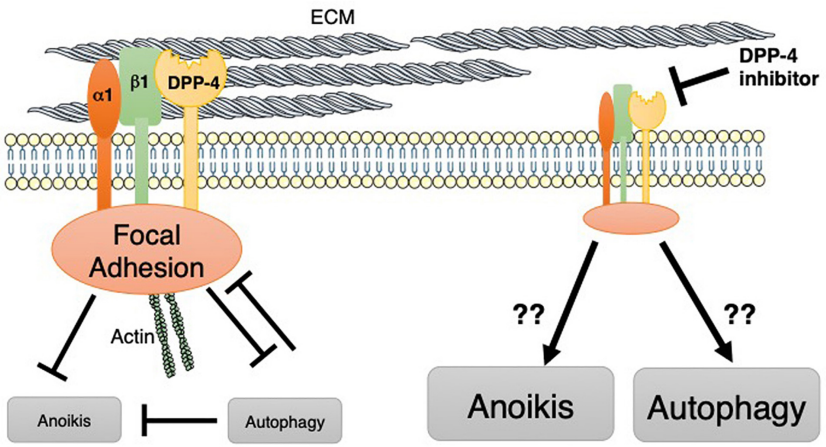

FIGURE 3 | DPP-4 may target integrin β1-mediated suppression of autophagy. On the cell membrane, DPP-4 displays an interaction with integrin β1. Integrin β1 is important for focal adhesion complexes. These integrin β1-mediated cell adhesion complexes potentially inhibit both anoikis and autophagy. Indeed, autophagy is an important biological mechanism for protecting cells from anoikis as well. Autophagy also targets and inhibits focal adhesion complexes.

of a "hub integrin" and acts as a receptor for specific ECM components, revealed in kidney epithelial cells (Glynne et al., 2001) or in T cell lymphoma (Elias et al., 2014). The loss of membrane-bound DPP-4 has been associated with suppression of the phosphorylation of integrin β1 S785, which plays a key role in the cellular adhesion of integrin β1 to the ECM (Sato et al., 2005). We have shown that the DPP-4 inhibitor linagliptin suppressed the interaction between DPP-4 and integrin β1, subsequently inhibiting the endothelial to mesenchymal transition program (Shi et al., 2015; Kanasaki, 2016), the fibrogenic programs associated with the inhibition of autophagy (Singh et al., 2015). Interestingly, in addition to the biological importance of integrin β1 on the suppression of autophagy, the autophagy pathway targets integrin β1 during nutrient starvation (Vlahakis and Debnath, 2017). Autophagy degrades focal adhesion proteins and promotes turnover of those molecules (Vlahakis and Debnath, 2017). Additionally, integrin-mediated cell adhesion to the ECM has been shown to protect cells from anoikis, the apoptosis induced by the lack of correct cell/ECM attachment. Once integrin-mediated interaction with the ECM is lost, cells induce autophagy for survival (**Figure 3**). Autophagy induction has been shown to promote the survival of epithelial cells and adjustments in the absence of cell–matrix contact, resulting in the anoikis resistance (Yang et al., 2013; Chen et al., 2017; Talukdar et al., 2018) (**Figure 3**). After autophagy was inhibited by either RNA interference or harboring of oncogenes, cells lost their ability to combat anoikis (**Figure 3**).

PERSPECTIVE: BARIATRIC SURGERY AND AUTOPHAGY INDUCTION

Bariatric surgery, including Roux-en-Y (RYGB), gastric banding, sleeve gastrectomy (SG), and biliopancreatic diversion (BPD), has been a beneficial intervention in the treatment of obesity for reduction in body weight and is associated with the amelioration of liver steatosis and metabolic defects. However, the detailed molecular mechanisms of the beneficial metabolic effects of bariatric surgery have not been completely established.

Some reports indicate that amelioration of metabolic profiling by gastrectomy was associated with autophagy induction (Soussi et al., 2016). Several possible explanations were made for this interesting phenomenon. After bariatric surgery, significant alterations in anatomical structure induced changes in the integrated responses during eating, including cephalic phase, chewing and tasting, gastric phase, intestinal phase and gut peptides, absorptive phase, glucose metabolism, liver and bile acid phase, and large intestine and microbiota phase [summarized in ref (Quercia et al., 2014)]. The molecules described in this review could be also notable for their significance in autophagy induction by bariatric surgery (**Figure 4**).

Glucagon

In the stomach, gastric emptying is regulated by gastric content and neural and hormonal influences and is altered after bariatric surgery as described earlier. Accelerated gastric emptying time for liquids but slower gastric emptying time for solids have been reported after RYGB (Horowitz et al., 1982). Kotler et al. (1985) reported faster intestinal transit time and increased enteroglucagon levels in patients with greater weight loss compared to weight-stable patients. The term "enteroglucagon" in this report did not necessarily include "glucagon" because of cross reaction with several glucagon sequence-containing polypeptides at the time of this report. The presence of extrapancreatic glucagon secretion in humans has been the focus of intense discussion for years, and even though evidence was conflicting, some investigators reported that glucagon responses after total pancreatectomy were present in animals (Sutherland and De Duve, 1948; Matsuyama and Foa, 1974; Vranic et al., 1974; Muller et al., 1978; Doi et al., 1979; Gotoh et al., 1989) and humans (Unger et al., 1966;

Barnes and Bloom, 1976; Villanueva et al., 1976; Boden et al., 1980; Karesen et al., 1980; Sudo et al., 1980; Dammann et al., 1981; Holst et al., 1983; Yasui, 1983; Polonsky et al., 1984; Bajorunas et al., 1986a,b; Ohtsuka et al., 1986; Tanjoh et al., 2003). The most challenging point was that until recently, analytical methods for glucagon have not been sufficiently sensitive or specific to justify decisive statements about the absence or presence of extrapancreatic fully processed 29–amino acid glucagon (Tanjoh et al., 2003). Recently, sandwich enzyme-linked immunosorbent assays (ELISA) utilizing a combination of C- and N-terminal antiglucagon antibodies have been emerged. Such ELISA system theoretically could eliminate cross-reactivity with truncated or elongated forms of glucagon containing polypeptides (Wewer Albrechtsen et al., 2014). Lund et al. (2016) studied patients who underwent total pancreatectomy and analyzed plasma glucagon levels. As expected, the gastrointestinal anatomy was remarkably changed, including the removal of the pyloric sphincter and duodenum after total pancreatectomy. Therefore, following the ingestion of a meal, nutrients are rerouted and delivered directly from the stomach to the jejunum in a manner similar to bariatric surgery as previously described. The unique point of the study by Lund et al. (2016) is that they utilized not only novel sandwich enzyme-linked immunosorbent assays of plasma glucagon but also mass spectrometry-based proteomics to confirm 29-amino acid circulatory glucagon levels in patients without a pancreas. Basal glucagon levels in these patients exhibited a lower trend, and glucose challenge of the gastrointestinal tract exerted significant hyperglucagonemia in these patients. Lund et al. (2016) also confirm that the intravenous glucose infusion attenuated plasma glucagon levels, and directs focus on the gastrointestinal tract. Unfortunately, there is no direct evidence indicated the hyperglucagonemia after bariatric surgery yet, but higher glucagon release within the first 2 h and higher trend of peak level of glucagon in post RYGB patients when compared to SG or neither operation group has been recently reported (Svane et al., 2019). These findings suggest that alteration in glucagon secretion or possibly hyperglucagonemia could be induced by bariatric surgery and be relevant in systemic physiological alterations, including autophagy (**Figure 4**). Subsequently such induction of autophagy could influence on the health in individuals with obesity but also in lean and in malnourished patients after gastrectomy. Further research would be required in this field.

GLP-1

The potential contribution of GLP-1 to the pathobiological alteration that occurs after bariatric surgery was seen in adverse outcomes of bariatric surgery. Late dumping syndrome, a well-known hypoglycemic event, occurs 2–3 h after a meal, after gastric surgery, and as a complication after surgery for obesity as well. The underlying cause of late dumping syndrome is not completely understood, but in general, it is hypothesized that postoperative elevation of incretin hormone, especially GLP-1 levels, leads to pancreatic β-cell hypertrophy (**Figure 4**). Such β-cell hypertrophy stimulates insulin secretion and hypoglycemic symptoms. These theories are supported by the observation that hyperinsulinemic hypoglycemia most frequently occurred

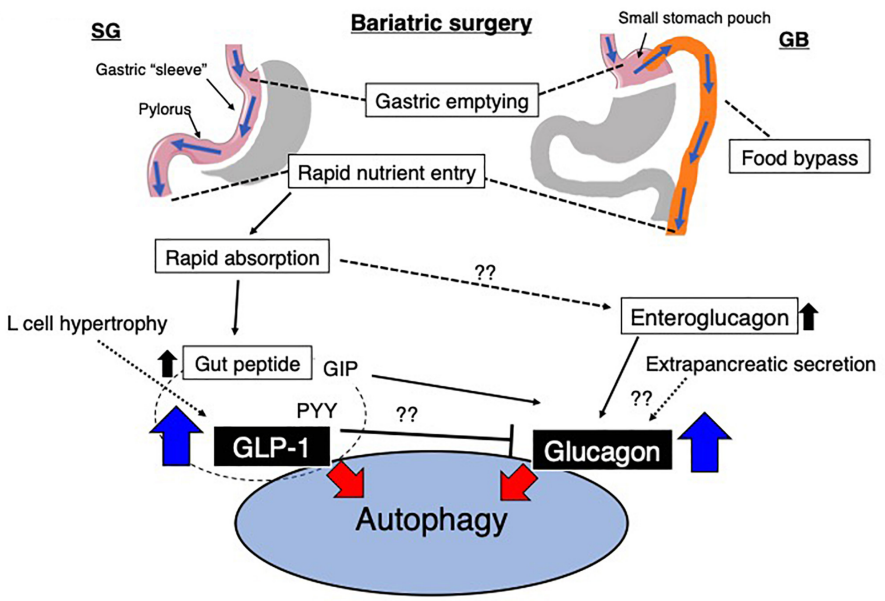

FIGURE 4 | Possible alterations in the gut environment and the relationship to autophagy after bariatric surgery. Sleeve gastrectomy (SG) and Roux-en-Y gastric bypass (GB) are the most common surgical procedures in bariatric surgery. After bariatric surgery, the rapid entry of nutrients likely induces the secretion of GLP-1. Additionally, glucagon is potentially increased by various/unknown mechanisms. GLP-1 and other gut-derived peptides, such as PYY, could inhibit glucagon expression, and GIP might stimulate glucagon expression. However, these observations were all investigated in pancreatic cells, and the regulation of 'gut glucagon' was not found. GIP, gastric inhibitory peptide; PYY, peptide YY; GLP-1, glucagon-like peptide-1.

in patients who underwent resection of parts of the stomach, in which the gastrojejunostomy bypasses the pylorus. Therefore, induced levels of GLP-1 during an oral glucose or meal test have been consistently reported after RYGB (Yi et al., 2008), BPD (Jin and Weng, 2016), and SG (Moellmann et al., 2018) (**Figure 4**). Even though none of these theories have been mechanistically proven during a hypoglycemic event, an increase in GLP-1 could be associated with hepatic autophagy. He et al. (2015) reported that in a study of rats with obesity and diabetes, RYGB led to significant induction of autophagy in the liver, restored autophagy levels in the liver and was associated with reducing the level of hepatic lipids. Increased autophagy in the liver after RYGB was well correlated with plasma GLP-1 levels (He et al., 2015). Therefore, induction of GLP-1 after bariatric surgery would also lead to significant stimulation of autophagy (**Figure 4**).

CONCLUSION

In this review, we focused on glucagon/GLP-1 and associated drugs in the physiology of autophagy. We propose that changes in the gastrointestinal tract that induces food to bypass the intestine would be relevant to the induction of autophagy via secretion of GLP-1 and/or glucagon.

AUTHOR CONTRIBUTIONS

KK concept, design, and major contribution to writing the manuscript. EK made figures and discussion. DK intellectual input in the projects.

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BRIEF RESEARCH REPORT
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The Impact of Mevastatin on HCV Replication and Autophagy of Non-Transformed HCV Replicon Hepatocytes Is Influenced by the Extracellular Lipid Uptake

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Statins efficiently inhibit cholesterol synthesis by blocking 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase in the mevalonate pathway. However, the effect of statins on intracellular cholesterol is partially counterbalanced by a consequent increased uptake of extracellular lipid sources. Hepatitis C virus (HCV) infection induces intracellular accumulation of cholesterol by promoting both new synthesis and uptake of circulating lipoproteins, which is required for HCV replication and release. Hepatocytes respond to the increase in intracellular cholesterol levels by inducing lipophagy, a selective type of autophagy mediating the degradation of lipid deposits within lysosomes. In a cellular system of HCV replication based on HuH7 hepatoma cells, statin treatment was shown to be sufficient to decrease intracellular cholesterol, which is accompanied by reduced HCV replication and decreased lipophagy, and has no apparent impact on endocytosis-mediated cholesterol uptake. To understand whether these results were influenced by an altered response of cholesterol influx in hepatoma cells, we analyzed the effect of statins in non-transformed murine hepatocytes (MMHD3) harboring subgenomic HCV replicons. Notably, we found that total amount of cholesterol is increased in MMHD3 cells upon mevastatin treatment, which is associated with increased HCV replication and lipophagy. Conversely, mevastatin is able to reduce cholesterol amounts only when cells are grown in the presence of delipidated serum to prevent extracellular lipid uptake. Under this condition, HCV replication is reduced and autophagy flux is severely impaired. Altogether, these results indicate that both *de novo* synthesis and extracellular uptake have to be targeted in non-transformed hepatocytes in order to decrease intracellular cholesterol levels and consequently limit HCV replication.

Keywords: hepatitis C virus, cholesterol, autophagy, RNA replication, lipid uptake

INTRODUCTION

Hepatitis C virus (HCV) dysregulates lipid metabolism to accomplish several steps of its life cycle (Paul et al., 2014; Strating and van Kuppeveld, 2017). HCV associates with lipoproteins to circulate in the bloodstream, to enter into and to be released from target cells. It induces the formation of a membranous web in close proximity to the endoplasmic reticulum (ER) where its replication complex is assembled. In this regard, a crucial role is played by cholesterol, whose intracellular levels are increased during HCV infection by stimulating both new synthesis and uptake from extracellular milieu (Ye, 2007; Bassendine et al., 2011; Grassi et al., 2016; Lavie and Dubuisson, 2017).

Cholesterol is synthesized from acetyl CoA through the mevalonate pathway at the ER, mainly in the liver and intestines (Edwards and Ericsson, 1999). The conversion of 3-hydroxy-3-methylglutaryl (HMG)-CoA in mevalonate by HMG-CoA reductase is the rate-limiting step of the cholesterol biogenesis. Once synthesized, cholesterol is rapidly targeted to biological membranes or distributed to intracellular deposits, such as lipid droplets, where it undergoes fatty acylation to generate cholesterol esters. An alternative source of cholesterol is represented by the uptake of low-density lipoproteins (LDLs) from serum through LDL receptor-mediated endocytosis (Ikonen, 2008; Arab et al., 2018). Cholesteryl esters that enter the cells through LDL are hydrolyzed in the lysosomes to provide unesterified cholesterol for cellular needs.

Statins inhibit cholesterol synthesis by blocking mevalonate production *via* inhibition of HMG-CoA reductase (Davies et al., 2016). Statin-treated cells respond to the reduction in the synthesis rate by increasing the number of LDL receptors on cell surface to ensure the uptake of cholesterol from serum (Davies et al., 2016).

Studies based on HCV RNA replicon or HCV-infected cells showed that HCV stimulates cholesterol synthesis, and statins markedly reduce RNA replication and viral particle infection (Ye et al., 2003; Kapadia and Chisari, 2005; Kapadia et al., 2007). This inhibition is caused by the reduced availability of cholesterol for membranous web formation and for viral lipoprotein assembly, as well as by the impairment of post-translational modifications of host proteins involved HCV replication (e.g., FBL2 geranylgeranylation), which depends on mevalonate pathway intermediates (Wang et al., 2005).

In addition to increased cholesterol synthesis, HCV is also able to induce cholesterol uptake from serum *via* upregulation of LDL receptor expression (Syed et al., 2014; Zhang et al., 2017). Blocking this pathway affects HCV replication, similar to what was observed with statins, suggesting that both sources of cholesterol are important in the viral cycle. It has been recently reported that inhibition of cholesterol transport through the endosomal–lysosomal pathway impairs the formation of the membranous web, where RNA replication occurs (Stoeck et al., 2017).

Autophagy is a lysosome-mediated catabolic process that ensures cellular integrity and homeostasis (Mizushima and Komatsu, 2011) in response to several stress stimuli, such as nutrient deprivation, accumulation of harmful substrates, or infection (Kroemer, 2015). In this process, double-membrane vesicles, called autophagosomes, engulf portions of cytoplasm and transport them to lysosomes for degradation (Antonioli et al., 2017). Different types of autophagy selective for specific cargos have been described (Khaminets et al., 2016). Among them, lipophagy controls intracellular lipid homeostasis, allowing the hydrolysis of intracellular triglycerides and cholesterol esters stored in lipid droplets (Liu and Czaja, 2013; Vescovo et al., 2014; Madrigal-Matute and Cuervo, 2016). Accordingly, lipophagy is induced in hepatocytes treated with statins to compensate for cholesterol synthesis decrease (Wang et al., 2015).

We have previously reported that HCV-infected HuH7 cells have high rate of lipophagy, whose inhibition results in a significant accumulation of cholesterol (Vescovo et al., 2012). Statin treatment in these cells is sufficient to reduce intracellular cholesterol levels, which is accompanied by a reduction of both lipophagy and HCV replication. However, a limitation of this study was the use of hepatoma cells, which may have alterations in the regulation of cholesterol homeostasis. Here, we analyzed how statins impact HCV replication, cholesterol levels, and autophagy in the non-transformed mouse hepatocytes MMHD3. These cells were previously reported to support replication of a JFH1-derived subgenomic replicon (HCV Rep), although species restriction impedes both viral entry and production of new viral particles (Uprichard et al., 2006).

MATERIALS AND METHODS

Cell Culture

HuH7 HCV-Rep is a human hepatoma cell line harboring the HCV genotype 1b (Con1) subgenomic replicon carrying a neomycin resistance gene (Vescovo et al., 2012). Immortalized Met Mouse Hepatocytes D3 (MMHD3) HCV-Rep is a mouse cell line harboring an HCV subgenomic replicon derived from JFH1 genotype 2a carrying a neomycin resistance gene (Uprichard et al., 2006). Cells were cultured at 37°C in 5% CO_2, and HuH7 HCV-Rep and HEK293T were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO) while MMHD3 HCV-Rep cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with the addition of 30 ng/ml insulin-like growth factor II (IGFII), 50 ng/ml epidermal growth factor (EGF) (Peprotech), and 10 μg/ml insulin (Roche). DMEM and RPMI were supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, 100 g/mL streptomycin, and 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO). To maintain HCV replication, culture medium of HuH7 and MMHD3 HCV replicon cells was added with 500 μg/ml and 250 μg/ml G418 (Sigma-Aldrich, St Louis, MO), respectively. G418 was removed in the course of the experiments.

To inhibit cellular cholesterol uptake, cells were cultured in RPMI supplemented with 10% delipidated FBS (Pan-Biotech, Aidenbach, Germany). To inhibit cholesterol synthesis, cells were incubated in complete or delipidated FBS medium in the presence of 5 μM mevastatin (Sigma-Aldrich, St Louis, MO) for 72 h before the assay.

To block lysosomal activity, cells were incubated in complete or delipidated FBS medium in the presence of 5 nM Bafilomycin A1 (Sigma-Aldrich, St Louis, MO) for 4 and 8 h before the assay. RFP-LC3 and RFP-GFP-LC3 retroviral reporters and methods for retrovirus production were previously described (Antonioli et al., 2014).

Lipid Staining and Quantification

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed thoroughly with PBS, and incubated with filipin (50 μg/ml;Sigma-Aldrich, St Louis, MO) or Bodipy 493/503 (2 μg/ml; Invitrogen, Carlsbad, CA) for 1 h at room temperature.

For luminometric quantification, 6×10^5 cells were trypsinized, washed thoroughly with PBS, and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed twice with PBS, split in two tubes, and incubated with fluorescence probes indicated above. Cells were finally washed thoroughly with PBS and distributed in flat-bottom 96-well plates (OptiPlate-96 F; Perkin Elmer, Waltham, MA). Fluorescence was measured using a Victor 3 1420 multilabel plate reader (Perkin Elmer, Waltham, MA) and the following filter pairs: 485/535 nm for Bodipy and 355/460 nm for 4′,6-diamidino-2-phenylindole and filipin. Fluorescence intensities were normalized with respect to 4′,6-diamidino-2-phenylindole. Assays were performed in triplicate.

For cholesterol quantitation, a Cholesterol Quantitation Kit (MAK043, Sigma-Aldrich, St Louis, MO) was used, and 1×10^6 cells were processed according to the manufacturer's recommendations. Cholesterol measurement was normalized with respect to the quantity of DNA extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Assays were performed in triplicate.

Antibodies and Immunoblotting Analysis

The primary antibodies used in this study were rabbit anti-LC3 (Cell Signaling Technology, Inc., Danvers, MA, for Western blot analysis) and α-glyceraldehyde-3-phosphate dehydrogenase (Calbiochem, Merck, Darmstadt, Germany). Cells were lysed in Cell Lytic (Sigma-Aldrich, St Louis, MO) complemented with protease and phosphatase inhibitors (Protease inhibitor cocktail plus 5 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1 mM sodium molibdate, and 0.5 mM phenylmethylsulfonyl fluoride; Sigma-Aldrich, St Louis, MO). Ten micrograms of protein extracts was separated on 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and electroblotted onto polyvinylidene difluoride (Millipore, Billerica, MA) membranes. Blots were incubated with primary antibodies in 5% nonfat dry milk in PBS plus 0.1% Tween 20 overnight at 4°C. Detection was achieved using horseradish peroxidase–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), visualized with ECL (Millipore, Billerica, MA), and chemiluminescent signals were analyzed using a Chemidoc Touch Imaging System (Bio-Rad, Hercules, CA). All samples were run in triplicate.

Lentivirus Generation and Infection

Lentiviral infections were performed as previously described (Di Rienzo et al., 2019). Briefly, HEK 293T cells were cotransfected with 10 μg of lentiviral vector targeting Beclin 1 messenger ribonucleic acid (mRNA) (Sigma-Aldrich, St Louis, MO), 2.5 μg of pVSV-G plasmid, and 7.5 μg of psPAX2 plasmid using the calcium phosphate method. After 48 h, lentiviral particles were recovered from the supernatants by ultracentrifugation at 19,800 RPM on SW28 rotor for 2 h and resuspended in PBS (500 μl for 20 ml of culture media). Cells were infected with 40 μl of viral suspension supplemented with 4 μg/ml polybrene (Sigma-Aldrich, St Louis, MO) overnight. Transduced cells were selected for puromycin resistance (250 μg/ml; Sigma-Aldrich, St Louis, MO).

Real-Time Polymerase Chain Reaction

Analysis of HCV RNA was performed as previously described (Refolo et al., 2019). Briefly, RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA synthesis was generated from 2 μg of RNA using the reverse transcription kit (Promega, Madison, WI) according to the manufacturer's recommendations. Real-time polymerase chain reactions (PCRs) were performed with the Corbett Research Rotor Gene 6000 analyzer using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Two point five microliters of 1:5 complementary deoxyribonucleic acid (cDNA) was used as template and cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Levels of RNAs were normalized to the L34 level using the equation $2^{-\Delta Ct}$.

Primer sets for all amplicons were designed using the Primer-Express 1.0 software system (Roche, Basel, CH).

Replicon genotype 1b forward: 5'-TACTCCCAACAGACGCG AGG-3'
Replicon genotype 1b reverse: 5'-GCAGGTCGCCAGGAAAG ATT-3'
Replicon genotype 2a forward: 5'-TCTGCGGAACCGGTGAG TA-3'
Replicon genotype 2a reverse: 5'-TCAGGCAGTACCACAAG GC-3'
L34 human forward: 5'-GTCCCGAACCCCTGGTAATAG-3'
L34 human reverse: 5'-GGCCCTGCTGACATGTTTCTT-3'
L34 mouse forward: 5'-GGTTGGGAAAGCACCTAAA-3'
L34 mouse reverse: 5'-GACGTGCTTCTGTGTCTTAG-3'
Beclin 1 mouse forward: 5'-GGCCAATAAGATGGGTCTGA-3'
Beclin 1 mouse reverse: 5'-GCTGCACACAGTCCAGAAAA-3'

Statistical Analysis

Statistical analysis was performed using unpaired, two-tailed Student's *t* test (Excel software). Values are shown as mean ± standard deviation of at least three independent experiments. *P* values <0.05 were marked by *. Densitometric analysis of immunoblots was performed using the Adobe Photoshop software.

RESULTS

MMHD3 HCV Rep was treated with mevastatin for 3 days and HCV replication was evaluated by real-time PCR. HuH7 HCV replicon cells were also included in this study as a positive control. Unexpectedly, while mevastatin impairs HCV replication in HuH7 HCV Rep cells, a significant increase of HCV RNA levels was observed in MMHD3 Rep cells (**Figure 1A**).

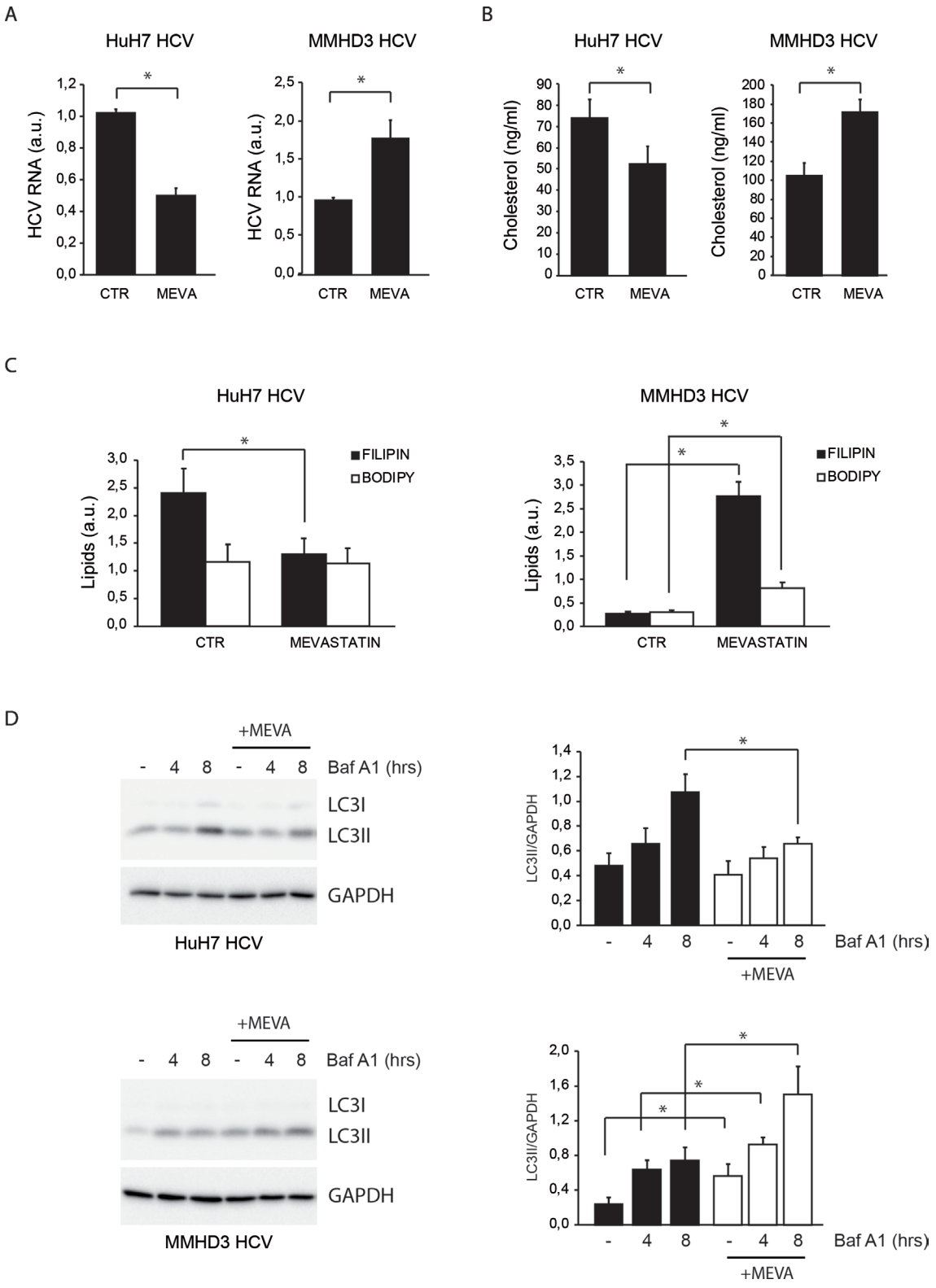

FIGURE 1 | Continued

FIGURE 1 | Comparison of the response to mevastatin treatment of HuH7 and MMHD3 hepatitis C virus (HCV) Rep cell lines in terms of HCV replication, cholesterol, and autophagy levels. **(A)** HuH7 HCV-Rep (left panel) and MMHD3 HCV-Rep (right panel) cells were treated with mevastatin for 3 days and HCV replicon levels were analyzed by real-time polymerase chain reaction (PCR). A.U.: Arbitrary units. **(B)** HuH7 HCV-Rep (left panel) and MMHD3 HCV-Rep (right panel) cells were treated with mevastatin for 3 days and intracellular cholesterol content was measured using cholesterol quantitation kit (Sigma-Aldrich). **(C)** HuH7 HCV-Rep (left panel) and MMHD3 HCV-Rep (right panel) cells were treated with mevastatin for 3 days and intracellular lipid content was analyzed by staining cells with filipin (cholesterol) and Bodipy 493/503 (neutral lipids) and measuring fluorescence intensity using a fluorimeter. **(D)** HuH7 HCV-Rep (top panel) and MMHD3 HCV-Rep (bottom panel) cells were incubated with mevastatin (MEVA) for 3 days and autophagic flux was analyzed by treating, or not, cells with 5 nM Bafilomycin A1 (Baf A1) for 4 or 8 h. Protein extracts were prepared and subjected to immunoblotting to determine LC3 protein levels. Glyceraldehyde 3-phospate dehydrogenase (GAPDH) was used as a protein loading control. Accompanying graphs show LC3II values normalized to GAPDH levels from three independent experiments. $^{*}P < 0.05$.

To elucidate whether the discordant effect of mevastatin on HCV replication was due to a different impact on the intracellular cholesterol amount, cholesterol levels were analyzed upon mevastatin incubation by both enzymatic assays and filipin staining (**Figures 1B** and **C** and **S1A**). Results show that mevastatin treatment reduces cholesterol levels in HuH7 Rep cells, while it augments them in MMHD3 Rep cells. Neutral lipid levels were also analyzed by Bodipy 493/503 staining, showing no changes in HuH7 Rep cells and a significant increase in MMHD3 Rep cells (**Figures 1C** and **S1A**).

We previously described that high levels of a lipid-selective autophagy are present in HuH7 Rep cells as a response to cholesterol accumulation triggered by HCV replication, which can be diminished by statin treatment (Vescovo et al., 2012). In light of the different response of MMHD3 Rep cells to mevastatin, we analyzed autophagy flux in these experimental conditions by measuring the autophagic marker LC3-II in the presence or absence of the lysosomal inhibitor Bafilomycin A1. As shown in **Figure 1D**, mevastatin treatment decreases autophagy levels in HuH7 Rep cells, while this was further increased in MMHD3 Rep cells in accordance with increased levels of cholesterol. We also confirmed that, in MMHD3 Rep cells, autophagosomes largely localized with cholesterol deposits, as shown by LC3 and filipin colocalization (**Figure S1B**). Altogether, these results suggests that, in non-transformed HCV replicating hepatocytes, statins cause an increase rather than a decrease of total cholesterol levels, which is accompanied by increased levels of autophagy.

Statin treatments are known to stimulate the extracellular uptake of lipoproteins as a cellular attempt to compensate for the cholesterol synthesis inhibition (Davies et al., 2016). The increase of neutral lipids associated with that of cholesterol suggests that extracellular lipid uptake is potentiated in MMHD3 Rep cells upon mevastatin incubation. To elucidate the role of extracellular cholesterol in the response of MMHD3 Rep cells, mevastatin treatments were repeated in cells cultured in medium containing delipidated serum. As shown in **Figure 2A**, HCV RNA levels were efficiently reduced by mevastatin when extracellular sources of cholesterol were also diminished. In parallel, cholesterol levels were analyzed by both enzymatic assays and filipin staining, showing that mevastatin is able to reduce cholesterol levels in MMHD3 Rep cells when cultured with delipidated serum (**Figures 2B** and **S1C**). Of note, lipid-deprived medium alone results in lower intracellular cholesterol levels; however, this decrease is not sufficient to impair HCV replication.

Then, we analyzed the autophagy flux in MMHD3 Rep cells cultured in serum-delipidated medium and mevastatin, either individually or in combination, and in the presence or absence of the lysosome inhibitor Bafilomycin A1 (**Figure 2C**). As expected, elimination of extracellular lipid sources result in autophagy induction, with nutrient starvation being a well-known inducer of autophagy. Interestingly, when cholesterol synthesis is also inhibited by mevastatin, we observed a block of LC3II degradation (autophagy flux) rather than a decrease of LC3II lipidation (autophagy induction), suggesting that, when total cholesterol becomes limiting, autophagosome–lysosome fusion or lysosomal degradative activity is affected. The impairment of autophagy flux in cholesterol-deficient conditions was also confirmed by using the tandem fluorescent-tagged LC3 (mRFP-EGFP-LC3, **Figure S1D**).

We further explored the role of autophagy in the control of cholesterol levels in MMHD3 Rep cells by inhibiting the process through downregulating the expression of the autophagy regulator Beclin 1 (**Figure 2D** and **E**). Beclin 1 silencing results in an accumulation of cholesterol in cells cultured either in complete or in delipidated serum. However, when Beclin 1-silenced cells were incubated with mevastatin, cholesterol accumulation was reduced only if extracellular sources of cholesterol were depleted, while a further accumulation was observed in cells incubated in complete medium. These results confirmed that when intracellular sources of cholesterol (new synthesis and autophagic degradation) are both inhibited, the uptake of extracellular cholesterol is significantly increased (**Figure 2F**).

DISCUSSION

HCV replication relies on the formation of the membranous web, a massive rearrangement of ER-associated membranes highly enriched in cholesterol (Paul et al., 2014). HCV promotes intracellular accumulation of cholesterol both by inducing new synthesis through the mevalonate pathway and by stimulating extracellular lipoprotein uptake (Bassendine et al., 2011; Strating and van Kuppeveld, 2017). The contribution of different sources of cholesterol to sustain viral replication remains partially characterized. Statins are powerful inhibitors of cholesterol synthesis. However, their effect on the total

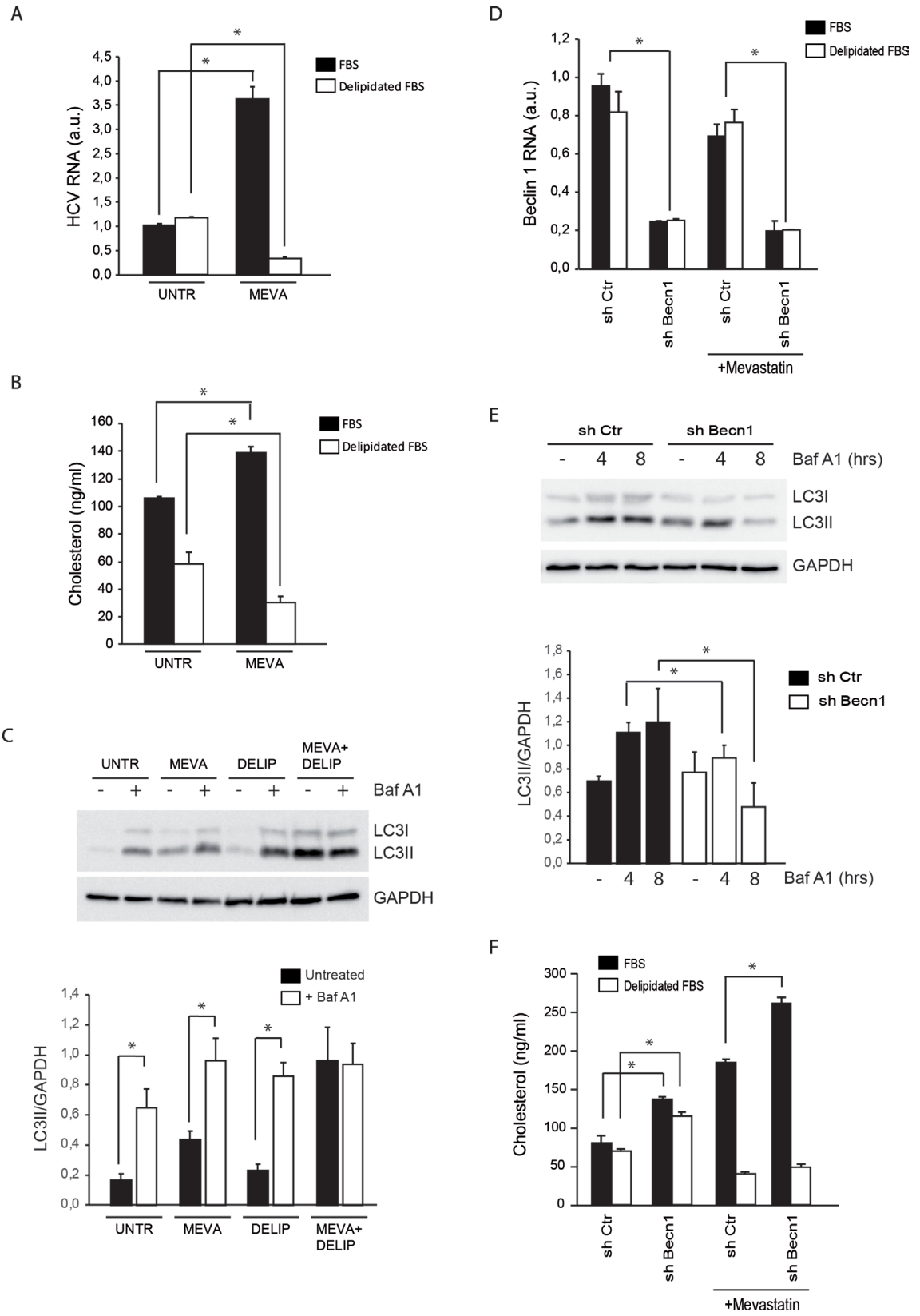

FIGURE 2 | Continued

FIGURE 2 | Analysis of HCV replication, cholesterol, and autophagy levels in MMHD3 HCV Rep cells treated with mevastatin and delipidated serum. **(A)** MMHD3 HCV-Rep cells were treated with mevastatin for 3 days in the presence of normal or delipidated fetal bovine serum (FBS). HCV replicon levels were analyzed by real-time polymerase chain reaction (PCR). **(B)** MMHD3 HCV-Rep cells were treated with mevastatin for 3 days in the presence of normal or delipidated FBS. Intracellular cholesterol content was measured using cholesterol quantitation kit (Sigma-Aldrich). **(C)** In MMHD3 HCV-Rep cells treated with mevastatin for 3 days in the presence of normal or delipidated FBS, autophagy flux was measured by treating, or not, cells with 5 nM of Bafilomycin A1 (Baf A1) for 8 h. Protein extracts were subjected to immunoblotting to determine LC3 protein levels. Glyceraldehyde 3-phospate dehydrogenase (GAPDH) was used as a protein loading control. The accompanying graph shows LC3II values normalized to GAPDH levels from three independent experiments. $^*P < 0.05$. **(D)** MMHD3 HCV-Rep cells were infected with a sh-Control (Ctr) or a sh-Beclin 1 (Becn1) lentivirus and Beclin 1 levels were analyzed by real-time PCR. **(E)** Autophagy flux in Beclin 1-silenced cells were analyzed by immunoblotting as described in **(C)**. **(F)** Intracellular cholesterol content was measured in Beclin 1-silenced cells cultured in delipidated FBS and treated, or not, with mevastatin for 3 days using a cholesterol quantitation kit (Sigma-Aldrich). $^*P < 0.05$.

levels of intracellular cholesterol is attenuated by the ability of hepatocytes to upregulate the uptake of extracellular cholesterol by inducing LDL receptor expression (Davies et al., 2016).

Previous studies showed that inhibition of cholesterol synthesis by statins is sufficient to significantly lower total cholesterol levels in HuH7 HCV replicon cells, which is associated with reduced HCV replication (Ye, 2007; Vescovo et al., 2012). However, this observation also suggests that the compensatory increase of extracellular cholesterol uptake triggered by statins may be somehow impaired in HuH7 cells, raising the question if these cells properly reflect what occurs in normal hepatocytes.

Here, we reported that, in non-transformed HCV replicon hepatocytes, the impact of statins on cholesterol levels is profoundly influenced by the availability of extracellular lipid sources. Indeed, treatment of immortalized murine hepatocytes with mevastatin is not able to decrease intracellular cholesterol levels unless these cells are cultured in the presence of delipidated serum. In line with this observation, mevastatin alone causes an increase rather than a decrease of HCV replication, while it shows antiviral properties only when combined with the delipidated serum.

Statins were described to inhibit HCV replication not only by reducing cholesterol levels but also by inhibiting prenylation of host factors required for HCV replication (Ye et al., 2003; Kapadia and Chisari, 2005). Since mevastatin treatments alone do not impair HCV replication in immortalized hepatocytes, it remains to be assessed at which extent protein prenylation is inhibited. In this regard, it has to be noted that the maximum concentration of mevastatin at which immortalized hepatocytes can be treated is 5 μM, which is lower than what is commonly used in HuH7, since higher doses induce massive cell death (data not shown).

We previously reported that lipophagy, a lipid-selective form of autophagy, is induced by HCV infection in HuH7 cells, which is required to prevent excessive accumulation of intracellular cholesterol, and decreases when cells are treated with statins. As in the case of cholesterol levels and HCV replication, we observed that the effect of statins on autophagy in non-transformed HCV replicon hepatocytes is dependent on the presence of extracellular sources of lipids. At variance with HuH7 replicon cells, autophagy is increased by statin treatment. Interestingly, we observed that delipidated serum induces autophagy flux, probably as a response to nutrient starvation, while a dramatic inhibition of LC3II degradation was observed when combined to mevastatin, suggesting that profound cholesterol depletion may affect autophagosome degradation, as also described in breast cancer cells (Lin et al., 2017).

The impact of autophagy inhibition on cholesterol levels is also determined by extracellular lipid availability. An increase of cholesterol levels was observed when autophagy was inhibited by downregulating Beclin 1 expression in non-transformed hepatocytes. However, also in this case, mevastatin is able to reduce cholesterol levels only if Beclin 1-silenced cells are cultured in the presence of delipidated serum, while a further increase was observed when extracellular lipid sources were available.

Although our observations need to be validated using more physiological cellular models, such as HCV-infected primary hepatocytes, they highlighted alterations in transformed hepatocytes with respect to lipid metabolism that may have limited our understanding of the effect of inhibition of cholesterol synthesis in the context of HCV infection. Non-transformed HCV replicon hepatocytes may therefore represent a more physiological cell system to elucidate how lipid metabolism is altered by HCV, which is a key aspect of HCV-induced pathogenesis (Patel and Harrison, 2012; Vescovo et al., 2016).

AUTHOR CONTRIBUTIONS

TV performed most experiments with crucial help from GR (immunoblotting) and MM (cholesterol assays). GT, MP, and GF conceived and designed the research. TV and GF wrote the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00718/full#supplementary-material

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REVIEW
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Mitophagy in Hepatic Insulin Resistance: Therapeutic Potential and Concerns

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Metabolic syndrome, characterized by central obesity, hypertension, and hyperlipidemia, increases the morbidity and mortality of cardiovascular disease, type 2 diabetes, nonalcoholic fatty liver disease, and other metabolic diseases. It is well known that insulin resistance, especially hepatic insulin resistance, is a risk factor for metabolic syndrome. Current research has shown that hepatic fatty acid accumulation can cause hepatic insulin resistance through increased gluconeogenesis, lipogenesis, chronic inflammation, oxidative stress and endoplasmic reticulum stress, and impaired insulin signal pathway. Mitochondria are the major sites of fatty acid β-oxidation, which is the major degradation mechanism of fatty acids. Mitochondrial dysfunction has been shown to be involved in the development of hepatic fatty acid–induced hepatic insulin resistance. Mitochondrial autophagy (mitophagy), a catabolic process, selectively degrades damaged mitochondria to reverse mitochondrial dysfunction and preserve mitochondrial dynamics and function. Therefore, mitophagy can promote mitochondrial fatty acid oxidation to inhibit hepatic fatty acid accumulation and improve hepatic insulin resistance. Here, we review advances in our understanding of the relationship between mitophagy and hepatic insulin resistance. Additionally, we also highlight the potential value of mitophagy in the treatment of hepatic insulin resistance and metabolic syndrome.

Keywords: hepatic insulin resistance, metabolic syndrome, mitochondrial dysfunction, hepatic fatty acid accumulation, mitophagy

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INTRODUCTION

Metabolic syndrome is characterized by hyperglycemia, hyperlipidemia, hypertension, and obesity (Bonamichi et al., 2017; Mohammadbeigi et al., 2018). The increasing prevalence of metabolic syndrome is posing a great threat to human health worldwide (Mohammadbeigi et al., 2018). Insulin resistance is a pathological manifestation that target tissues including liver, muscle, and adipose tissues are less sensitive to the effect of insulin (Barseem and Helwa, 2015). There is a consensus that insulin resistance including liver, skeletal muscle, and adipose tissue insulin resistance is the leading risk factor for metabolic syndrome, obesity, and type 2 diabetes (Bonamichi et al., 2017; Tahrani, 2017). In view of the vital role of liver in glycometabolism and lipid metabolism, hepatic insulin resistance (central insulin resistance) is regarded as the more important risk factor for the development of whole-body insulin resistance and metabolic syndrome (Perseghin, 2009; Ibarra-Reynoso et al., 2014). Therefore, a better understanding of the mechanism by which insulin resistance

develops in liver tissue may offer novel therapeutic directions for the treatment or prevention of metabolic syndrome (Ibarra-Reynoso et al., 2014).

Mitochondria are the major sites of fatty acid β-oxidation, which is the major degradation mechanism of fatty acids in hepatocytes and skeletal muscle cells (Chow and From, 2010; Crescenzo et al., 2016). Recent studies have proposed that mitochondrial dysfunction can impair mitochondrial fatty acid β-oxidation, which may cause fatty acid accumulation in liver and skeletal muscle tissues (Chow and From, 2010; Crescenzo et al., 2016). Moreover, accumulating studies have recognized that free fatty acid–induced mitochondrial dysfunction can cause accumulation of hepatic fatty acids, which in turn leads to hepatic insulin resistance (Gonzalez-Franquesa and Patti, 2017; Wu et al., 2018). In the initial study, a decreased number of mitochondria have been found in insulin-resistant skeletal muscle cells, suggesting that mitochondrial function is impaired in insulin-resistant skeletal muscle cells (Perreault et al., 2018). As research progresses, hepatic fatty acid–induced mitochondrial dysfunction has also been proved to play an important role in the development of hepatic insulin resistance (Wang et al., 2017b; Wang et al., 2018; Wang et al., 2019b). Recently, it has been widely recognized that mitochondrial autophagy (mitophagy), a catabolic process, can selectively remove damaged mitochondria by autophagolysosomes to maintain mitochondrial function and energy metabolism (Redmann et al., 2018; Li et al., 2018a). As a mitochondrial quality control mechanism, mitophagy can target and degrade damaged mitochondria to suppress damaged mitochondria-derived reactive oxygen species (ROS), which can dramatically impair healthy mitochondria, leading to mitochondrial dysfunction. Theoretically, mitophagy can preserve mitochondrial function to accelerate fatty acid oxidative degradation and suppress hepatic fatty acid accumulation, which may be conducive to the treatment of hepatic insulin resistance. However, the therapeutic potential and molecular mechanism of mitophagy on hepatic insulin resistance are still unclear.

The purpose of this review is to investigate the complex association between mitophagy and hepatic insulin resistance. First, we review the role and molecular mechanism of insulin-mediated glycometabolism and the associated abnormalities observed in insulin resistance. We then discuss the pivotal role of hepatic insulin resistance in whole-body insulin resistance and metabolic syndrome. Moreover, we discuss the role and molecular mechanism of hepatic fatty acid accumulation on hepatic insulin resistance. Next, we discuss the relationship among mitophagy, mitochondrial dysfunction and hepatic fatty acid accumulation. We also briefly review the related signaling pathways that regulate mitophagy. After discussing the potential role of mitophagy on hepatic insulin resistance with a focus on mitochondrial function and fatty acid oxidation, we put forward a novel idea that mitophagy can preserve mitochondrial function to suppress hepatic fatty acid accumulation, which is conducive to the prevention or treatment of hepatic insulin and metabolic syndrome. Therefore, our major objective is to summarize the role of mitophagy on hepatic insulin resistance and also discuss whether mitophagy is a potential target for the treatment of insulin resistance and metabolic syndrome.

INSULIN RESISTANCE

Insulin, an important endocrine hormone secreted by pancreatic β cells, acts on the insulin receptors (IRs) to regulate the metabolic process of carbohydrate, protein, and lipid in liver, muscle, and adipose tissues (Nicholas et al., 2017; Honka et al., 2018). Under normal circumstances, pancreatic β cells can secrete insulin in response to meal-induced increase in blood glucose (Kang et al., 2017). First, insulin promotes muscle tissue to assimilate blood glucose and convert it into muscle glycogen and protein (Kleinert et al., 2013; Ruby et al., 2017). Second, insulin not only promotes hepatocytes to absorb blood glucose and convert it into liver glycogen, but also inhibits glycogenolysis and gluconeogenesis to reduce postprandial blood glucose (Unger, 2011). Thirdly, insulin stimulates adipose cells to absorb blood glucose and converts it into fat (Gastaldelli, 2011; Sears and Perry, 2015). Collectively, insulin commonly acts on hepatocytes, skeletal muscle, and adipose cells to maintain glycemic homeostasis (**Figure 1**) (Sears and Perry, 2015).

Under insulin-resistant conditions, reduced insulin sensitivity of hepatocytes, skeletal muscle, and adipose cells is found. Insulin resistance is caused by plenty of risk factors such as fatty acid accumulation in liver and skeletal muscle tissues, inflammation, changes in intestinal flora, endoplasmic reticulum stress responses, and environment–gene interactions (Liu et al., 2014; Zhang et al., 2015a; Kikuchi et al., 2018; Vatner et al., 2018; Xu et al., 2018; Crossland et al., 2019). Recently, study has focused on the cause–effect relationship between hepatic fatty acid accumulation and hepatic insulin resistance (He et al., 2013; Finck and Hall, 2015; Dallak, 2018; Vatner et al., 2018).

MECHANISM OF HEPATIC INSULIN RESISTANCE

Liver is the most important organ in the regulation of glycometabolism and lipid metabolism (Ding et al., 2018). Generally, insulin binds to the IR and stimulates the autophosphorylation of IR tyrosine residues, which further activates tyrosine kinase in hepatocytes (Brunetti, 2014; Wang et al., 2015). And then tyrosine kinase phosphorylates insulin receptor substrate 1 and 2 (IRS-1 and IRS-2), which can bind to and activate phosphatidylinositol 3-kinase (PI3K). PI3K then activates the serine/threonine kinase AKT and further increases glucose transporter 2 expression. This process promotes hepatocytes to absorb blood glucose and maintain normal lipid and glucose homeostasis (Cai et al., 2016; Li et al., 2017). Taken together, insulin signal pathway plays a major role in maintaining cell energy metabolism.

Growing evidence has revealed that hepatic lipid accumulation may impair hepatic insulin action (Petersen et al., 2017). Additionally, disordered liver and adipose tissue lipolysis can elevate fatty acid flux to liver and further deteriorate

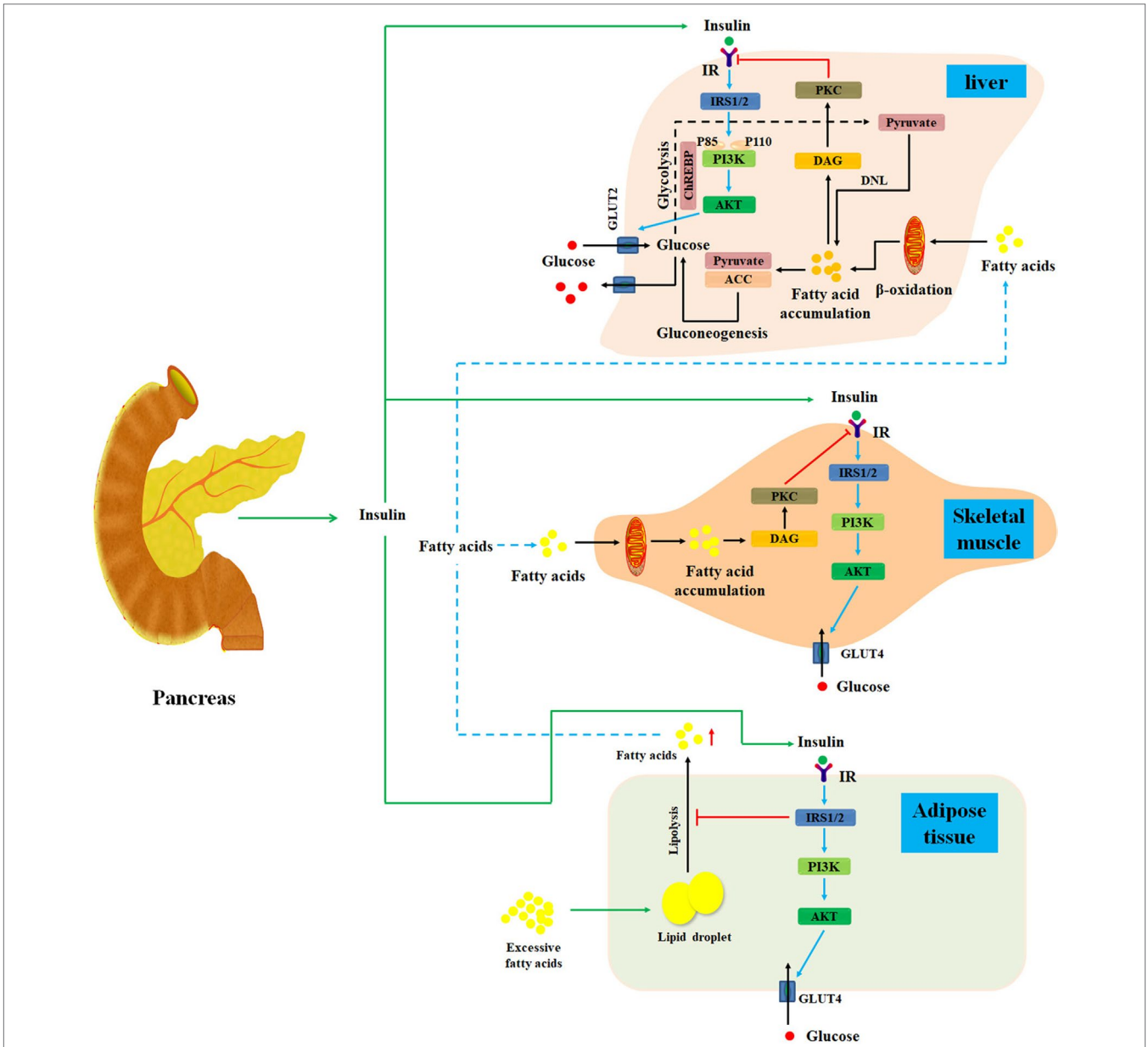

FIGURE 1 | Diagram illustrates the mechanisms of insulin resistance. Normally, after a meal, pancreatic β cells secrete insulin in response to meal-induced increase in blood glucose. First, insulin promotes muscle tissue to assimilate blood glucose and convert it into muscle glycogen and protein. Additionally, insulin also promotes hepatocytes to absorb blood glucose and converts it into liver glycogen. Meanwhile, insulin can inhibit glycogenolysis and gluconeogenesis to reduce postprandial blood glucose in liver tissues. Moreover, insulin can stimulate adipose cells to assimilate blood glucose and convert it into fat. Under starvation conditions, the secretion of insulin will be inhibited, and the hypoglycemic activity of insulin on liver, muscle, and adipose is also suppressed. Under insulin-resistant conditions, lipolysis is promoted in adipose tissue, thereby releasing excess fatty acids into the blood and then transporting them to the liver and skeletal muscle tissue. Then, fatty acids can activate PKC, which may markedly impair insulin signal pathway to cause hepatic and skeletal muscle insulin resistance. IR, insulin resistance; DNL, *de novo* lipogenesis; DAG, diacylglycerol; IRS, insulin receptor substrate; PKC, protein kinase C; ChREBP, carbohydrate responsive element-binding protein; ACC, acetyl-CoA carboxylase; GLUT, glucose transporter.

hepatic lipid accumulation, which significantly impair insulin signal pathway and cause hepatic insulin resistance (Yang et al., 2016). In the meantime, the expression of acetyl-CoA carboxylase and pyruvate carboxylase, two specific risk factors for insulin resistance, is also promoted in hepatocytes (**Figure 1**) (Perry et al., 2015; Goedeke et al., 2018). Collectively, hepatic lipid accumulation can cause hepatic insulin resistance through multiple mechanisms (Konstantynowicz et al., 2011). Therefore, it is widely accepted that suppression of hepatic lipid accumulation is a promising approach for the therapy of hepatic insulin resistance (Konstantynowicz et al., 2011; Mohamed and Jornayvaz, 2013; Kubo et al., 2017).

THE LIVER IS THE FIRST ORGAN TO DEVELOP INSULIN RESISTANCE

Accumulating evidence has demonstrated that hepatic insulin resistance is the primary event in free fatty acid–induced whole-body insulin resistance. And muscle and adipose tissue insulin resistance may be the consequence of hepatic insulin resistance (Perry et al., 2014; Badi et al., 2019; Medak and Townsend, 2019). Recent evidence has shown that muscle- and adipose tissue–specific IR knockout mice only show muscle or adipose tissue insulin resistance, but total body glucose metabolism still remains normal (Perseghin, 2009). It is surprising that hepatic IR knockout mice are characterized by liver, muscle, and adipose tissue insulin resistance, as well as fasting and postprandial hyperglycemia (Perseghin, 2009). Further research has confirmed that hepatic lipid accumulation is responsible for hepatic insulin resistance and whole-body insulin resistance (Ye et al., 2016; Sabater et al., 2017; Santos et al., 2017). Furthermore, Lee et al. (2015) have discovered that obese adolescents with fatty liver have a higher risk of systemic insulin resistance than obese adolescents with normal liver. Consistent with previous studies, Rotman and Neuschwander-Tetri (2017) have also proved that hepatic insulin resistance is most likely to be caused by minimal stimulation from hepatic fatty acid accumulation. However, skeletal muscle insulin resistance is less likely to be caused by ectopic fatty acid accumulation in skeletal muscle tissues, and adipose tissue insulin resistance is least likely to be caused by adipose tissue fatty acid accumulation (Rotman and Neuschwander-Tetri, 2017). In summary, growing evidence supports the viewpoint that hepatic fatty acid accumulation can give rise to hepatic insulin resistance and subsequent development of systemic insulin resistance and metabolic disorders.

WHAT ARE FATTY ACIDS?

Fatty acids are carboxylic acids with a long hydrocarbon chain (Tamil and Nagarajan, 2013). There are various kinds of fatty acids such as essential fatty acid, omega-3 fatty acids, *trans*-fatty acids, and free fatty acids (Williams et al., 2017; Shahidi and Ambigaipalan, 2018).

Essential fatty acids mainly consisting of polyunsaturated fatty acids are very important constituents of the human body. It cannot be generated by the human body, while it can only be absorbed from the diet (Amjad et al., 2017).

Omega-3 fatty acids, kind of polyunsaturated fatty acids, are found in deep sea fishes, sea dog, and linseed. A growing body of study has shown that omega-3 fatty acids have positive effects on cardiovascular disease, diabetes, cancer, Alzheimer disease, dementia, and depression (Shahidi and Ambigaipalan, 2018).

Trans-fatty acids, kind of unsaturated fatty acids, can increase the level of cholesterol and low-density lipoprotein in the blood and have a negative effect on coronary heart disease (Takeuchi and Sugano, 2017; Ahmed et al., 2018).

Free fatty acids are by-products of fat metabolism in adipose tissues. The cause–effect relationship between hepatic free fatty acid accumulation and hepatic insulin resistance has been well described (Liu et al., 2015; Kubo et al., 2017).

Given the definite role of free fatty acids on mitochondrial dysfunction and hepatic insulin resistance, this review aims to explore the complex relationship among hepatic fatty acid accumulation, mitochondrial dysfunction, hepatic insulin resistance, and mitophagy (Garcia-Ruiz et al., 2013; Chen et al., 2015; Mohamad et al., 2015).

THE ROLE OF HEPATIC FREE FATTY ACID ACCUMULATION ON HEPATIC INSULIN RESISTANCE

In general, fatty acids are mainly stored in adipose tissue, and liver tissue is not the physiologic reservoir of fatty acids (Barry and Marry, 2015; Rotman and Neuschwander-Tetri, 2017). Nevertheless, long-term consumption of high-fat diet can lead to excess fatty acids flowing into adipose tissue (Wensaas et al., 2010; Barry and Marry, 2015). When the storage capacity of adipose tissue becomes saturated, superfluous fatty acids will overflow to the blood and further accumulate in nonadipose tissues such as liver, muscle, bone, pancreas, and heart as free fatty acids. This is the process of ectopic fatty acid accumulation (Hagberg et al., 2012; Malinska et al., 2015; Xu et al., 2017). Free fatty acids are the primary sources of hepatic lipid accumulation (Chen et al., 2017c). Under fasting conditions, circulating free fatty acids are the main fuel sources for various tissues except the brain tissue. Hence, plenteous free fatty acids are secreted into the blood by adipose tissue and further transported into the mitochondria of liver and skeletal muscle cells, in which free fatty acids will be oxidized and degraded to provide energy for cellular activity (Grattagliano et al., 2012; Peter and Georg, 2013; Zoladz et al., 2017). Therefore, as one of the important fuel sources, moderate levels of fatty acids in liver and muscle tissues are essential for cellular activities. However, excessive fatty acid accumulation in liver and muscle tissues is a pathological condition (Kubo et al., 2017).

Lipotoxicity is regarded as a pathological state in which high-fat diet impairs the normal metabolism of free fatty acids, resulting in accumulation of free fatty acids in the plasma and tissues (Lee et al., 2018). These fatty acids may impair glucose oxidation and glycogen synthesis and inhibit glucose transport or phosphorylation by suppressing insulin signal pathway and multiple steps of intracellular glucose metabolism (Yazıcı and Sezer, 2017). Therefore, markedly elevated hepatic fatty acids accumulation is a risk factor for hepatic insulin resistance. And the cause–effect relationship between hepatic fatty acid accumulation and hepatic insulin resistance has been confirmed in numerous human and animal researches (Wang et al., 2016; Sabater et al., 2017). The proposed mechanisms of hepatic fatty acid accumulation on hepatic insulin resistance include elevated gluconeogenesis, lipogenesis and chronic inflammation, impaired insulin signal pathway, excessive oxidative stress, and endoplasmic reticulum stress (Pereira et al., 2014; Yoon and Cha, 2014; Wang et al., 2017b).

Hepatic gluconeogenesis, a process of converting nonsugar substances into glucose, is an important regulatory mechanism of blood glucose homeostasis. Hepatic insulin resistance is characterized by overactive gluconeogenesis in liver, which can significantly increase blood glucose level. Glucose-6-phosphatase and glucokinase are two pivotal enzymes that regulate gluconeogenesis (Qi et al., 2018; Song et al., 2018b). Initially, free fatty acids are converted to long-chain fatty acyl coenzyme A by acyl-CoA synthase and then transformed into acetyl CoA in mitochondria of hepatocytes. This will activate hepatic gluconeogenesis to increase hepatic glucose production (Estee and Fiona, 2013; Adeva-Andany et al., 2019). Furthermore, free fatty acids evidently promote the expression of glucose-6-phosphatase to activate gluconeogenesis in liver tissue (Petersen et al., 2017; Tyrrell et al., 2017). Taken together, free fatty acids have a tremendous ability to activate hepatic gluconeogenesis to promote hepatic glucose production, which results in hyperglycemia and hepatic insulin resistance.

De novo lipogenesis is another mechanism of free fatty acid–induced hepatic insulin resistance. Free fatty acids promote lipogenic gene expression by regulating transcription factors including sterol regulatory element-binding protein-1c (SREBP-1c), carbohydrate responsive element-binding protein, and peroxisome proliferator-activated receptor γ2 (PPARγ2) (Siculella et al., 2016). Qin et al. (2016) have further identified that free fatty acids increase intracellular lipid accumulation by inhibiting PPARα expression and increasing SREBP-1c level (**Figure 1**).

Moreover, free fatty acid–induced inflammation also plays a central role in the development of hepatic insulin resistance (Wang et al., 2013; Chen et al., 2017c; Zhang et al., 2018). Free fatty acids promote the activity of IKK-β and nuclear factor κB through degrading IκB-α, which further increases the expression of interleukin 1β (IL-1β), tumor necrosis factor α, and IL-6 in liver tissues (Zhang et al., 2015b; Peng et al., 2017). These inflammatory factors can inhibit the activation of PI3K and IRS-1/2 tyrosine phosphorylation to impair insulin signaling pathway (Sharma et al., 2015; Yuan et al., 2016). Additionally, free fatty acids also trigger the expression of hepatic diacylglycerol and protein kinase C-δ (PKCδ) to prevent IRS-1/2 tyrosine phosphorylation, resulting in the occurrence of hepatic insulin resistance (**Figure 1**) (Pereira et al., 2014; Ter Horst et al., 2017).

It is well established that oxidative stress is a crucial risk factor for hepatic insulin resistance. Interestingly, PKCδ, an activator of nicotinamide adenine dinucleotide phosphate (NADPH), can promote the generation of ROS to increase oxidative stress. And ROS in turn promotes the activation of PKCδ, IKK-β, and c-Jun N-terminal kinase (JNK) to exacerbate oxidative stress. In line with previous researches, antioxidants like N-acetyl-L-cysteine and taurine can significantly prevent hepatic insulin resistance through suppression of excessive oxidative stress in rats (Guo-Guang et al., 2013; Pereira et al., 2014; Pereira et al., 2015; Cui et al., 2017; Villagarcía et al., 2018). Moreover, Pereira et al. (2014) have reported that hepatic fatty acid accumulation may activate PKCδ to increase NADPH oxidase-dependent oxidative stress, which further promotes the expression of IKK-β/JNK to impair hepatic insulin signaling pathway and finally cause hepatic insulin resistance. In summary, the pathway of free fatty acid–induced hepatic insulin resistance is free fatty acids → PKCδ → NADPH oxidase → oxidative stress → IKK-β/JNK → insulin signaling pathway → hepatic insulin resistance (Pereira et al., 2014).

MITOPHAGY AND LIPID METABOLISM

Autophagy widely existing in eukaryotic cells is essential for maintaining cellular energy homeostasis (Vargas et al., 2017). It is a conservative self-digestion process that relies on lysosomes, by which excessive fatty acids, damaged cell structures, and organelles can be degraded by lysosomal enzymes (Miyamoto and Heller, 2016; Chu et al., 2018). Depending on how they bind to lysosome, autophagy can be divided into macroautophagy, microautophagy, and chaperone-mediated autophagy (Cerri and Blandini, 2018). Moreover, autophagy can be also classified as selective autophagy and nonselective autophagy (Kim et al., 2016). Mitophagy is a highly selective autophagy, which can selectively degrade damaged mitochondria through macroautophagy (McWilliams et al., 2018). To be more specific, damaged mitochondria are swallowed up by LC3-positive autophagosomes, which subsequently fuse with lysosomes and degrade these mitochondria (McWilliams et al., 2018). Recently, a growing body of research has shown that mitophagy is of vital importance for mitochondrial quality control and mitochondrial dynamics including biosynthesis and degradation (Youle and Narendra, 2011; Michael et al., 2015; Kim et al., 2016).

There is a consensus that mitochondrial dysfunction is closely involved in the development of Parkinson disease and Alzheimer disease (Ganguly et al., 2017). Fortunately, these diseases can be reversed by mitophagy (Kerr et al., 2017; Sliter et al., 2018). Recent research has shown that impaired mitophagy is responsible for the development of insulin resistance diseases, such as metabolic syndrome, type 2 diabetes, obesity, and hyperlipidemia (Seillier et al., 2015; Rovira-Llopis et al., 2017; Che et al., 2018). Nevertheless, whether mitophagy can improve insulin resistance diseases is still not clear (Gonzalez-Franquesa and Patti, 2017). Therefore, we then explore the potential role and molecular mechanism of mitophagy on insulin resistance with a focus on lipid metabolism. We hope to elucidate the connection between mitophagy and insulin resistance and finally reveal a novel therapeutic target for insulin resistance.

SIGNALING PATHWAYS INVOLVED IN MITOPHAGY

Mitophagy selectively degrades damaged mitochondria, which contributes to mitochondrial quality control and maintenance of mitochondrial function. There are three main types of mitophagy: PINK1/Parkin-mediated mitophagy, BNIP3/NIX-mediated mitophagy, and FUN14 domain-containing 1 (FUNDC1)–mediated mitophagy (Sato and Furuya, 2017; Yuan et al., 2017; Li et al., 2018c).

PINK1/Parkin-Mediated Mitophagy

It is well recognized that the mitochondrial serine/threonine kinase PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin are two important proteins mediating mitophagy in mammalian cells (Williams et al., 2015; Harper et al., 2018). In healthy mitochondria, PINK1 is transported into the inner mitochondrial membrane and then cleaved by the inner membrane protease PARL. Ultimately, the truncated form of PINK1 is released into the cytoplasm for N-terminal recognition and then degraded by the proteasome to remain at a low basal level (Wu et al., 2015; Wang et al., 2019a). In damaged mitochondria, mitochondrial membrane potential ($\Delta\Psi m$) is insufficient to transport PINK1 to the inner mitochondrial membrane. Therefore, PINK1 mainly locates on the outer mitochondrial membrane of depolarized mitochondria and then phosphorylates some outer mitochondrial membrane protein (Greene et al., 2012; Lemasters and Zhong, 2018). This process can recruit autophagy adaptors including NDP52 and OPTN, which can bind to the double membrane vacuoles (autophagosomes). Therefore, damaged mitochondria can be captured by autophagosomes, which then fuse with lysosomes to degrade these mitochondria (Michael et al., 2015; Moreira et al., 2017). In order to further activate mitophagy, PINK1 located on the outer mitochondrial membrane of damaged mitochondria recruits cytosolic Parkin to damaged mitochondria to activate mitophagy by Mnf1 and Mnf2 phosphorylation. Additionally, PINK1 can phosphorylate Ser65 in ubiquitin and ubiquitin-like domain of Parkin to enhance Parkin E3 ubiquitin ligase activity to induce mitophagy (Wang et al., 2019a).

Generally, Parkin promotes mitophagy through two main pathways: First, Parkin ubiquitinates mitochondrial GTPase such as Miro and Mitofusin proteins including Mnf1 and Mnf2 to cause mitochondrial fragmentation and motility arrest, followed by sequestration and degradation of damaged mitochondria by autophagolysosomes (Eid et al., 2016; Moreira et al., 2017). Additionally, Parkin induces the ubiquitination of mitochondrial outer membrane proteins such as voltage-dependent anion channel, which can be identified by ubiquitin-binding adaptors including histone deacetylase 6 and SQSTM1/p62. This process contributes to propelling damaged mitochondria to the autophagic isolation membrane, for subsequent degradation of damaged mitochondria by mitophagy (Moreira et al., 2017).

Recent study has found that hepatic fatty acid accumulation can cause damaged mitochondria accumulation, which can impair mitochondrial respiratory chain function and fatty acid oxidative degradation (Ashrafi and Schwarz, 2013; Wu et al., 2015). Surprisingly, PINK1/Parkin-mediated mitophagy can reverse mitochondrial dysfunction and preserve mitochondrial function through eliminating damaged mitochondria in time (Wu et al., 2015; Nguyen et al., 2016; Xiong et al., 2018; Song et al., 2018a; Wang et al., 2019a).

BNIP3/NIX -Mediated Mitophagy

In response to hypoxia and nutrient deprivation, programmatic elimination of mitochondria by mitophagy is induced to inhibit excessive mitochondrial mass and maintain mitochondrial function. This kind of stress-responsive mitophagy is regulated by two key mitophagy adaptors: BCL-2/adenovirus E1B interacting protein 3 (BNIP3) and Nip-like protein X (NIX). BNIP3 and NIX, two pivotal BCL-2 homology domain 3–only proteins, play important roles in mitophagy–mediated mitochondrial quality control (Lampert et al., 2019; Xu et al., 2019). It is noteworthy that PINK1 and Parkin activate mitophagy through indirect binding to autophagosomes, while BNIP3 and NIX activate mitophagy through direct binding to autophagosomes (Moreira et al., 2017).

Initially, BNIP3/NIX may promote the moderate expression of ROS to enhance mitophagy (Scherz-Shouval and Elazar, 2011). Second, phosphorylation is an important process in BNIP3-induced mitophagy. The phosphorylation can promote BNIP3 to bind to LC3 II, a molecule critical for autophagosome formation, and phosphorylation of Ser24 on BNIP3 can further promote the affinity (Moreira et al., 2017). Additionally, Beclin-1, the mammalian ortholog of yeast Atg6, activates autophagy in the form of Beclin-1–Vps34 (lipid kinase Vps-34 protein)–Vps15 (lipid kinase Vps-15 protein) complexes (Ma et al., 2014; Wang et al., 2017a). Bcl-2 and Bcl-XL can bind to the BH3 domain of Beclin-1 in the form of Beclin-1–Bcl-2 and Beclin-1–Bcl-XL complexes to inhibit mitophagy. However, BNIP3 and NIX can compete with Beclin-1 to bind to Bcl-2 and Bcl-XL. And then Beclin-1 is liberated from these complexes to activate mitophagy (Chiara Maiuri et al., 2014; Chiang et al., 2018). Thirdly, Ras homolog enriched in brain (Rheb) can suppress mitophagy through activating mammalian target of rapamycin (mTOR), while BNIP3 can block Rheb-mTOR signaling pathway to enhance mitophagy (Lin et al., 2014; Gong et al., 2017). Moreover, NIX can directly bind to LC3. LC3 can bind to γ-aminobutyric acid receptor–associated protein (GABARAP) to form LC3–GABARAP complex, which promotes the mobilization of autophagosomes to damaged mitochondria (Moreira et al., 2017).

Taken together, metabolic stress, including hypoxia, nutrient deprivation, and fatty acid–induced dysfunctional mitochondria accumulation, can induce BNIP3/NIX-mediated mitophagy to clear damaged mitochondria and preserve mitochondrial integrity and function (Danielle et al., 2012; Moreira et al., 2017).

FUNDC1-Mediated Mitophagy

FUN14 domain-containing 1 located on the mitochondrial outer membrane contains a motif of Y (18) xxL (21), an LC3-interacting region (LIR), at the N-terminal (Lei et al., 2012). Under hypoxic conditions, similar to BNIP3 and NIX, FUNDC1 can directly bind to LC3 through its LIR motif to induce mitophagy (Wenxian et al., 2014; Yu et al., 2019a). Under nonstress conditions, Sc and CK2 kinases can phosphorylate Tyr-18 in LIR motif of FUNDC1 to interfere with the interaction between FUNDC1 and LC3, which subsequently impairs FUNDC1-mediated mitophagy (Wenxian et al., 2014). Additionally, FUNDC1 regulates mitophagy-mediated mitochondrial quality control through interacting with fission and fusion machinery components. For example, under hypoxic conditions, phosphoglycerate mutase 5, a mitochondrial Ser/Thr protein phosphatase, can dephosphorylate FUNDC1 and subsequently impair the interaction of FUNDC1 with mitochondrial fusion protein OPA1 to suppress mitochondrial fusion. Under

normoxic conditions, bits of FUNDC1 have been identified in the endoplasmic reticulum–mitochondria contact sites. Surprisingly, in response to hypoxic stress, FUNDC1 substantially interacts with the endoplasmic reticulum resident protein calnexin and further recruits mitochondrial fission protein DRP1 to activate mitochondrial fission (Palikaras et al., 2018). Moreover, under hypoxic conditions, ULK1 (UNC-51 like kinase 1), a pivotal component of autophagy initiation complex, can phosphorylate Ser-17 in LIR motif of FUNDC1 to promote the interaction between FUNDC1 and LC3 to induce mitophagy (Springer and Macleod, 2016). Similar to FUNDC1, hypoxia-induced mitophagy is also regulated by BNIP3 and NIX. Under hypoxic conditions, hypoxia inducible factor 1 can increase the expression of BNIP3 and NIX to enhance mitophagy (Palikaras et al., 2018). Notwithstanding the crosstalk among FUNDC1, BNIP3, and NIX is still confusing, their coordinated action is pivotal for mitophagy-based mitochondrial quality control.

Recent study has shown that BNIP3-, NIX-, and FUNDC1-mediated mitophagy plays a critical role in the treatment of lipid metabolism, hepatocellular carcinoma, hepatic insulin resistance, alcoholic liver disease, hepatic steatosis, and liver injury (Danielle et al., 2012; Williams and Ding, 2015; Chao et al., 2018; Liu et al., 2018; Li et al., 2019; Yu et al., 2019b). Nevertheless, research of mitophagy on liver diseases is still in its infancy, and its role and molecular mechanism involved in liver diseases are still needed to be confirmed by extensive experiments. Given the central role of mitochondrial quality control on energy homeostasis and adaptive response, pursuit of the role and molecular mechanism of mitophagy on liver diseases should be a field full of surprises, which may provide a novel perspective for the prevention and treatment of insulin resistance and metabolic syndrome.

MITOPHAGY PREVENTS HEPATIC FATTY ACID ACCUMULATION

Mitochondrial fatty acid oxidation is an important physiological process for fatty acid degradation and ATP production. Human body can absorb abundant fatty acids from diets and store them in adipose tissue. In the fasting state, these fatty acids are released from adipose tissue and further transported into the mitochondria of hepatocytes and skeletal muscle cells, in which fatty acids are oxidized for ATP production (Zhang et al., 2012). Therefore, normal mitochondrial function plays a pivotal role in regulating lipid metabolism and cellular energy supply. It is worth noting that healthy mitochondria can suppress fatty acid accumulation in liver and muscle tissue through accelerating fatty acid β-oxidation (Rambold et al., 2015; Sharma et al., 2018).

Accumulating evidence has shown that defective hepatic mitochondrial respiration characterized by damaged mitochondria accumulation can impair mitochondrial fatty acid β-oxidation, which sequentially causes various adverse consequences, such as excessive ROS, reduced ATP production, and hepatic fatty acid accumulation (Serviddio et al., 2010; Chistiakov et al., 2014; Crescenzo et al., 2016). There is a consensus that mitophagy, one of the prominent approaches for mitochondrial quality control, can remove damaged mitochondria to restore mitochondrial quality and mitochondrial function (Chakraborty et al., 2018; Saxena et al., 2019). The positive role of mitophagy on the scavenging of ectopic fatty acid accumulation has been clearly demonstrated. In an animal model of alcoholic fatty liver, Eid et al. (2016) have demonstrated that Parkin-mediated mitophagy can selectively clear damaged mitochondria to maintain mitochondrial quality, which is pivotal to the inhibition of hepatic lipid accumulation. In line with Eid and colleagues' standpoint, Williams et al. (2015) have found that Parkin knockout mice are more susceptible to alcohol-induced liver steatosis than wild-type mice. They have speculated that Parkin-mediated mitophagy plays a pivotal role in maintaining mitochondrial function, which can accelerate fatty acid oxidation and sequentially suppress fatty acid accumulation in liver tissue (Williams et al., 2015). In an animal model of high-fat diet–induced nonalcoholic fatty liver, the results have indicated that impaired PINK1/Parkin-dependent mitophagy may be responsible for hepatic fatty acid accumulation (Liu et al., 2018). As expected, quercetin, a common flavonoid, can activate PINK1/Parkin-mediated mitophagy to accelerate mitochondrial fatty acid oxidation and inhibit hepatic fatty acid accumulation in an animal model of high-fat diet–induced nonalcoholic fatty liver. Additionally, quercetin also activates PINK1/Parkin-dependent mitophagy to prevent oleic acid/palmitic acid–induced lipid accumulation in HepG2 cells (Liu et al., 2018). Moreover, linseed oil, exenatide, melatonin, akebia saponin D, and sirtuin 3 have also been shown to suppress hepatic lipid accumulation through activating mitophagy (**Table 1**).

Moreover, recent study has shown that macrophage can infiltrate into white adipose tissue to promote lipolysis and then release fatty acids into the bloodstream. These fatty acids may be transported to liver tissue and then promote lipid synthesis by esterification. Fatty acids can promote the activation of PKC to impair insulin signal pathway, subsequently causing hepatic insulin resistance (Samuel and Shulman, 2016). Wu et al. (2019) have found that impaired mitophagy can accelerate macrophage infiltration in white adipose tissue in high-fat diet–fed mice with FUNDC1 knockout. They have also demonstrated that impaired mitophagy can promote macrophage infiltration to activate MAPK signal pathway and inflammatory response to impair mitochondrial quality control, subsequently causing insulin resistance and hepatic steatosis (Wu et al., 2019). Collectively, these evidences have suggested that mitophagy can suppress macrophage-induced inflammatory response and improve mitochondrial quality control to suppress hepatic insulin resistance and steatosis.

In a word, mitophagy can prevent hepatic fatty acid accumulation *via* maintaining mitochondrial function and accelerating mitochondrial fatty acid oxidation. However, the study of mitophagy on liver diseases is still in its infancy, and its role and molecular mechanism are also still needed to be confirmed by extensive experiments.

MITOPHAGY AS THERAPEUTIC INTERVENTION IN HEPATIC INSULIN RESISTANCE

Mitochondrion is the major site of aerobic respiration and the main energy production center. Glucose, fatty acids and amino acids are

TABLE 1 | The role and molecular mechanisms of natural or synthesized compounds-induced mitophagy on liver diseases.

Compound	Disease model	Mechanism	Protein	Reference
Quercetin	Mice with nonalcoholic fatty liver disease (NAFLD) and free fatty acid–treated HepG2 cells	Activating mitophagy to improve hepatic steatosis	PINK1 ↑, Parkin ↑, Beclin-1↑, LC3-II/I ↑, and p62 ↓	(Liu et al., 2018)
Linseed oil	Obese mice	Activating mitophagy to improve hepatic insulin resistance, hepatic mitochondrial biogenesis, hepatic lipid accumulation	Parkin ↑, FUNDC1 ↑, LC3-II/I ↑, and p62 ↓	(Yu et al., 2019b)
Exenatide	Mice with NAFLD	Activating mitophagy to reduce oxidative stress and NLRP3 inflammasome in liver tissue	LC3-II/I ↑, Beclin-1 ↑, Parkin ↑, BNIP3 ↑, NLRP3↓, and IL-1β ↓	(Shao et al., 2018)
Melatonin	Mice with NAFLD and palmitic acid (PA)–treated primary hepatocytes	Promoting Drp1-mediated mitochondrial fission and BNIP3-dependent mitophagy to rescues mitochondrial respiratory function	Drp1 ↑, Atg5 ↑, Beclin-1 ↑, mito-LC3II ↑, and BNIP3 ↑	(Zhou et al., 2018)
Akebia saponin D	Oleic acid–treated BRL cells	Alleviating hepatic steatosis through promoting BNIP3-mediated mitophagy	mTOR ↓, LC3II ↑, and BNIP3 ↑	(Gong et al., 2018)
Sirtuin 3	Mice with NAFLD and PA -treated primary hepatocytes	Alleviating hepatic steatosis through promoting BNIP3-mediated mitophagy	Mito-LC3II ↑, Atg5 ↑, Beclin-1 ↑, and BNIP3 ↑.	(Li et al., 2018b)

substrates for mitochondrial energy production. Mitochondria play central roles in substrate oxidation, tricarboxylic acid cycle, oxidative phosphorylation, cell proliferation, cell metabolism, and programmed cell death (Montgomery and Turner, 2015). There are plenteous methods for the determination of mitochondrial function, including the protein and mRNA expressions of mitochondrial-encoded genes CYTB and COX1; respiratory chain complexes I, II, and III; nuclear-encoded genes PGC1α; mitochondrial enzyme activity; mitochondrial size and shape; mitochondrial quantity; and ROS production (Montgomery and Turner, 2015; O'brien et al., 2017; Trotta and Chipuk, 2017; Lima et al., 2018; Lv et al., 2019). However, there is no recognized method available for the detection of mitochondrial respiratory chain function. Therefore, it is urgent to develop a recognized method for mitochondrial respiratory chain function evaluation.

Given the central role of mitochondria in cellular energy metabolism, mitochondrial dysfunction is predominantly referred to as defective mitochondrial oxidative phosphorylation, which is characterized by impaired substrate oxidation and damaged mitochondria accumulation (Crescenzo et al., 2016). In general, damaged mitochondria accumulation can cause excessive ROS production, which will destroy mitochondrial oxidative phosphorylation and substrate oxidation, thereby inhibiting fatty acid oxidative degradation and accelerating fatty acid accumulation (Finck and Hall, 2015; Crescenzo et al., 2016; Horst et al., 2017). As a result, fatty acids especially diacylglycerol and ceramide may excessively accumulate in liver tissue. Previous report has shown that diacylglycerol can activate PKC to impair insulin signal pathway (Ditte et al., 2016; Ter Horst et al., 2017). Moreover, ceramide also activates PKCζ to suppress protein kinase AKT and further inhibits insulin signal pathway (Montgomery and Turner, 2015; Sajan et al., 2015; Chen et al., 2017b). In conclusion, mitochondrial dysfunction gives rise to hepatic fatty acid accumulation, which ultimately impairs insulin signal pathway and causes hepatic insulin resistance.

Insulin plays a central role in lipid and glucose metabolism and also promotes mitochondrial function characterized by enhanced mitochondrial oxidative metabolism and ATP production in hepatocytes (Kim et al., 2015; Ruegsegger et al., 2019). However, insulin resistance also leads to mitochondrial dysfunction. Previous evidence has demonstrated that hepatic insulin resistance exacerbates lipid deposition, oxidative stress, lipid peroxidation, and mitochondrial dysfunction in liver tissues (Goodpaster, 2013). Therefore, a vicious circle between dysfunctional mitochondrial respiration and hepatic insulin resistance is established.

Growing evidence has shown that mitophagy may be a promising therapeutic target for hepatic insulin resistance. Marycz et al. (2018) have speculated that mitophagy is a repair mechanism responsible for cellular energy homeostasis and cell survival in insulin resistance hepatocytes and adipose cells. Qi et al. (2016) have also found that impaired mitophagy dramatically exacerbates high-fat diet–induced insulin resistance. Fortunately, enhanced mitophagy remarkably protects mice from high-fat diet–induced insulin resistance. Therefore, they consider that mitophagy should be considered as a protective response to high-fat diet–induced insulin resistance (Qi et al., 2016).

Currently, mitochondrial dysfunction is recognized as a pivotal risk factor for insulin resistance and metabolic syndrome (Crescenzo et al., 2016; Sarparanta et al., 2017). Autophagy is responsible for eliminating misfolded proteins and dysfunctional organelles such as aged or dysfunctional mitochondria and endoplasmic reticulum (Che et al., 2018). Therefore, as a selective autophagy, impaired mitophagy may fail to eliminate damaged mitochondria in time, which further causes excessive accumulation of damaged mitochondria in hepatocytes and triggers mitochondrial dysfunction (Crescenzo et al., 2016). Therefore, researchers have suggested two therapeutic strategies for improving mitochondrial dysfunction: One is to increase the quantity of mitochondria by enhancing its biosynthesis. The other is to remove damaged mitochondria by mitophagy. Considering that clearance of dysfunctional mitochondria is more important for mitochondrial homeostasis

including mitochondrial function and biosynthesis, more attention has been focused on the removal mechanism of dysfunctional mitochondria in insulin-resistant conditions (Gonzalez-Franquesa and Patti, 2017; Kalavalapalli et al., 2018; Marycz et al., 2018). There is no doubt that mitophagy-mediated removal of dysfunctional mitochondria should be the center of attention. Growing evidence has also shown that mitophagy may be a potential therapeutic target for hepatic insulin resistance (Mottillo et al., 2016; Zhang et al., 2017). Very recently, a limited number of researches have reported that mitophagy may improve mitochondrial quality and accelerate fatty acid oxidative degradation to suppress hepatic insulin resistance and lipid accumulation. However, the cause–effect relationship between hepatic insulin resistance and mitophagy is also still needed to be confirmed by substantial experiments (Pickrell et al., 2013; Chen et al., 2017a).

CONCLUSION

This review focuses on the complex relationship among hepatic insulin resistance, hepatic fatty acid accumulation, mitochondrial dysfunction, and mitophagy. Mitochondria play pivotal roles in the regulation of fatty acid metabolism. Extensive studies have indicated that mitochondrial dysfunction plays a central role in hepatic fatty acid–induced hepatic insulin resistance (Montgomery and Turner, 2015; Wang et al., 2017b). Interestingly, mitophagy contributes to mitochondrial quality control and the maintenance of mitochondrial function by selectively degrading defective mitochondria. At present, the protective mechanism of mitophagy on hepatic insulin resistance still needs further experimental confirmation. In this review, we summarize some evidences: (1) Influx of fatty acids into liver tissue can cause mitochondrial dysfunction; (2) mitochondrial dysfunction can further obstruct the timely elimination of fatty acids and induce hepatic fatty acid accumulation; (3) hepatic fatty acid accumulation is responsible for the pathogenesis of lipotoxicity and inflammation; (4) lipotoxicity and inflammation can interfere with insulin signaling pathway to cause hepatic insulin resistance; (5) interestingly, enhanced mitophagy can selectively remove damaged mitochondria to improve mitochondrial dysfunction and restore mitochondrial function, which is beneficial to the elimination of hepatic fatty acids; (6) mitophagy-mediated recovery of mitochondrial function can reverse hepatic fatty acid accumulation–induced hepatic insulin resistance. The association between mitophagy and hepatic insulin resistance is still not completely understood, in part due to various methods for detecting mitochondrial function (Chow and From, 2010;

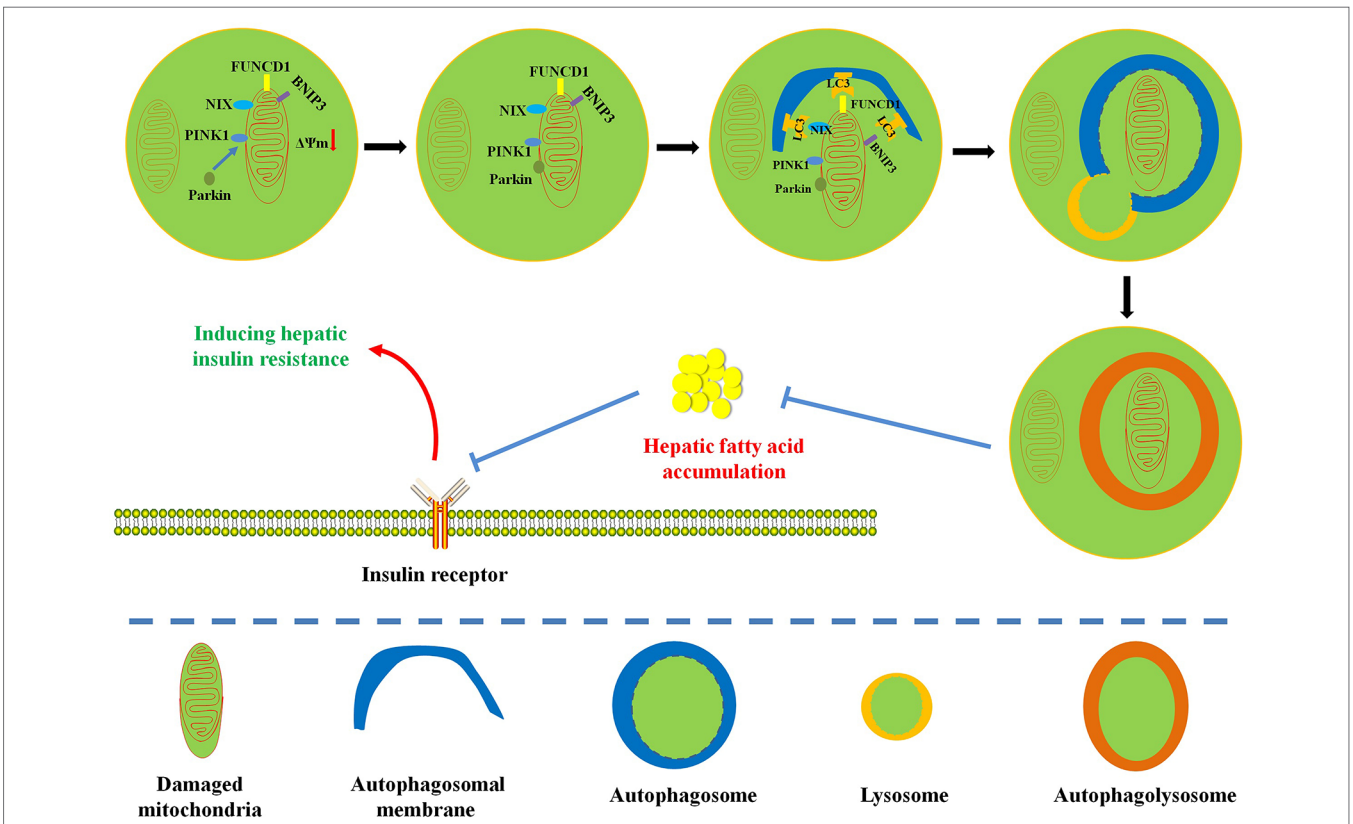

FIGURE 2 | Diagram illustrates the possible protective mechanism of mitophagy on hepatic fatty acid–induced hepatic insulin resistance. Enhanced mitophagy can degrade damaged mitochondria to restore mitochondrial function and accelerate mitochondrial fatty acid oxidation, which is beneficial to the reversion of hepatic fatty acid accumulation and the improvement of hepatic insulin resistance.

Montgomery and Turner, 2015). Therefore, given the confirmed relationship between hepatic fatty acid–induced mitochondrial dysfunction and hepatic insulin resistance, we hypothesize that mitophagy can remove damaged mitochondria to restore mitochondrial function and promote the oxidative degradation of fatty acids (**Figure 2**). This physiological process is conducive to the inhibition of hepatic fatty acid accumulation and hepatic insulin resistance (**Figure 2**). A limited number of studies have proved that enhanced mitophagy can inhibit hepatic lipid accumulation to improve hepatic insulin resistance. However, the definite relationship between mitophagy and hepatic insulin resistance still needs further experimental confirmation (Yang et al., 2014; Seillier et al., 2015; Corsa et al., 2019). Moreover, energy metabolism and organismal homeostasis attribute to tight coordination between mitochondrial biogenesis and degradation. Certainly, pursuit of natural or synthesized compounds possessing mitochondrial biogenic and mitophagic activities may provide novel insights into the therapeutic interventions for mitochondria-related diseases such as hepatic insulin resistance and metabolic syndrome.

AUTHOR CONTRIBUTIONS

ZS, YN, and XH drafted the manuscript. ZS, XH, YZ, BF, and LT created the figures and performed literature searches. ZS and GZ revised the manuscript and edited the final draft.

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Methylprednisolone Decreases Mitochondria-Mediated Apoptosis and Autophagy Dysfunction in Hepatocytes of Experimental Autoimmune Hepatitis Model *via the* Akt/mTOR Signaling

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Autoimmune hepatitis (AIH) is characterized by massive immune cell-mediated hepatocyte destruction. Glucocorticoids, particularly methylprednisolone (MP), are the most effective treatment for AIH; however, the mechanism underlying the effects of glucocorticoid treatment has not been fully elucidated. The present study explored the effects of MP on damaged hepatocytes in mice with concanavalin A (ConA)–induced experimental autoimmune hepatitis (EAH). C57BL/6 mice were divided into three groups: a normal control group (injected with normal saline), a ConA (20 mg/kg) group, and a ConA + MP (3.12 mg/kg) group. The serum levels of liver enzymes, cytokines, activated T cells, and apoptosis– and autophagy–associated marker proteins were determined 12 h after ConA injection. Human hepatocyte cell line LO2 was used to verify the effects of ConA and MP *in vitro*. MP treatment significantly decreased inflammatory reactions in the serum and liver tissues and activated the Akt/mTOR signaling pathway to inhibit apoptosis and autophagy in hepatocytes *in vivo*. Transmission electron microscopy (TEM) revealed fewer autophagosomes in the MP-treated group than in the ConA-treated group. MP treatment obviously suppressed apoptosis and mitochondrial membrane potential ($\Delta\Psi m$) loss in hepatocytes *in vitro*. Furthermore, ConA treatment increased the levels of LC3-II, p62/SQSTM1, and Beclin-1, while bafilomycin A1 did not augment the levels of LC3-II. MP treatment decreased the levels of LC3-II, p62/SQSTM1, and Beclin-1 and upregulated the levels of phosphorylated (p)-Akt and p-mTOR. In conclusion, MP ameliorated mitochondria-mediated apoptosis and autophagy dysfunction in ConA-induced hepatocyte injury *in vivo* and *in vitro via* the Akt/mTOR signaling pathway.

Keywords: autoimmune hepatitis, methylprednisolone, LO2 cell, apoptosis, autophagy, Akt/mTOR signaling pathway

INTRODUCTION

Autoimmune hepatitis (AIH) is a self-perpetuating inflammatory liver disease, and the misdiagnosis or delayed treatment of AIH can lead to liver cirrhosis, liver cancer, transplantation, and rapid death

(European Association for the Study of the Liver, 2015; Manns et al., 2015; Montano-Loza and Czaja, 2015).

Methylprednisolone (MP) is a physiological inhibitor of inflammatory responses that is widely used as an anti-inflammatory and immunosuppressive agent in the treatment of numerous autoimmune and allergic diseases, mainly through suppressing CD4+ T cell activation indirectly by modulating dendritic cell function and directly by regulating T cell receptor signaling (Cain and Cidlowski, 2017). Glucocorticoids have been reported to prevent the progression of various liver diseases by the following mechanisms: (i) protection of normal human liver cells from apoptosis induced by tumor necrosis factor (Zhao et al., 2015), (ii) suppression of calpain μ activation and talin degradation in ischemia-induced liver injury in rats (Wang et al., 2008), (iii) increased ability of hepatocytes to accumulate cAMP due to protein synthesis-dependent processes (Thoresen et al., 1989). Longhi *et al.* found that corticosteroids used to treat AIH can reconstitute the regulatory T cell population, which, in turn, suppresses the proliferation of CD8+ lymphocytes and induces production of the anti-inflammatory cytokine IL-4 (Longhi et al., 2005). Furthermore, Moser *et al.* revealed that glucocorticoids affect the capacity of dendritic cells to sensitize naive T cells *in vivo* (Moser et al., 1995). However, the mechanisms by which glucocorticoids alleviate AIH remain to be elucidated.

Apoptosis and autophagy, which are overactivated following ConA treatment, contribute to liver injury (Yin et al., 2008; Czaja, 2014; Li et al., 2016c; Feng et al., 2018b). Apoptosis, the predominant mechanism of liver cell death in interface hepatitis (Kerr et al., 1979), is a double-edged sword, as it can both inhibit inflammatory responses and lead to tissue damage when overactivated. Autophagy is an evolutionarily conserved process that plays an important role in responses to different types of cellular stress (Yu et al., 2018). We hypothesized that MP may ameliorate the pathological changes in liver injury by regulating apoptosis and autophagy in hepatocytes and subsequently relieve AIH progression. The present study was designed to evaluate whether MP affects the levels of apoptosis and autophagy *in vivo* and *in vitro* and the possible mechanism underlying this effect.

MATERIALS AND METHODS

Drugs and Reagents

ConA was purchased from Sigma–Aldrich (St. Louis, MO, USA). MP (HY-B0260, analytical standard >99.0%) and bafilomycin A1 (HY-100558, analytical standard >99.0%) were obtained from MedChemExpress (NJ, USA). Antibodies p62/SQSTM1 (cat# 5114), Beclin-1 (cat# 3495), p-mTOR (Ser 2448, cat# 5536), mTOR (cat# 2983), p-Akt (Ser 473, cat# 4060), Akt (cat# 4691), Bax (cat# 2772), Bcl-2 (cat# 3498), and cleaved caspase-3 (cat# 9664) were purchased from Cell Signaling Technology (Danvers, MA, USA). LC3B (sc-376404) was purchased from Santa Cruz Biotechnology (Europe), and β-actin was purchased from ZSGB-BIO (Beijing, China). Monodansylcadaverine (MDC, G0170) was purchased from Solarbio (Beijing, China). The tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining kit was purchased from Beyotime (Shanghai, China). Other reagents were of high analytical grade and were commercially available unless otherwise specified.

Animals

Female C57BL/6 mice (aged 8–10 weeks; 19–22 g) were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). The mice were housed in a specific pathogen-free (SPF) facility at a constant room temperature and humidity and had unlimited access to standard laboratory chow and water one week before the experiments. All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China.

Experimental Design and Treatment Schedule

An experimental autoimmune hepatitis (EAH) mouse model was established 12 h after the injection of ConA at a dose of 20 mg/kg, which was chosen based on our previous studies (Wang et al., 2018a; Ye et al., 2018). The animals were randomly divided into three groups (n = 8 per group): (1) a normal control group (NC), (2) an EAH group (EAH), and (3) an EAH group with MP (3.12 mg/kg). The distribution of mice in all the groups was random. Mice in the MP and NC groups received MP or saline by intragastric administration once 0.5 h after the ConA injection. Finally, all of the mice were sacrificed 12 h after the ConA injection (**Figure 1A**).

Liver Function and Cytokine Assay

Retro-orbital blood samples were collected by removing the eyeball. Plasma was separated by centrifugation at 2,000 rpm for 10 min, and alanine transaminase (ALT) and aspartate transaminase (AST) measurements were performed using an automatic dry biochemical analyzer (Hitachi Auto Analyzer7170, Japan). The levels of IL-6 and IFN-γ in the murine plasma were analyzed by ELISA kits (MultiSciences, Hangzhou, China) according to the manufacturer's instructions.

Histopathological Analysis

Liver tissues were isolated, and the samples were fixed in 4% buffered paraformaldehyde for 48 h and then embedded in paraffin. The sections (4–5 μm) were mounted on slides, deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and subjected to hematoxylin and eosin (H&E) staining.

Immunohistochemistry (IHC) Analysis

Paraffin-embedded tissue samples were dewaxed, rehydrated, and incubated with a primary antibody overnight at 4°C. The samples were incubated with a secondary antibody (Bioss, Beijing, China) according to the manufacturer's instructions. A brown color in the cell membrane indicated positive staining. The integrated optical densities (IODs) of the different indicators were calculated using Image-Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA).

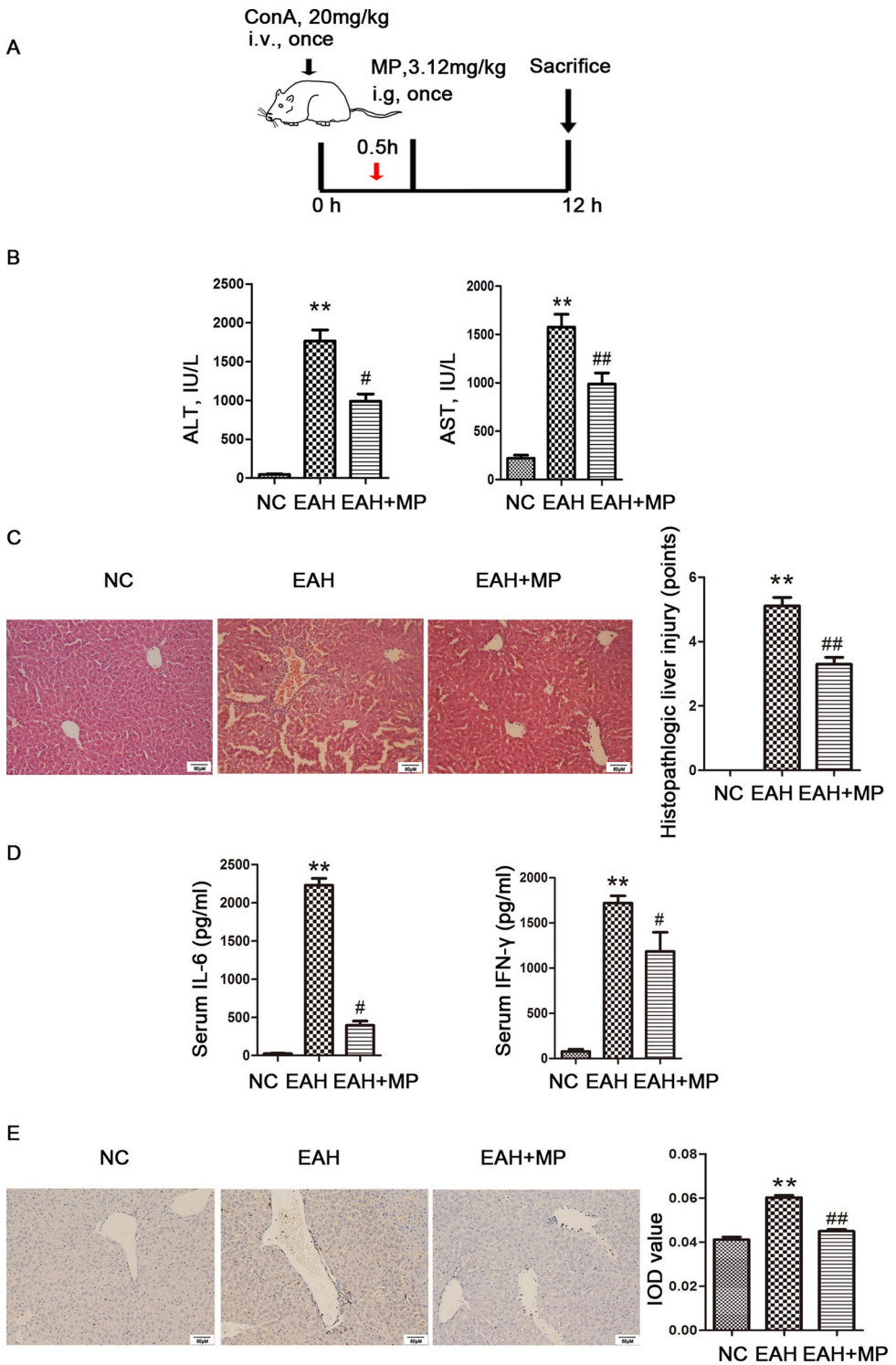

FIGURE 1 | Effects of MP on ConA–induced EAH. **(A)** Animal study protocol. **(B)** Serum ALT and AST. **(C)** Pathological liver specimens stained with H&E (×200): The control group showed normal hepatic architecture, while ConA injection induced marked pathological damage, including dramatic inflammatory cell infiltration in the portal area, massive cloudy swelling, blood vessel congestion and dilatation, and disordered hepatic sinusoid structures. MP-treated animals showed improvements of these lesions. **(D)** Serum IL-6 and IFN-γ. **(E)** Immunohistochemistry was used to show CD4+ T cell infiltration (original magnification, ×200). ConA injection in the EAH group induced a significant increase in the number of infiltrating CD4+ T cells compared to that in the control group. The percentage of CD4+ T cells was significantly decreased after MP treatment and almost returned to normal levels compared to that in the EAH group. Data are presented as the mean ± SD (n = 8). $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. EAH group. ConA, concanavalin A; MP, methylprednisolone; NC, normal control; EAH, experimental autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IL-6, interleukin-6; IFN-γ, interferon-γ; IOD, integrated optical density.

TUNEL Assay

A TUNEL assay was performed according to the protocol provided in the TUNEL kit. Paraffin-embedded liver sections sliced at a 5-μm thickness were dewaxed in xylene for 10 min twice and dehydrated in ethanol. Then, the sections were incubated with 20 μg/ml DNase-free proteinase K at room temperature for 15 min to digest the sections. After washing 4 times, a TUNEL reaction mixture was added to the sections, which were then incubated at 37°C in a humidified atmosphere for 1 h and observed by light microscopy.

Transmission Electron Microscopy (TEM)

Fragments from the liver parenchyma of each mouse were fixed in 2.5% glutaraldehyde, fixed in 1% osmium acid, dehydrated in an alcohol gradient, embedded in epoxy resin, and sliced by a microtome. The sections were stained, and images were taken by TEM (HT7700, Hitachi, Japan). For quantification of autophagosomes in hepatocytes of each group, 10 lower-magnification photomicrographs from the hepatocytes of hepatic tissues (×2,500, each image containing at least one hepatocytes nucleus) were applied (Watanabe et al., 2009; Eid et al., 2013).

Cell Culture and Treatments

Human foetal hepatocyte cell line LO2 was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin-streptomycin solution at 37°C in a humidified 5% CO_2 incubator. To induce excessive inflammation and develop an *in vitro* model of hepatocyte injury, LO2 cells were exposed to medium supplemented with ConA for 12 h. To test the effects of MP, cells were incubated with 10 μM MP 2 h before ConA treatment and then incubated with bafilomycin A1 (10 nM) 3 h before harvesting. ConA and MP were dissolved in saline and distilled water, respectively, and bafilomycin A1 was dissolved in DMSO according to the manufacturer's instructions.

Cell Proliferation Assay

Cells (2-5×10^3/100 μl/well) were seeded in 96-well plates and treated with different concentrations of MP for 12 h. Next, 20 μl of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. The purple formazan crystal was dissolved in 150 μl of DMSO, and the absorbance at 570 nm was subsequently recorded using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA).

Morphological Analysis by Hoechst 33258 Staining

To conduct staining experiments, LO2 cells at 50% confluence were plated on 18-mm coverslips in a six-well plate and incubated for 12 h. After treatment with the different reagents for 12 h, the cells were washed with ice-cold phosphate-buffered saline (PBS) twice and fixed in ice-cold methanol for approximately 15 min. The cells were stained with a Hoechst 33258 solution (KeyGen Biotech) and then photographed by a fluorescence microscope to observe the nuclear morphology of the apoptotic bodies.

Apoptosis Analysis by Flow Cytometry (FCM)

Further observation of apoptosis was carried out using an Annexin V-FITC/PI apoptosis detection kit (KeyGEN BioTECH). After treatment with the different reagents, the cells were collected, washed twice with ice-cold PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's guidelines. The distributions of viable (FITC-/PI-), early-apoptotic (FITC+/PI-), late-apoptotic (FITC+/PI+), and necrotic (FITC-/PI+) cells were analyzed *via* FCM. Both early- and late-apoptotic cells were defined as apoptotic cells in this study. Data were analyzed using FlowJo software according to the manufacturer's instructions.

Examination of Mitochondrial Membrane Potential (Δψm) and Reactive Oxygen Species (ROS)

To detect changes in the ΔΨm and ROS level, LO2 cells treated with the different reagents were incubated with 10 μM rhodamine 123 (Rh123) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) diluted in PBS at 37°C in the dark for 30 min. Finally, the stained cells were washed with cold PBS and analyzed *via* FCM. The data are shown as the mean values from independent experiments that were conducted in triplicate.

ΔΨm was further verified by tetraethyl benzimidazolyl carbocyanine iodide (JC-1) staining (Pourahmad et al., 2003). LO2 cells were seeded at a density of 5×10^4 cells/well on coverslips and incubated in 24-well plates. Before incubation with ConA for 12 h, the cells were pretreated with 10 μM MP for 2 h. After drug treatment, the cells were washed with PBS and incubated in medium containing 5 g/ml JC-1 stain for 20 min in the dark at 37°C. The cells were then directly observed under a fluorescence microscope at an excitation wavelength of 514–529 nm and an emission wavelength of 585–590 nm.

Monodansylcadaverine (MDC) Staining

Autophagic vacuoles formed in the cells were detected by MDC staining. LO2 cells were seeded at a density of 5×10^4 cells/well on cover slips in 24-well plates and incubated overnight to allow adherence. Then, the cells were treated with saline or ConA (10 μg/ml) alone or in combination with MP for 12 h. The cells were then washed three times with PBS and incubated with MDC (50 μM) for 30 min in the dark at 37°C. Next, excess MDC was washed away, and the cells on the cover slips were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Autophagic vacuoles formed in the cells were analyzed by fluorescence microscopy at an excitation wavelength of 460–500 nm and an emission wavelength of 512–542 nm.

Western Blot Analysis

Liver specimens and LO2 cells were lysed using RIPA lysis buffer in the presence of protease and phosphatase inhibitors for 30 min on ice, followed by centrifugation at 13300 rpm at 4°C for 15 min to clear the lysates; the lysate supernatant was then harvested. Protein concentrations were determined with the Bradford Protein Assay Kit, and known amounts of BSA were

used to standardize and equalize protein concentrations before loading. Equivalent amounts of total protein (usually 30–40 μg) were separated on SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ). After blocking, the membranes were incubated overnight at 4°C with primary antibodies including cleaved caspase-3, Bax, Bcl-2, LC3B, Beclin-1, p62, Beclin-1, Akt, p-Akt, mTOR, p-mTOR, and β-actin. After washing with TBST, the membranes were incubated with an HRP-conjugated secondary antibody (ZSBIO, Beijing, China) at 37°C for 1 h. Finally, the membranes were washed twice and detected using an enhanced chemiluminescence system. The IODs of the different indicators were calculated using Image-Pro Plus software.

Statistical Analysis

Descriptive and analytical statistical analyses were performed using the SPSS (version 22.0) software package. Statistical significance between groups was determined by a two-tailed Student's *t*-test. The data are presented as the mean ± SD, and $P < 0.05$ indicates statistical significance.

RESULTS

MP Treatment Attenuates Liver Injury Induced by ConA in Mice

An important characteristic of AIH is an obviously elevated serum level of transaminase, which indicated the extent of liver injury. A ConA-induced injury model was established as previously described with or without treatment with MP (3.12 mg/kg), as shown in **Figure 1A**. We determined the levels of liver enzymes in mouse serum 12 h after ConA injection. As shown in **Figure 1B**, the liver enzyme levels in the EAH group were significantly higher than those in the NC group, indicating the successful simulation of human AIH. In addition, the ALT and AST levels were reduced by MP treatment, demonstrating the protective effect of MP in the EAH liver. As the progression of liver injury is associated with proinflammatory cytokines, the serum levels of IL-6 and IFN-γ were examined and determined by ELISA (**Figure 1D**). As expected, the levels of these cytokines in the EAH group were significantly higher than those in the NC group and were reduced by MP treatment.

We then examined the effect of MP on EAH liver damage using H&E staining. As shown in **Figure 1C**, mice in the EAH group suffered severe liver damage, as indicated by massive inflammatory cell infiltration in areas around the central vein. In contrast, those in the EAH+MP group exhibited minor liver injury, indicating that treatment with MP significantly reduced liver injury.

As the T cell-mediated immune response plays a vital role in AIH, the T cell infiltration in the livers in the different groups were analyzed by IHC. As indicated by CD4 staining, portal areas showed T cell infiltration upon ConA treatment, while MP treatment decreased the presence of T cells in the liver tissues (**Figure 1E**).

The above data verified that MP treatment alleviates inflammation and hepatocyte injury and changes the T cell-mediated immune response in the liver.

MP Treatment Attenuates Hepatocyte Apoptosis and Autophagy in ConA-Induced Liver Injury

Hepatocyte apoptosis was strongly induced in the EAH group compared to the control, while MP reduced this effect as shown by a reduction in the TUNEL positive staining (**Figure 2A**). This finding was further supported by analyzing the indicators of cellular apoptosis. B-cell lymphoma-2 (Bcl-2) is an antiapoptotic protein, while Bcl-2-associated X protein (Bax) and caspase-3 are markers of apoptosis. In the EAH group, the expression of cleaved caspase-3 and Bax was increased, while the production of Bcl-2, which inhibits apoptosis, was dramatically reduced compared to that in the NC group. Conversely, MP treatment downregulated the proapoptotic markers Bax and cleaved caspase-3 and upregulated the expression of Bcl-2 (**Figure 2B**). The IHC staining results were consistent with those of western blot (**Figure 2C**). The results of the TUNEL assay and apoptosis-related protein analysis implied that ConA-induced apoptosis in hepatocytes and that MP treatment alleviated apoptosis.

LC3-B, p62, and Beclin-1 are markers of autophagy. Western blot analyses indicated that ConA injection significantly upregulated the LC3-II, Beclin-1, and p62 expression levels compared with those in the NC group. Treatment with MP reversed this effect (**Figure 3A**), which was further confirmed by IHC staining (**Figure 3B**). As shown in **Figure 3C**, TEM images showed more autophagosomes in the EAH group than in the NC group; conversely, MP treatment decreased the number of autophagosomes (**Supplementary Figure 1**).

These results provide strong evidence that MP treatment attenuates hepatocyte apoptosis and autophagosomes and reduces pathological liver damage following ConA administration in mice.

Pretreatment With MP Ameliorates Liver Injury in EAH At Least in Part Through the Akt/mTOR Signaling Pathway

As mentioned above, MP treatment protected against liver injury by inhibiting hepatocyte apoptosis and autophagosomes. However, the mechanism underlying this effect remains unclear. To determine whether the protective effect of MP on EAH injury is mediated by the Akt/mTOR signaling pathway, the protein levels of Akt, p-Akt, mTOR, and p-mTOR in liver tissues were detected. ConA injection downregulated the p-Akt/Akt and p-mTOR/mTOR levels in the liver tissues of the EAH group. MP treatment, however, partly suppressed this effect, which was significant (**Figure 3D**). Similar results were observed for p-Akt in the IHC analysis (**Figure 3E**). In summary, MP treatment may activate the Akt/mTOR signaling pathway in ConA-induced liver injury.

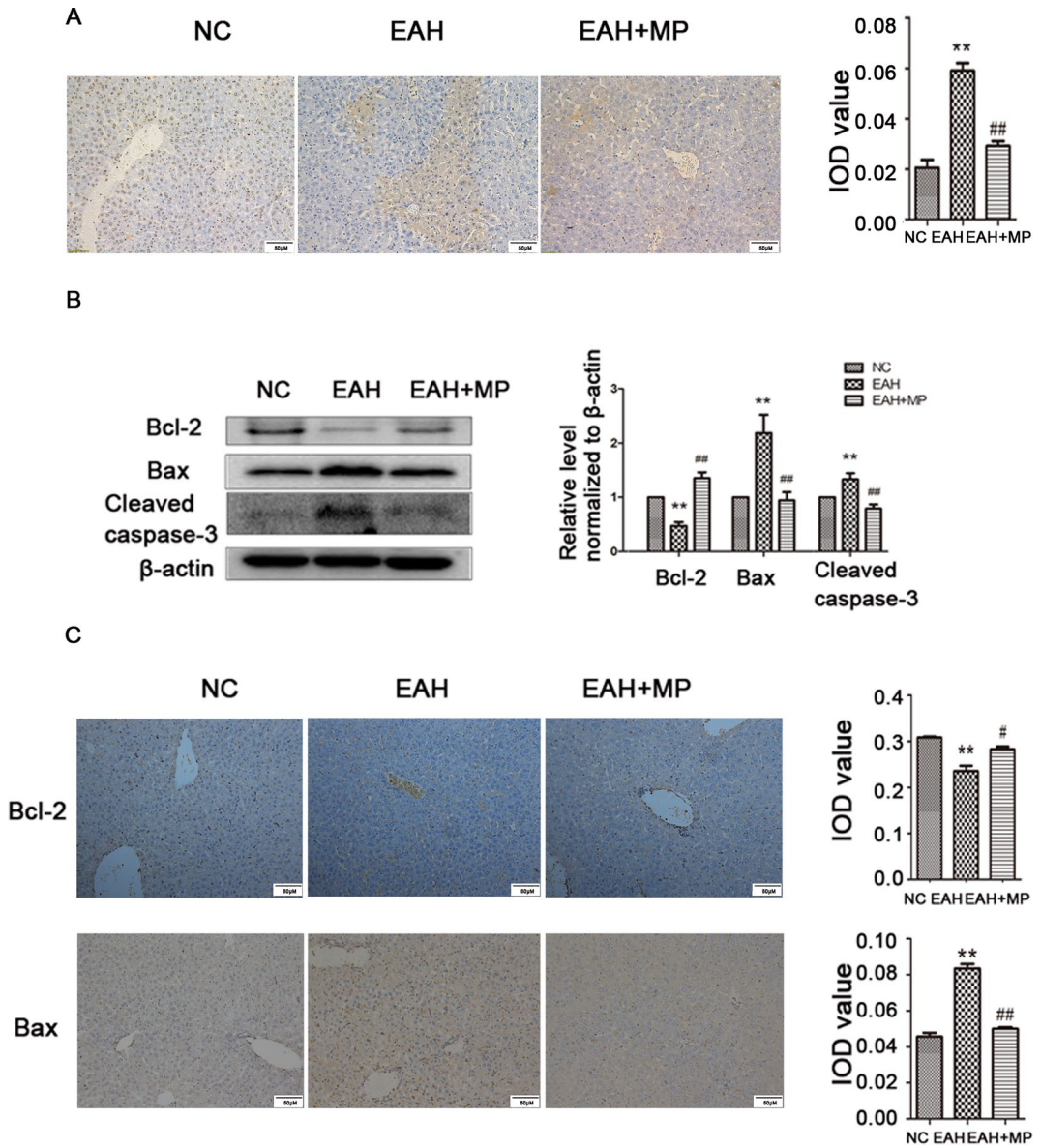

FIGURE 2 | MP pretreatment attenuates hepatocyte apoptosis and autophagy in ConA–induced EAH, partially through the Akt/mTOR signaling pathway. **(A)** TUNEL staining indicated apoptotic hepatocytes in the three groups after ConA injection (original magnification, ×400). **(B)** Protein expression of Bcl–2, Bax, and cleaved caspase–3 in liver tissues. **(C)** Immunohistochemistry was used to detect Bcl–2 and Bax levels in liver tissues (original magnification, ×200). ConA, concanavalin A; MP, methylprednisolone; NC, normal control; EAH, experimental autoimmune hepatitis; IOD, integrated optical density. **P < 0.01 vs. NC group; #P < 0.05 vs. ConA group (20 mg/kg); ##P < 0.01 vs. ConA group (20 mg/kg).

ConA Treatment Aggravates Apoptosis and Autophagy Dysfunction in LO2 Cells

The animal experimental results suggested that the mechanism underlying MP treatment needed to be further explored *in vitro*. LO2 cells treated with ConA for 12 h were stained with Annexin V-FITC/PI, and the levels of apoptosis were determined. As shown in **Figure 4A**, the mean apoptosis rates (from three independent experiments) were 4.0% (0 μg/ml ConA group), 15.4% (5 μg/ml ConA group), 16.9% (10 μg/ml ConA group) and 26.5% (20 μg/ml ConA group). LO2 cell apoptosis significantly increased as the dose of ConA increased. Furthermore, the apoptosis-related proteins Bax, Bcl-2, and cleaved caspase-3 in LO2 cells were examined *via* western blot, as shown in **Figure 4B**. The expression of Bcl-2, an antiapoptotic marker, was dramatically reduced, while that of Bax and cleaved caspase-3 was increased.

To investigate whether ConA disrupts ΔΨm, the mitochondria-specific and voltage-dependent dye Rh123 was used to monitor alterations in ΔΨm in LO2 cells. As displayed in **Figure 4C**, ΔΨm in the three ConA-treated groups was vitally different from that in the NC group. Previous studies demonstrated that ROS levels

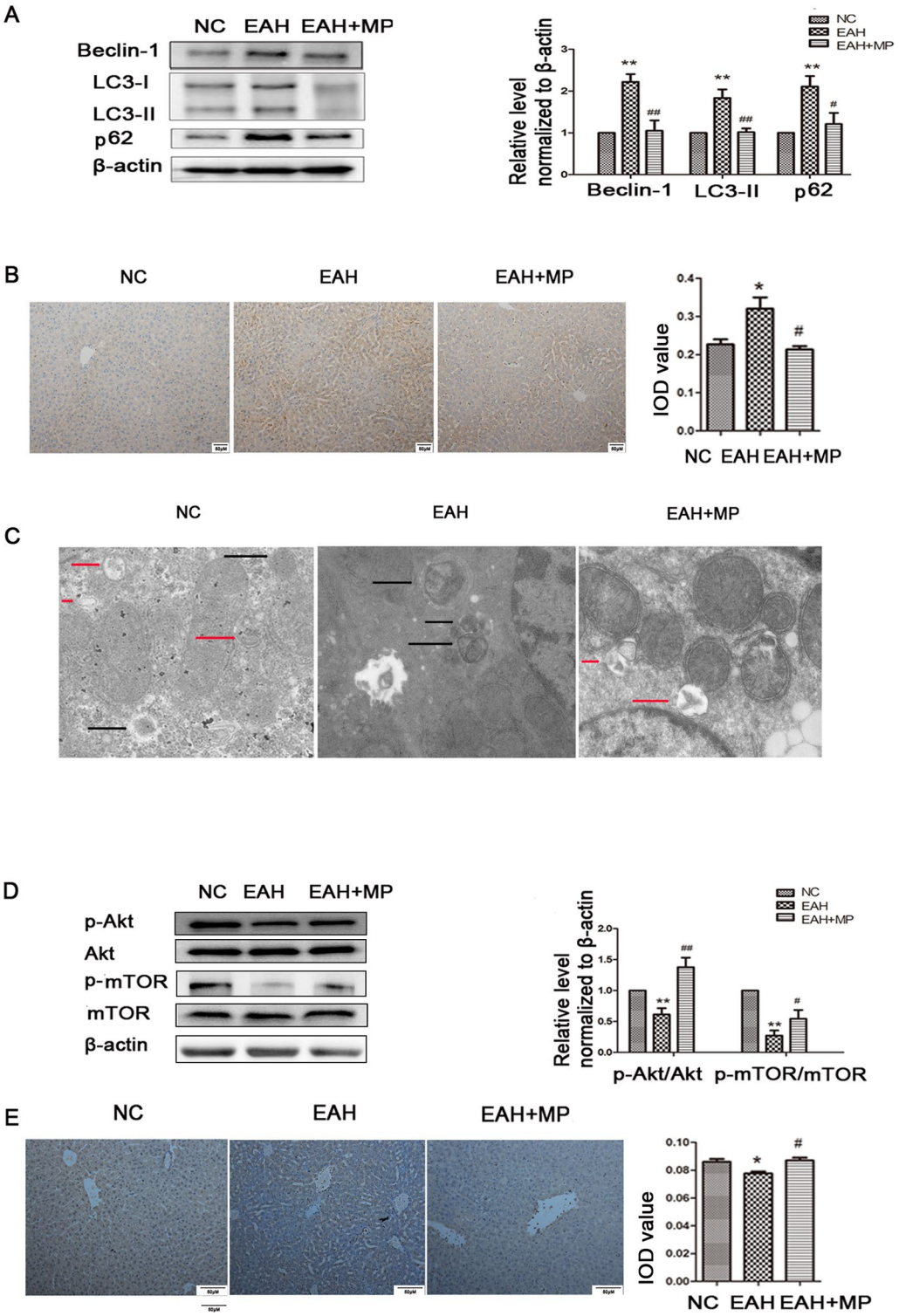

FIGURE 3 | (A) Protein expression of beclin–1, LC3 and p62. **(B)** Immunohistochemistry was used to detect LC3 levels in liver tissues (original magnification, ×200). **(C)** Transmission electron microscopy examination of autophagosomes and autolysosomes the hepatocytes of hepatic tissues. Black arrows denote autophagosomes and red arrows denote autolysosomes (original magnification, ×7k). **(D)** Protein expression of Akt, p-Akt and p-mTOR, mTOR. **(E)** Immunohistochemistry was used to detect p–Akt levels in liver tissues (original magnification, ×200). In western blot analyses, β–actin was used as the internal control; the relative protein expression level in the treated group was calculated after setting the control group expression (0 μg/ml) value to 1.00. Data are presented as the mean ± SD. *P < 0.05 vs. NC group; **P < 0.01 vs. NC group; #P < 0.05 vs. EAH group; ##P < 0.01 vs. EAH group. ConA, concanavalin A; MP, methylprednisolone; NC, normal control; EAH, experimental autoimmune hepatitis; IOD, integrated optical density.

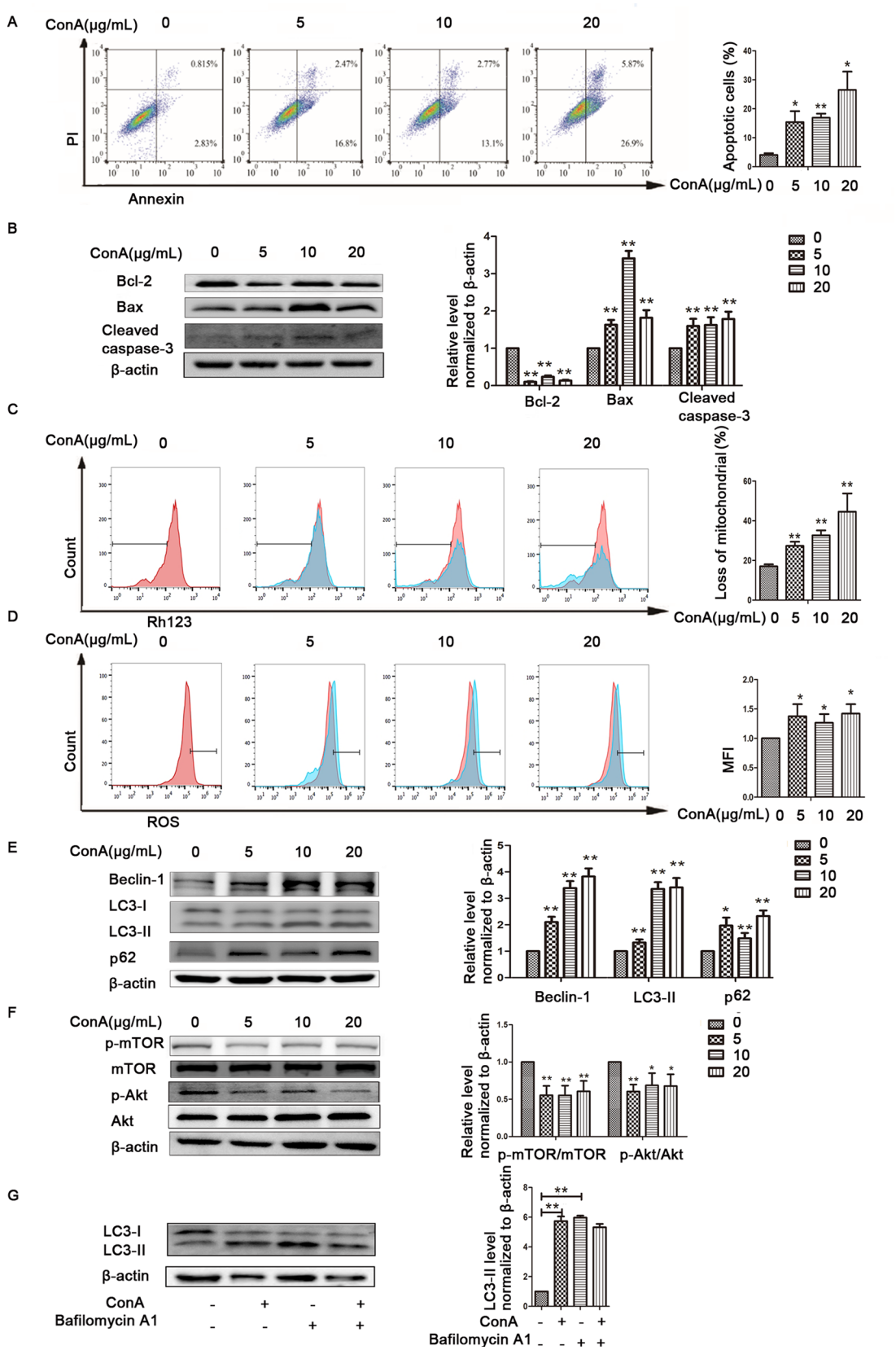

FIGURE 4 | Continued

FIGURE 4 | ConA treatment aggravated mitochondria-mediated apoptosis and autophagy dysfunction in LO2 cells. **(A)** The Annexin V and PI dual-labeling technique was used to analyze the effect of ConA treatment on LO2 cell apoptosis. **(B)** The protein expression of Bcl–2, Bax, and cleaved caspase–3 was measured by western blot. **(C)** LO2 cells were treated with various concentrations of ConA for 12 h and then incubated with 10 μM Rh123. The red branches represent the control. **(D)** LO2 cells treated with various concentrations of ConA for 12 h were incubated with 10 mM DCFH-DA for 30 min, and ROS levels were then analyzed by flow cytometry. The red branches represent the control; the relative MFI level in the treated group was calculated after setting the control group expression (0 μg/ml) value to 1.00. **(E)** The protein expression of beclin–1, LC3 and p62 was measured by western blot. **(F)** The protein expression of Akt, p-Akt and p-mTOR, mTOR was measured by western blot. In western blot analyses, β–actin was used as the internal control; the relative protein expression level in the treated group was calculated after setting the control group expression (0 μg/ml) value to 1.00. **(G)** Autophagic flux was calculated by dividing the levels of LC3-II in the presence of bafilomycin A1 by that without bafilomycin A1.*$P < 0.05$ vs. control group; **$P < 0.01$ vs. control group. ConA, concanavalin A; MFI, mean fluorescence intensity; DCFH-DA, dichlorodihydrofluorescein diacetate.

are substantially affected by the inhibition of mitochondrial function (Li et al., 2016b; Yang et al., 2018). We further evaluated the ROS levels in LO2 cells after ConA treatment by DCFH-DA staining. As shown in **Figure 4D**, the ROS levels in LO2 cells treated with ConA were observably increased compared to those in untreated cells.

The effects of ConA on the expression of LC3-II, p62, and Beclin-1 in LO2 cells were detected by western blot. After treatment with 0-20 μg/ml ConA for 12 h, the protein levels of the autophagy markers, including LC3-II, Beclin-1, and p62, were upregulated (**Figure 4E**).

Additionally, the expression levels of p-Akt/Akt and p-mTOR/mTOR were significantly decreased in LO2 cells treated with ConA compared to those in untreated cells (**Figure 4F**). Hence, 10 μg/ml ConA was used for subsequent *in vitro* experiments.

Bafilomycin-A, a specific inhibitor of vacuolar-type H+ ATPase, which inhibits late stages of autophagy by preventing autophagosome-lysosome fusion was used to monitor the effect of ConA on autophagy flux (**Figure 4G**) (**Supplementary Figure 2**). The LC3-II levels were not further augmented in the presence of bafilomycin-A1 treatment indicating that the upregulation of LC3-II was not caused by activation of autophagy pathway, but due to possible impairment of autophagy flux.

The Protective Effects of MP Treatment on Mitochondria-Mediated Apoptosis and Autophagy in LO2 Cells

The *in vitro* results were the same as those observed in liver tissues described above. First, hepatocyte viability following treatment with MP was determined by the MTT assay. MP caused a dose-dependent decrease in LO2 cell viability (**Figure 5A**). Thus, 10 μM MP was chosen for subsequent *in vitro* experiments. The variation in apoptosis was evaluated by Hoechst 33258 staining. After LO2 cells were treated with ConA with or without MP for 12 h, several apoptotic bodies were observed in the ConA group, whereas MP treatment decreased apoptosis (**Figure 5B**). The FCM and western blot results indicated that ConA treatment significantly induced mitochondria-mediated apoptosis; however, pretreatment with MP significantly reduced apoptosis (**Figures 5C, D**). The results of $\Delta\Psi$m transformation and the ROS level also verified that MP pretreatment may improve mitochondria-mediated apoptosis in LO2 cells (**Figures 6A–C**).

The expression of LC3-II, p62, and Beclin-1 was detected by western blot. After pretreatment with 10 μM MP, the expression of autophagy markers in LO2 cells was decreased (**Figure 7A**). Next we used MDC, an acidotropic dye which tends to accumulate in late stage autophagosome like vacuoles to monitor the effect of MP on autophagy (Klionsky et al., 2016). As shown in **Figure 7B**, significantly more MDC-labeled vacuoles accumulated in the cytoplasm of cells in the ConA group than in the control group, while MP pretreatment decreased the accumulation of MDC-labeled vacuoles. To study the regulatory effect of MP treatment on the Akt/mTOR pathway, we examined the levels of phosphorylated Akt1 and mTOR in LO2 cells following MP treatment. MP treatment increased the phosphorylation of AKT1 and mTOR (**Figure 7C**). These *in vitro* data verify that apoptosis and autophagy in hepatocytes are redulated by MP treatment partly through the Akt/mTOR signaling pathway.

DISCUSSION

AIH is currently viewed as an important component of noninfectious liver disease and it has attracted much attention in recent years, although the etiology and pathogenesis of AIH are still uncertain. Aberrant autoimmunity is thought to induce an interaction between genetic susceptibility and unknown environmental triggers to cause persistent hepatocyte damage. Precision medicine for AIH, including diagnosis, medicinal therapy, and drug monitoring, is a novel therapeutic pattern distinct from evidence-based medicine that aims to develop a personalized treatment strategy (Bossen et al., 2018; Chen et al., 2019; Fan et al., 2019). However, ~20% of AIH patients exhibit a poor response to standard treatment with glucocorticoids alone or in combination with azathioprine, and the number of effective second-line therapeutic options for AIH is limited (2015; Cropley and Weltman, 2017; Shen et al., 2018). Thus, exploring the mechanisms underlying the effects of known drugs on therapeutic targets is necessary for research and drug development in AIH.

Glucocorticoids are suggested to protect several types of cells, including human fibroblasts and auditory hair cells, from apoptosis (Haake et al., 2009; Nieuwenhuis et al., 2010). Zhao et al. revealed that dexamethasone (DEX) protected LO2 cell hepatocytes from apoptosis induced by a tumor necrosis factor-related ligand that induces apoptosis by upregulating the expression of P-glycoproteins. In addition, several studies have also found an association between the pharmacological mechanism of DEX with up- or downregulated autophagy and apoptosis in other disease models (He et al., 2016; Wang et al., 2018b). In view of these observations, the present study investigated whether MP

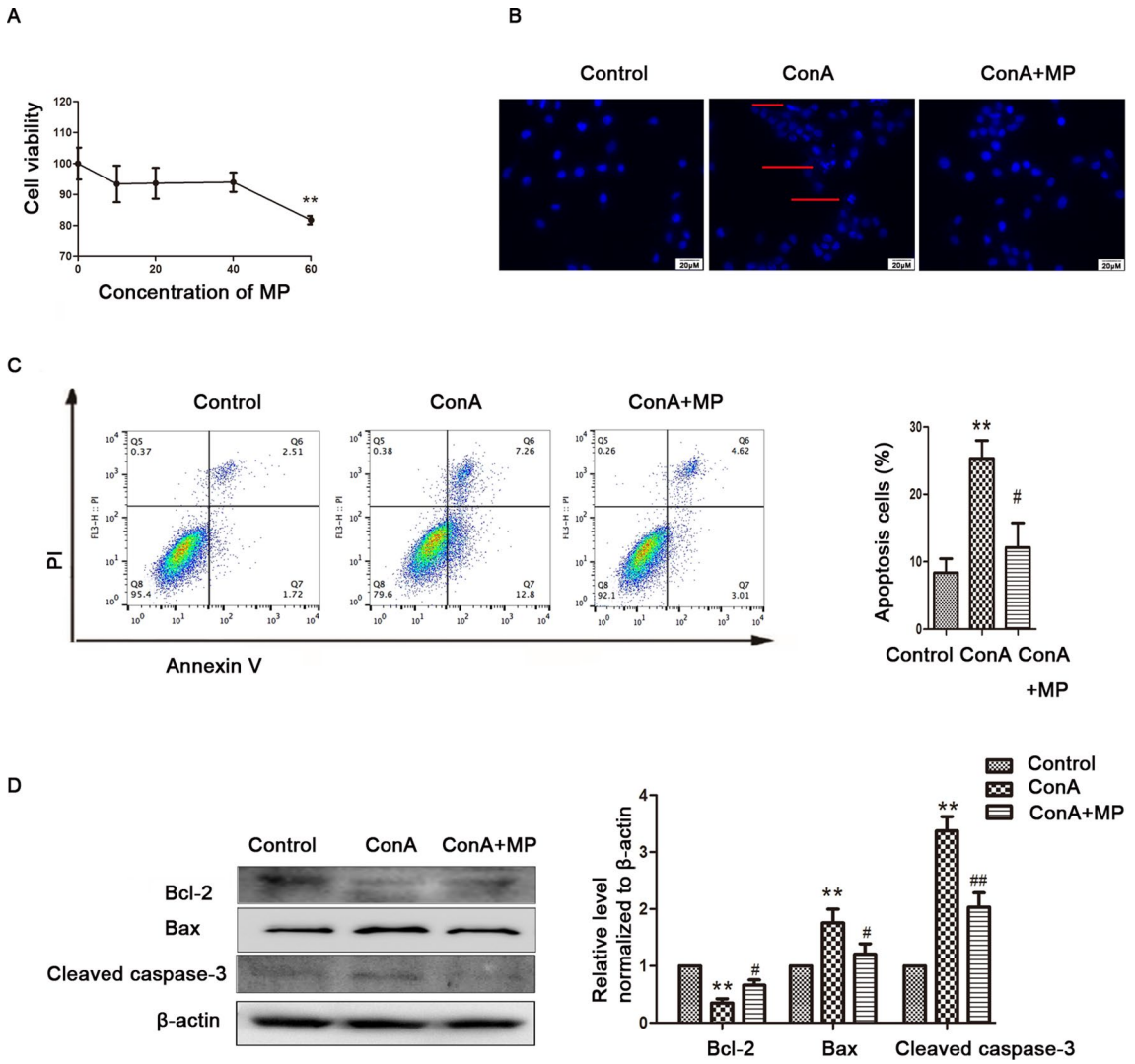

FIGURE 5 | The protective effects of MP treatment on mitochondria-mediated apoptosis in LO2 cells. **(A)** LO2 cells were incubated with the indicated concentrations (10-60 μM) of MP for 12 h. Cell viability was determined by the MTT assay. **(B)** The appearance of apoptotic bodies in the fluorescence microscopy Hoechst 33258 staining assay of LO2 cells. Red arrows denote apoptotic bodies (original magnification, ×400). **(C)** The Annexin V and PI dual-labeling technique was used to analyze the effect of MP treatment on LO2 cell apoptosis. **(D)** The protein expression of Bcl–2, Bax, and cleaved caspase–3 was measured by western blot. β–actin was used as the internal control. The relative protein expression level in the treated group was calculated after setting the control group expression value to 1.00. *P < 0.05 vs. control group; **P < 0.01 vs. control group; #P < 0.05 vs. ConA group; ##P < 0.01 vs. ConA group. ConA, concanavalin A; MP, methylprednisolone. **P<0.01 vs. normal control group; #P < 0.05, ##P < 0.01 vs. EAH group

protects hepatocytes from mitochondria-mediated apoptosis and autophagy dysfunction in EAH mouse livers. ConA injection in mice caused increased cytokines and evident hepatic injuries, which manifested as increased serum levels of transaminases, cytokines, and histopathological changes in the liver. MP treatment alleviated liver damage in mice by increasing the serum levels of ALT and AST. Furthermore, MP treatment inhibited mitochondria-mediated apoptosis and ameliorated autophagy dysfunction in ConA-induced hepatocyte injury at least in part through the Akt/mTOR signaling pathway. The results from experiments in LO2 cells verified these *in vivo* results.

Apoptosis is the predominant mechanism of hepatocyte death in AIH (Czaja, 2014). Interventions that directly modulate apoptosis in AIH have not been studied in the clinic but have been shown to promote malignancy in animals. A number of potential drugs were shown to have protective effects in an AIH model partly through the inhibition of hepatocyte apoptosis (El-Agamy et al., 2018; Feng et al., 2018a; Kim et al., 2018; Yu et al., 2019). Most studies on AIH have found that the antiapoptotic effects of these drugs depend on cytokines secreted by inflammatory cells, including activated T cells and macrophages. In the present study, MP functioned as an antiapoptotic agent that prevented apoptosis in the livers of EAH model mice through a mitochondria-mediated pathway. ConA is a powerful stimulus that triggers an *in vitro* immune response (Taniguchi et al., 1989); Leist *et al.* found that ConA is directly toxic to cultured hepatocytes due to

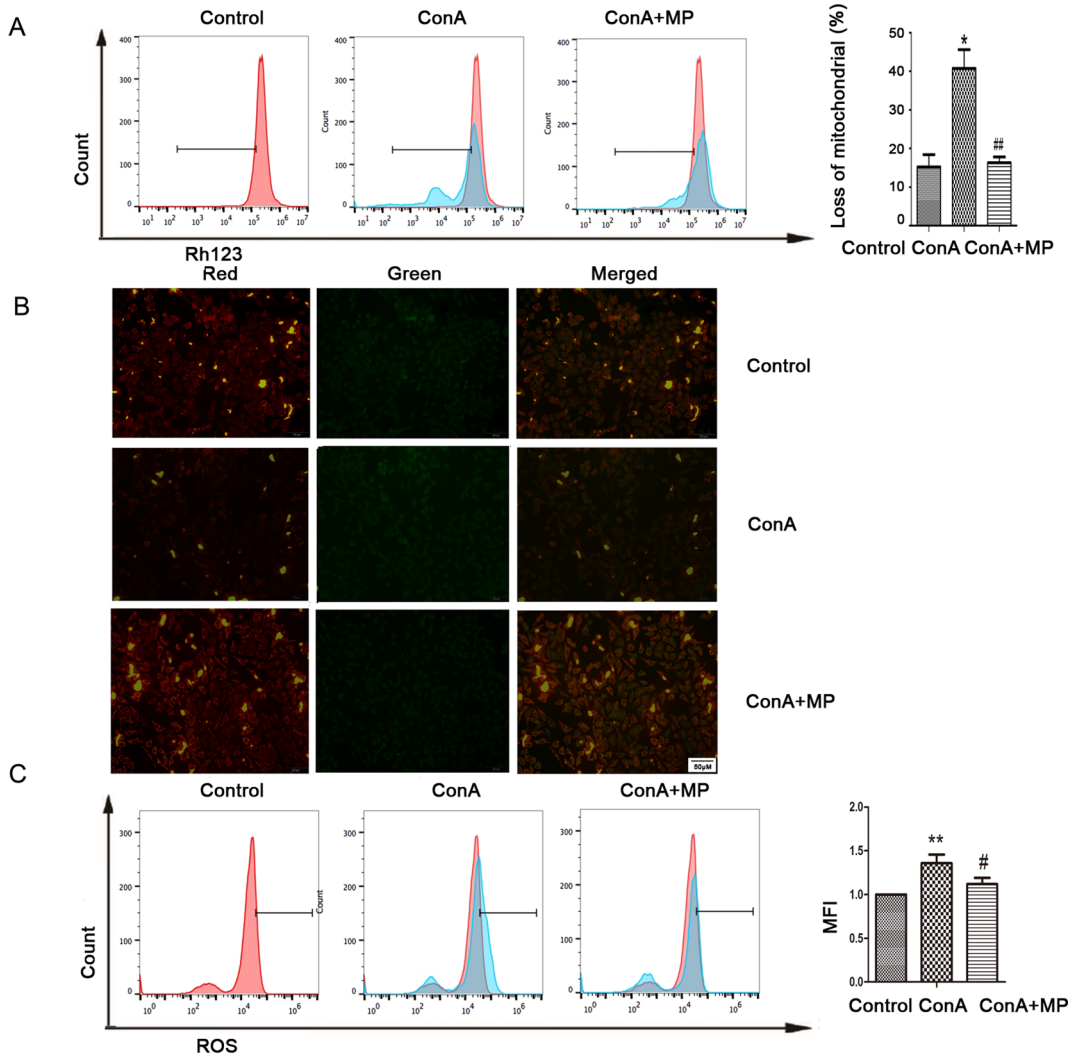

FIGURE 6 | Variation in the Ψm and ROS level. **(A)** LO2 cells were treated with ConA with or without MP for 12 h. The red branches represent the control. Data are shown as the mean ± SD, *P < 0.05 vs. control group; # P < 0.05 vs. ConA group; **(B)** The Δψm (red/green) alteration in LO2 cells was determined by fluorescence microscopy analysis after staining with JC-1. **(C)** LO2 cells treated with ConA with or without MP for 12 h were incubated with 10 mM DCFH-DA for 30 min, and the ROS level was then analyzed by flow cytometry. The control groups are presented as lines filled with red color; the relative MFI level in the treated group was calculated after setting the control group expression (0 μg/ml) value to 1.00. Data are shown as the mean ± SD, *P < 0.05 vs. control group; **P < 0.01 vs. control group; #P < 0.05 vs. ConA group; ##P < 0.01 vs. ConA group. ConA, concanavalin A; MP, methylprednisolone; Ψm, mitochondrial membrane potential; ROS, reactive oxygen species; MFI, mean fluorescence intensity.

the excessive activation of membrane receptors and subsequent disturbance of the cytoskeleton, which is associated with a strong affinity for the hepatocyte vascular membrane (Leist and Wendel, 1996). Hence, we used direct ConA treatment to mimic the inflammation microenvironment. Our *in vitro* experiments provide unequivocal evidence for the direct protective role of MP in ConA-induced hepatocyte injury.

Autophagy, a lysosomal degradative pathway that is often used to eliminate damaged or unnecessary organelles and intracellular microbial pathogens, can be simulated by various factors, including starvation, growth factor deprivation, and hypoxia. Although autophagy plays a significant role in the pathogenesis of liver disease, its influence on the pathogenesis of EAH remains controversial (Rautou et al., 2010; Puri and Chandra, 2014). Previous studies have found that increased autophagosome formation may be generated by activation of the autophagy process, blockage of autophagy flux caused by the inefficient fusion of autophagosomes and lysosomes, or lysosomal dysfunction (Rothermel and Hill, 2007; Singh et al., 2009). In the present study, the *in vivo* data were obtained using several widely used and complementary approaches to detect autophagy at the tissue level; electron microscopy was to identify autophagic vesicles at the cellular level, and autophagy-related protein expression was evaluated by immunoblotting and immunohistochemistry. An increased number of autophagic vesicles was observed in the hepatocytes of ConA-induced EAH mice, while the expression of p62, a protein degraded by autophagy, was also increased. To provide

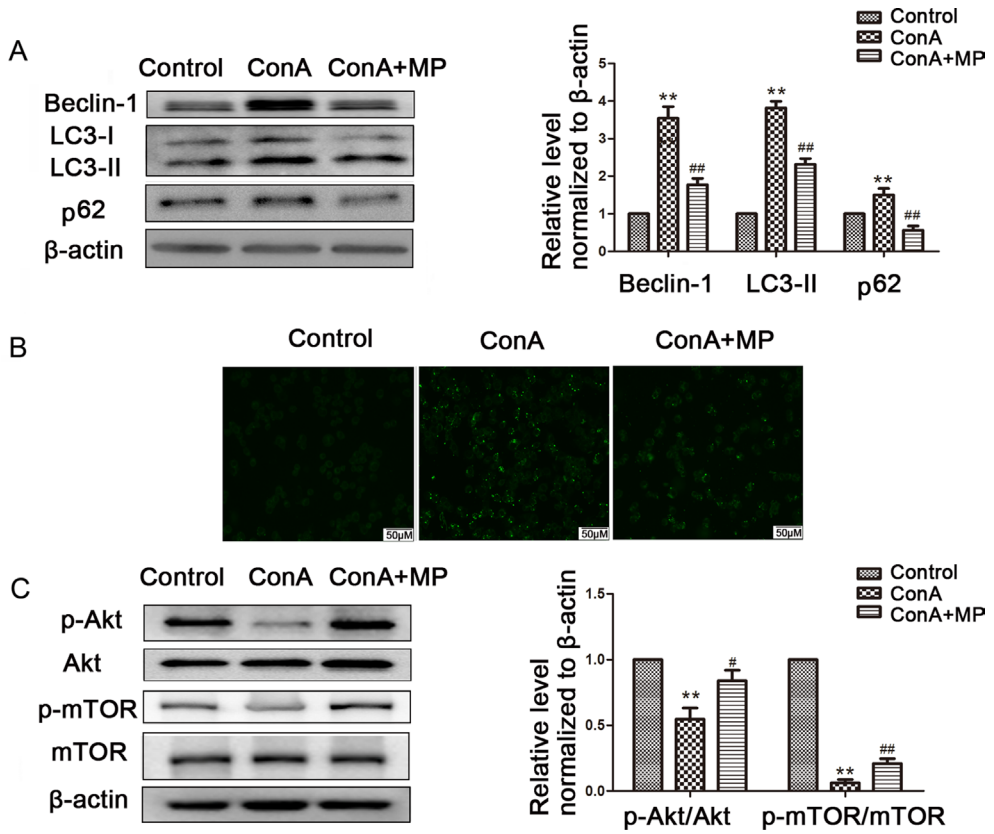

FIGURE 7 | The protective effects of MP treatment on autophagy function in LO2 cells. **(A)** The protein expression of beclin–1, LC3 and p62 was measured by western blot. **(B)** Following each treatment, acidic vacuoles in LO2 cells were stained with MDC and observed under a fluorescence microscope. **(C)** The protein expression of Akt, p-Akt and p-mTOR, mTOR was measured by western blot. β–actin was used as the internal control. The relative protein expression level in the treated group was calculated after setting the control group expression value to 1.00. Data are presented as the mean ± SD. **P < 0.01 vs. control group; #P < 0.05 vs. ConA group; ##P < 0.01 vs. ConA group. ConA, concanavalin A; MP, methylprednisolone; MDC, monodansylcadaverine.

an overview of the autophagic process, bafilomycin A1, commonly used *in vitro* to verify the patency of autophagy flux, was applied in the subsequent cell experiments. Bafilomycin A1 treatment did not augment the protein expression of LC3-II, indicating that the increased LC3-II expression was not caused by autophagy activation, but blockage of autophagy flux caused by the inefficient fusion of autophagosomes and lysosomes, or lysosomal dysfunction. This finding was in line with several recent studies using immortalized human hepatocytes or liver tissues from patients with liver disease, which implied autophagic protein degradation dysfunction and possible inefficient fusion between autophagosomes and lysosomes (Sir et al., 2008; Rautou et al., 2010; Rautou et al., 2011; Liu et al., 2014). The role of mTOR in the regulation of autophagic flux remains complicated, and the overall effect of mTOR on autophagic flux may largely depend on autophagic flux itself (Zhou et al., 2013). We found significantly increased levels of p-AKT and p-mTOR following MP treatment *in vivo* and *in vitro*. These results indicate that the amelioration of autophagy dysfunction by MP involves recovery of the Akt/mTOR signaling pathway; that is, activation of mTOR is correlated with the improvement of autophagic flux. Our data are in line with previous studies, which revealed that the activation of mTOR can accelerate the elimination of autophagic vacuoles by enhancing the function of the intracellular autophagy pathway in cells (Bains et al., 2009; Hu et al., 2015).

It should be noted that the present study had several limitations. First, the mechanisms involved in immune-induced liver injury are complex, and ConA-induced hepatocyte injury could partially mimic *in vivo* mechanisms rather than human AIH (Hardtke-Wolenski and Jaeckel, 2010; Ye et al., 2018; Christen, 2019). A few previous studies used ConA stimulation in hepatocytes or cell lines to explore the detailed mechanisms of immune-mediated liver injury (Chen et al., 2014; Li et al., 2016a; Ko et al., 2018; Yang et al., 2019), and ConA stimulation has also been applied to study monocytes and macrophages in immune reactions (Chanput et al., 2010; Zhuang et al., 2016; Nakamoto et al., 2017; Shi et al., 2019). Thus, this study aimed to evaluate the direct molecular mechanism of MP treatment on ConA-induced hepatocyte injury, and we believe that the *in vitro* data are relevant to the *in vivo* data. Further research is required to elucidate the exact mechanism of ConA–induced liver injury. Second, we did not explore more detailed mechanism of impaired autophagy flux during the process. Third, the present study was performed using mice and cellular experiments, and additional studies on human AIH are needed to verify the protective effects in the future.

In conclusion, both *in vivo* and *in vitro* experimental data suggest that MP treatment ameliorates mitochondria-mediated apoptosis and autophagy dysfunction, which is associated with the Akt/mTOR pathway. The current study suggests the clinical and rational use of MP to treat AIH. However, the detailed molecular mechanisms of the effect of MP on hepatocytes in AIH are not completely clear. These results provide insights into the mechanisms underlying the effect of MP on hepatocytes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Treatment Committee of Sichuan University.

AUTHOR CONTRIBUTIONS

XF and RM wrote the manuscript. LY and XL designed the research. TY, XF, RM, and HW performed the research and analyzed the data. TY, MS, and TW participated in the research design. XF performed the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01189/full#supplementary-material

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