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Basic Study

Angiotensin-converting enzyme 2 improves liver fibrosis in mice by regulating autophagy of hepatic stellate cells

ACE2 improves liver fibrosis through autophagy

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Abstract

BACKGROUND

Liver fibrosis is the common pathological process associated with the occurrence and development of various chronic liver diseases. At present, there is still a lack of effective prevention and treatment methods in clinical practice. Hepatic stellate cell (HSC) plays a key role in liver fibrogenesis. In recent years, the study of liver fibrosis targeting HSC autophagy has become a hot spot in this research field. Angiotensin-converting enzyme 2 (ACE2) is a key negative regulator of renin-angiotensin system (RAS), and its specific molecular mechanism on autophagy and liver fibrosis needs to be further explored.

AIM

To investigate the effect of ACE2 on hepatic fibrosis in mice by regulating HSC autophagy through the AMPK/mTOR pathway.

METHODS

Overexpression of ACE2 in a mouse liver fibrosis model was induced by injection of liver-specific recombinant adeno-associated virus ACE2 vector (rAAV2/8-ACE2). The degree of liver fibrosis was assessed by histopathological staining and the biomarkers in mouse serum were measured by Luminex multifactor analysis. The number of apoptotic HSCs was assessed by TUNEL and immunofluorescence staining. Transmission electron microscopy was used to identify the changes in the number of HSC autophagosomes. The effect of ACE2 overexpression on autophagy-related proteins was evaluated by multicolor immunofluorescence staining. The expression of autophagy-related indicators and AMPK pathway-related proteins was measured by western blotting.

RESULTS

A mouse model of liver fibrosis was successfully established after 8 wk of intraperitoneal injection of carbon tetrachloride (CCl_4). rAAV2/8-ACE2 administration reduced collagen deposition and alleviated the degree of liver fibrosis in mice. The serum levels of platelet-derived growth factor (PDGF-BB), angiopoietin-2, vascular endothelial growth factor (VEGF) and angiotensin II (Ang II) were decreased, while the levels of IL-10 and angiotensin-(1-7) were increased in the rAAV2/8-ACE2 group. In addition, the expression of alpha-smooth muscle actin (α-SMA), fibronectin (FN), and CD31 was down-regulated in the rAAV2/8-ACE2 group. TUNEL and immunofluorescence staining showed that rAAV2/8-ACE2 injection increased HSC apoptosis. Moreover, rAAV2/8-ACE2 injection notably decreased the number of autophagosomes and the expression of autophagy-related proteins (LC3I, LC3II, Beclin-1) , and affected the expression of AMPK pathway-related proteins (AMPK, p-AMPK, p-mTOR).

CONCLUSION

ACE2 overexpression can inhibit HSC activation and promote cell apoptosis by regulating HSC autophagy through the AMPK/mTOR pathway, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling.

Key Words: Angiotensin-converting enzyme 2; Hepatic stellate cells; Autophagy; Liver fibrosis; Portal hypertension; Mice

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Core Tip: Liver fibrosis and cirrhosis are the common outcomes of most chronic liver diseases, and there is a lack of effective treatment at present. ACE2, as the main target receptor for the novel coronavirus pneumonia (COVID-19) virus invasion into the human body, is one of the research hotspots. The involvement of autophagy in the activation mechanism of HSC during liver fibrosis has attracted increasing attention. Our study found that ACE2 can inhibit the activation and proliferation of HSCs by regulating autophagy, and promote apoptosis of HSCs, providing new ideas for the treatment of liver fibrosis and hepatic sinusoidal remodeling.

INTRODUCTION

Liver fibrosis and cirrhosis are the common pathological processes associated with the occurrence and development of various chronic liver diseases, and there is still a lack of effective prevention and treatment methods for these conditions. In liver fibrosis and cirrhosis, excessive deposition of extracellular matrix (ECM) in the liver and regenerating nodules compress blood vessels, resulting in structural changes. In addition, hepatic sinusoidal vasoconstriction and vascular remodeling cause functional changes that ultimately lead to increased intrahepatic vascular resistance and portal pressure[1]. Hepatic stellate cells (HSCs) are located in the perisinusoidal Disse space

between liver sinusoidal endothelial cells (LSECs) and hepatocytes[2]. The vasomotion of hepatic sinusoids greatly affects intrahepatic blood flow and portal venous resistance, and HSCs and LSECs play key roles in increasing intrahepatic vascular resistance and portal venous pressure. Hepatic sinusoidal vascular remodeling occurs in hepatic fibrosis and is characterized by capillarization of the hepatic sinusoids and surrounded by more contractile HSCs[3]. HSC activation is a complex and coordinated process. After activation, HSCs begin to proliferate and release excess collagen, proteoglycan and other ECM components, which in turn cause changes in the intrahepatic structure; furthermore, HSCs acquire contractility, reducing the diameter of the hepatic sinusoids and increasing resistance, leading to liver fibrosis and portal hypertension[4-6].

Autophagy is a metabolic process in which eukaryotic cells eliminate disposable or potentially dangerous cytoplasmic material. It plays a critical role in cell development, differentiation, and homeostasis. In this process, some damaged proteins or organelles are wrapped by autophagic vesicles with a double membrane structure and sent to lysosomes (animals) or vacuoles (yeast and plants) for degradation and recycling[7]. Autophagy, as a cellular housekeeper, can eliminate defective proteins and organelles, clear intracellular pathogens, and prevent the accumulation of abnormal proteins. Therefore, autophagy plays an active role in the pathology of many diseases. Growing evidence suggests that an adequate autophagic response in hepatocytes and nonparenchymal cells (HSCs, LSECs, Kupffer cells) is critical for the physiological function of the liver[8]. During hepatic fibrogenesis, the study of the mechanism of autophagy involved in HSC activation has attracted increasing attention. Autophagy increases the degradation of lipid droplets in HSCs, providing energy for HSC activation[9,10]. A study showed that after reducing HSC autophagy in mice, HSC activation was inhibited, and the degree of liver fibrosis was alleviated[11]. In recent years, the study of liver fibrosis targeting HSC autophagy has become a hot spot in this research field.

The renin-angiotensin system (RAS) is an important endocrine system that regulates vascular tone and water and electrolyte metabolism in the body. Our previous studies have confirmed that HSCs have local RAS, and activated HSCs increase the synthesis of angiotensin II (Ang II) in liver cirrhosis[12]. Under the action of angiotensin-converting enzyme 2 (ACE2), Ang II is converted to Ang-(1-7), which stimulates the Mas receptor (MasR) to cause vasodilation. ACE2 is a key negative regulator of RAS, and studies have shown that it can inhibit liver fibrosis by degrading Ang II[13,14], but its specific molecular mechanism needs to be further explored. We confirmed that carvedilol could inhibit Ang II-induced HSC proliferation and contraction and improve pathological hepatic sinusoidal remodeling in mice[12,15]. The study also indicated that HSCs are the main cells expressing ACE2 in the liver. In addition, our study demonstrated that carvedilol could notably reduce HSC autophagy and inhibit HSC activation and proliferation[16]. It has been reported that ACE2 alleviates the severity of acute lung injury by inhibiting autophagy[17]. Therefore, we hypothesized that ACE2 could inhibit HSC activation and proliferation by regulating autophagy, thus improving hepatic sinusoidal remodeling and ultimately alleviating liver fibrosis and portal hypertension.

5 The AMPK/mTOR signaling pathway is not only an important node in the intracellular energy metabolism monitoring system but also an important upstream pathway regulating autophagy. Studies have reported that ACE2 can improve vascular endothelial dysfunction in type 2 diabetic rats with insulin resistance by regulating the AMPK/mTOR pathway[18]. In addition, ACE2 was shown to effectively modulate the AMPK/mTOR signaling pathway in a mouse model of acute lung injury[17]. Our previous study confirmed that metformin could inhibit HSC proliferation, migration and angiogenesis through the Akt/mTOR and mTOR/HIF-1α pathways[19]. In this study, we evaluated the effect of ACE2 on liver fibrosis in mice and demonstrated the molecular mechanism by which ACE2 regulates HSC autophagy through the AMPK/mTOR pathway to improve liver fibrosis and hepatic sinusoidal remodeling.

The aim of this study was to determine the effect of ACE2 on HSC activation, proliferation, apoptosis and liver fibrosis by regulating autophagy. This study will provide a new direction for the prevention and targeted treatment of liver fibrosis and portal hypertension.

MATERIALS AND METHODS

Mouse model of liver fibrosis

Forty adult male C57BL/6J mice (6-8 wk, 18-20 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China). The mice were housed in an air-conditioned room at a defined temperature (23-25 ℃) for one week prior to the initiation of the experiments. All experimental protocols were approved by the Animal Care Committee of the Second Hospital, Cheeloo College of Medicine, Shandong University.

The liver fibrosis mouse model was established by intraperitoneal injection of carbon tetrachloride (CCl_4, 20%, 0.5 mL/100 g) twice a week for 8 wk. To evaluate the effect of ACE2 on liver fibrosis, the liver-specific recombinant adeno-associated viral vector rAAV-ACE2 (rAAV2/8-ACE2) was injected into the tail vein 4 wk after CCl_4 administration. Mice were randomly assigned to four groups (10 in each): Group 1, normal control (olive oil); Group 2, CCl_4-induced liver fibrosis (CCl_4); Group 3, rAAV2/8-ACE2+CCl_4; and Group 4, rAAV2/8-ACE2+CCl_4+rapamycin (mTOR inhibitor). Rapamycin (2 mg/kg) was administered at the 6th week after the intraperitoneal injection of CCl_4.

The mice were dissected after anesthesia administration, and liver tissues were removed and partially stored at -80 ℃ Another section was fixed in 4% paraformaldehyde and embedded in paraffin.

Cytokine ELISA and Luminex analysis

Mouse blood samples were centrifuged at 4 ℃ (3000 rpm) for 10 minutes, and the supernatant was collected. According to the manufacturer's instructions, the serum

levels of platelet-derived growth factor BB (PDGF-BB), angiopoietin-2, vascular endothelial growth factor (VEGF), IL-10, Ang II and Ang-(1-7) were measured using Luminex multifactor assay kits and ELISA kits. The data were analyzed using Graph Pad Prism 8.0.

Histopathological evaluation

The paraffin-embedded liver tissue sections were morphologically evaluated based on hematoxylin and eosin (H&E) staining. The degree of liver fibrosis in mice was measured by Masson trichrome and Sirius red staining. According to the METAVIR scale, the degree of liver fibrosis was divided into four stages from 0 to 4 (0 - No fibrosis; 1 - Portal fibrosis; 2 - Periportal fibrosis; 3 - Bridging fibrosis; 4 - Cirrhosis). The quantity of collagen production in each group after Sirius red staining was analyzed using Image-Pro Plus 6.0 software.

Immunohistochemical staining

Liver tissue sections were deparaffinized, serially dehydrated in ethanol, and then incubated overnight with primary antibody at 4 °Cafter antigen retrieval. The primary antibodies used in the experiment included anti-alpha-smooth muscle actin (α-SMA) antibody (1:400, Abcam, USA), anti-fibronectin (FN) antibody (1:2000, Abcam, USA), and anti-CD31 antibody (1:2000, Abcam, USA). After incubation with the appropriate biotinylated secondary antibody for 30 minutes, the liver sections were stained with diaminobenzidine (DAB) and hematoxylin. The positive staining areas appeared brownish yellow. The sections were observed under a light microscope, photographed, and then analyzed with Image-Pro Plus 6.0 software.

Transmission electron microscopy (TEM)

Fresh liver tissue sections were immobilized in electron microscopy fixative (Servicebio, Wuhan, China) for 2 h. The specimens were then immobilized in osmic acid buffer and dehydrated in ethanol. Finally, the ultrathin sections were photographed using TEM

(HT7800/HT7700, Hitachi, Tokyo, Japan) after staining with 2% uranium acetate in alcohol solution. The structure of autophagosomes in each group was observed by TEM.

Apoptosis detection by TUNEL and immunofluorescence staining

Apoptotic HSCs were localized with labeled nucleotides in TUNEL staining. The mouse liver sections were stained according to the in situ cell death detection kit (Roche, Germany) protocol. The sections were then incubated with an α-SMA primary antibody (1:500, Abcam, USA) and a CY3 goat anti-rabbit fluorescence secondary antibody (1:300, Servicebio, Wuhan, China). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and photographed under a fluorescence microscope. The relative number of apoptotic cells in each group was analyzed using Image-Pro Plus 6.0 software.

Multicolor immunofluorescence staining

Paraffin sections of mouse liver tissue were deparaffinized, subjected to antigen retrieval, and blocked with hydrogen peroxide and serum. The primary antibody, corresponding HRP-labeled secondary antibody, and fluorescently labeled tyramine were successively added. After microwave repair treatment, the first round of primary and secondary antibodies were eluted, and the fluorescently labeled tyramine was still attached to the target. When the second and third targets were detected, the previous steps were repeated for a new round of labeling and microwave repair processing. The fourth primary antibody and 594-labeled fluorescent secondary antibody were added, and the nuclei were then counterstained with DAPI. The slides were covered with anti-fade mounting medium. Finally, images were detected and collected with a slice scanner (pannoramic, 3Dhistech, Hungary). DAPI emits blue light; Fluorescein isothiocyanate (FITC-ACE2) emits green light; 647 (Desmin) is set to pink light; 594 (LC3) is set to purplish red light. The number of positive cells for each index was analyzed using Image-Pro Plus 6.0 software.

Western blot analysis

Mouse liver tissue proteins were extracted, and the concentration of each protein was determined. Equal amounts of protein samples were subjected to electrophoresis on 8-12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% nonfat dry milk for 1 h to block nonspecific sites and then incubated with the appropriate primary antibodies at 4 °Covernight. After incubation with the secondary antibody and membrane washing, the antibody-bound proteins were detected by chemiluminescence staining using an enhanced chemiluminescence assay kit (Millipore, USA). The density of each band was analyzed with ImageJ software.

Statistical analysis

The data are expressed as mean±S.D. Statistics were analyzed using GraphPad Prism 8.0 and SPSS19.0 software. Statistical significance was determined by one-way ANOVA followed by LSD-t test. For all experiments, $P<0.05$ was considered statistically significant.

RESULTS

Effect of ACE2 on CCl_4-induced liver injury and fibrogenesis

The effect of ACE2 on CCl_4-induced liver fibrosis was evaluated by H&E (Figure 1A), Masson trichrome (Figure 1B) and Sirius red staining (Figure 1C and 1D). Compared with those in the control group, inflammatory cell infiltration and fibrous tissue hyperplasia in the liver tissues of mice were increased after 8 wk of CCl_4 injection (METAVIR > F2) (Figure 1A). In the CCl_4 group, the liver architecture was widely disorganized, and the hepatic sinusoids could not be distinguished. In addition, notable collagen deposition and the formation of fibrous septa bridging the portal regions were observed in the CCl_4 group. However, fibrotic tissue and inflammatory cells in the rAAV2/8-ACE2+CCl_4 group were markedly reduced compared with those

in the CCl_4 and rapamycin groups (METAVIR ≤ F2) (Figure 1B, 1C and 1D). Masson trichrome and Sirius red staining showed that collagen deposition was significantly increased in mice treated with CCl_4 alone ($P < 0.05$). After rAAV2/8-ACE2 treatment, the degree of collagen deposition in the perisinusoidal spaces, interlobular septum and periportal zones was reduced ($P < 0.05$) (Figure 1B, 1C and 1D). The results indicated that rAAV2/8-ACE2 treatment could improve liver injury and fibrosis in mice, while rapamycin treatment increased the degree of liver fibrosis compared with that in the ACE2 overexpression group. This finding suggested that mTOR inhibitor could attenuate the antifibrotic effect of rAAV2/8-ACE2.

Analysis of serum biomarkers in mice with liver fibrosis

The levels of PDGF-BB, VEGF, angiopoietin-2, IL-10, Ang II and Ang-(1-7) in mouse serum were measured (Figure 2A-F). PDGF signaling plays a vital role in HSC activation and angiogenesis[20]. VEGF and angiopoietin-2 are the most important regulators in the process of angiogenesis[21,22]. As a potential anti-inflammatory factor, IL-10 has been reported to inhibit the expression of many proinflammatory mediators[23]. The results showed that the levels of PDGF-BB, angiopoietin-2, VEGF, and Ang II in the CCl_4 group were notably higher than those in the normal control group ($P < 0.001$), and rAAV2/8-ACE2 injection reduced the expression of these cytokines ($P < 0.001$) (Figure 2A, 2B, 2C and 2E). In addition, the results demonstrated that the levels of IL-10 and Ang-(1-7) were higher in the rAAV2/8-ACE2 group than in the CCl_4 group ($P < 0.001$, $P < 0.001$), while rapamycin decreased the expression levels of these two cytokines ($P < 0.01$, $P < 0.05$) (Figure 2D and 2F). The results indicated that rAAV2/8-ACE2 treatment could inhibit HSC activation and angiogenesis in mice with liver fibrosis.

Effect of ACE2 on HSC activation and apoptosis

α-SMA is a typical marker of HSC activation and proliferation. In the immunohistochemistry staining, α-SMA positive cells were distributed along the

endothelium of hepatic sinusoids in the liver tissues of CCl_4-induced fibrotic mice. The number of α-SMA positive cells in the rAAV2/8-ACE2 treatment group was notably lower than that in the CCl_4 and rapamycin groups ($P < 0.05$, $P < 0.01$) (Figure 3A).

Fibronectin (FN) is the primary protein constituting the basement membrane, and CD31 is commonly used as a vascular endothelial marker. These proteins are rarely expressed in normal liver tissues. Immunohistochemical staining revealed that the expression of these proteins was increased in the CCl_4-induced liver fibrosis group ($P < 0.001$, $P < 0.001$) and decreased in the rAAV2/8-ACE2 treatment group ($P < 0.001$, $P < 0.001$). However, rapamycin increased the protein expression of fibronectin and CD31 ($P < 0.01$, $P < 0.01$) (Figure 3B and 3C).

TUNEL and immunofluorescence staining were used to detect the number of apoptotic HSCs. Our results demonstrated that there were more apoptotic HSCs in the rAAV2/8-ACE2 treatment group than in the CCl_4 and rapamycin groups ($P < 0.05$, $P < 0.05$) (Figure 4).

The results showed that ACE2 overexpression inhibited HSC activation and induced HSC apoptosis in fibrotic mouse liver tissues, while the mTOR inhibitor attenuated the effect of rAAV2/8-ACE2 on HSCs.

Effect of ACE2 on HSC autophagy in mice

To further verify the effect of ACE2 on autophagy in liver fibrosis, a large number of autophagosomes were detected by ultrastructural analysis in the HSCs of mice in the CCl_4 group. TEM analysis showed that rAAV2/8-ACE2 injection decreased the number of autophagosomes in HSCs compared with that in the CCl_4 group ($P < 0.05$). However, autophagosomes were increased in the rAAV2/8-ACE2+CCl_4+rapamycin group ($P < 0.01$) (Figure 5A and 5B). The results of multicolor immunofluorescence staining demonstrated that the expression of the ACE2 protein was increased after rAAV2/8-ACE2 injection, and the expression of the autophagy protein LC3 was decreased compared with that in the CCl_4 group ($P < 0.01$). Treatment with rapamycin attenuated the inhibitory effect of ACE2 on LC3 protein expression ($P <$

0.05) (Figure 5C and 5D). These results suggested that ACE2 overexpression could reduce HSC activation and liver fibrosis by inhibiting HSC autophagy.

Effect of ACE2 on HSC autophagy and AMPK pathway proteins in mouse liver tissues

Autophagy is regulated by numerous autophagy-related genes (ATGs), such as LC3 and Beclin-1. LC3II is a marker protein on the autophagosome membrane and is often considered an indicator of autophagy formation. As an autophagy-specific substrate, p62 interacts with LC3 to infiltrate into autophagosomes and is efficiently degraded by autophagolysosomes[24]. To determine the effect of ACE2 on HSC autophagy, we detected the expression of HSC autophagy-related indicators (LC3I, LC3II, Beclin-1) in the liver tissues of mice in each group by western blotting. Moreover, we verified the correlation of ACE2 with autophagy and the AMPK pathway by assessing the expression of AMPK pathway-related proteins (AMPK, p-AMPK, p-mTOR) in mouse liver tissues. Compared with that in the control group, the p-AMPK/AMPK ratio was higher in the CCl_4 group ($P < 0.01$). However, the ratio of p-AMPK/AMPK in the rAAV2/8-ACE2-treated group was dramatically lower than that in the CCl_4 group ($P < 0.05$) (Figure 6A and 6C). In contrast, p-mTOR levels in mice in the rAAV2/8-ACE2-treated group were significantly higher than those in the CCl_4 group ($P < 0.01$) (Figure 6A and 6D). In addition, the results indicated that the protein levels of Beclin-1 and LC3II in the rAAV2/8-ACE2+CCl_4 group were markedly reduced compared to those in the CCl_4 alone group ($P < 0.001$, $P < 0.05$) (Figure 6B, 6E and 6F). The m-TOR inhibitor (rapamycin) affected mTOR phosphorylation and the level of autophagy proteins in liver tissues. The present study showed that rapamycin abolished the effect of rAAV2/8-ACE2 on the expression of the autophagy proteins LC3I, LC3II and Beclin-1. Compared with those in the rAAV2/8-ACE2 group, the relative Beclin-1 and LC3II levels were increased by rapamycin treatment ($P < 0.05$, $P < 0.05$) (Figure 6B, 6E and 6F). The western blot results

showed that ACE2 overexpression could inhibit the expression of autophagy-related proteins in mouse liver tissues through the AMPK/mTOR pathway.

DISCUSSION

Liver fibrosis has high morbidity and mortality worldwide, and it is a compensatory response to liver inflammation and injury caused by multiple pathogenic factors[25]. In liver fibrosis, excess fibrous extracellular matrix (ECM) proteins, such as collagens I and III, are deposited in the Disse space of the hepatic sinusoids[26]. Changes in ECM composition induce LSECs to lose their fenestrae and form a basement membrane, a process known as hepatic sinusoidal capillarization[27]. The activation of HSCs plays a crucial role in the process of liver fibrosis. Upon activation due to liver injury, quiescent HSCs lose their retinoid droplets, exhibit increased α-SMA expression, and release large amounts of ECM, ultimately resulting in liver fibrosis[28].

ACE2 is expressed in human alveolar epithelial cells, esophageal epithelial cells, small intestinal epithelial cells, and vascular endothelial cells[29]. Our present study found that ACE2 was also expressed in liver HSCs. In recent years, the COVID-19 virus (2019 nCoV, SARS CoV-2) that caused the outbreak has been proven to invade human alveolar epithelial cells mainly through ACE2[30]. SARS-CoV-2 infection can reduce ACE2 activity, leading to an imbalance in Ang II/ACE2 regulation[31]. ACE2, which is the main target receptor for SARS-CoV-2 invasion into the human body, is currently a research hotspot. A global registry study suggested that patients with chronic liver disease and cirrhosis had higher mortality after being infected with COVID-19[32]. The baseline liver disease severity of patients with chronic liver disease and cirrhosis is closely related to the COVID-19-related incidence rates and mortality. Therefore, SARS-CoV-2 infection may exacerbate the degree of cirrhosis and portal hypertension in patients with chronic liver disease by reducing the activity of ACE2 in the liver.

ACE2 is an endogenous negative regulator that acts as a RAS "brake" to limit fibrogenesis through Ang II degradation and Ang-(1-7) formation. It was reported that

the degree of liver fibrosis in ACE2 knockout mice increased after 21 days of bile duct ligation or chronic CCl_4 treatment[13]. In addition to its effect on the RAS, whether ACE2 can affect liver fibrosis through other mechanisms remains unclear. In our study, a liver fibrosis model was induced by the intraperitoneal injection of CCl_4 to investigate the effect of ACE2 on liver fibrosis by inhibiting autophagy. In addition, the liver-specific recombinant adeno-associated viral vector rAAV2/8-ACE2 was used in this study. ACE2 is specifically overexpressed in the liver with minimal systemic effects. Moreover, enhanced expression and activity of liver tissue-specific ACE2 can reduce local Ang II levels, increase local Ang- (1-7) levels, and minimize off-target effects[33].

Autophagy is the process of degrading defective proteins, damaged organelles, excess lipids and other harmful components in cells to maintain cellular components and homeostasis[34]. Autophagy levels are elevated in conditions of inflammation and oxidative stress, and excessive autophagy is involved in inflammatory and liver diseases[25]. Studies have demonstrated that inhibiting autophagy in HSCs reduces lipid droplet degradation, thereby preventing cell activation[9]. Autophagosome is composed of a small portion of the cytoplasm surrounded by double membranes. The digested substances are various components contained in the cytoplasm, such as mitochondria and fragments of endoplasmic reticulum, and the contents are degraded by fusion with lysosomes. Autophagy generally refers to macroautophagy, which includes two consecutive stages of autophagosome formation and degradation[16]. It has been reported that IL-10 inhibits oxidative stress-induced HSC autophagosome formation, HSC activation and liver fibrosis through the mTOR/STAT3 signaling pathway[35]. The TEM results indicated that the number of autophagosomes in the rAAV2/8-ACE2-treated group was decreased. To explore the relationship between ACE2 and autophagy, we detected the expression of the autophagy proteins LC3I, LC3II and Beclin-1 in the liver tissues of mice in each group. The results indicated that ACE2 overexpression effectively inhibited autophagy during mouse liver fibrosis.

Autophagy regulation is intricately associated with signaling pathways such as the AMPK/mTOR pathway. AMPK can inhibit mTORC1 activity by activating the TSC1/TSC2 protein heterodimer[36,37]. mTORC1 negatively regulates the initiation of autophagy through phosphorylation at Ser757 of ULK1 upon activation[37]. Compared with that in the CCl_4 group, the p-AMPK/AMPK ratio was decreased ($P < 0.05$), while the relative expression of p-mTOR was increased in the rAAV2/8-ACE2 group ($P < 0.01$). The results showed that ACE2 overexpression could influence the AMPK/mTOR signaling pathway. We treated mice with an m-TOR inhibitor (rapamycin), which effectively inhibited m-TOR phosphorylation. The findings of the study indicated that ACE2 overexpression could inhibit HSC autophagy in mouse liver tissues through the AMPK/mTOR pathway. The results suggested that the AMPK/mTOR signaling pathway was an important node for ACE2 to regulate HSC autophagy.

Pathological staining showed the successful establishment of a mouse model of liver fibrosis after 8 wk of intraperitoneal injection of CCl_4. rAAV2/8-ACE2 injection alleviated collagen deposition and fibrosis in the liver tissues of mice. We further investigated the mechanism by which ACE2 overexpression alleviated liver fibrosis. When liver injury occurs, HSCs are activated and proliferate, and the demand for intracellular energy increases. At this time, blocking autophagy can impair HSC activation and fibrotic activity[10]. α-SMA is an important indicator for evaluating HSC activation and proliferation. In the present study, rAAV2/8-ACE2 injection inhibited α-SMA expression and HSC activation. In addition, apoptosis plays a vital role in the proliferation, differentiation and death of HSCs, and HSC apoptosis is the key to reversing liver fibrosis[38]. TUNEL and immunofluorescence staining showed that rAAV2/8-ACE2 injection increased HSC apoptosis. Our previous study demonstrated a complex relationship between autophagy and apoptosis, and the inhibition of autophagy could induce HSC apoptosis[16]. Therefore, our findings indicated that ACE2 overexpression could alleviate liver fibrosis by regulating autophagy to inhibit HSC activation and promote apoptosis.

Intrahepatic angiogenesis and sinusoidal remodeling play an important role in the development of hepatic fibrosis and portal hypertension[39]. The inhibition of pathological angiogenesis can alleviate liver fibrosis. LSEC capillarization is associated with the accumulation of interstitial collagen in the Disse space of hepatic sinusoids and is the main pathological change in liver fibrosis[27]. The reversal of LSEC capillarization has been reported to promote HSC quiescence[40]. During cirrhosis, angiogenesis-related cytokines and receptors expressed in HSCs, such as VEGF, PDGF, and angiopoietin, can induce HSC migration, angiogenesis, and collagen production[41]. Our study demonstrated that the levels of VEGF, angiopoietin-2 and PDGF-BB were elevated in liver fibrosis, resulting in increased angiogenesis. rAAV2/8-ACE2 injection inhibited the expression levels of these angiogenesis-related factors. Therefore, the results indicated that ACE2 overexpression could effectively attenuate intrahepatic angiogenesis, thus alleviating hepatic sinusoidal resistance.

In the present study, adeno-associated viral vector technology, pathological staining, multifactor analysis, multicolor immunofluorescence staining, transmission electron microscopy and other advanced techniques were used to comprehensively explore the relationship and mechanism among ACE2, autophagy and liver fibrosis. However, there are still some limitations of this study. Whether ACE2 affects HSC autophagy and liver fibrosis through other pathways needs to be further explored. This study provides a new theoretical basis for the targeted treatment of liver fibrosis and portal hypertension, and its clinical application needs further research.

CONCLUSION

In summary, the study indicates that autophagy plays a crucial role in HSC activation and liver fibrosis. ACE2 overexpression can inhibit HSC activation and promote apoptosis by regulating HSC autophagy, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling. Our study also demonstrates that the AMPK/mTOR pathway is involved in the effect of ACE2 on autophagy. This study may provide new ideas for

exploring the molecular mechanism by which ACE2 inhibits liver fibrosis and hepatic sinusoidal remodeling.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis is a hallmark of end-stage chronic liver disease, which leads to millions of deaths each year. At present, the treatment options for liver fibrosis and cirrhosis are limited and often ineffective. ACE2-driven protective RAS provides an effective therapeutic target for liver fibrosis. In addition, the study of liver fibrosis targeting HSC autophagy has attracted more and more attention.

Research motivation

In addition to its effect on the RAS, whether ACE2 can affect liver fibrosis through other mechanisms remains unclear. Moreover, how to enhance the expression and activity of tissue-specific ACE2 to avoid its potential off-target effect is a problem to be solved. Using a suitable and efficient gene delivery system to achieve tissue-specific overexpression of ACE2 has pointed out a new direction for the targeted treatment of liver fibrosis.

Research objectives

The aim of this study is to determine the effect of ACE2 on HSC activation, proliferation, apoptosis and liver fibrosis by regulating autophagy. This study provides new ideas for exploring the molecular mechanism by which ACE2 inhibits liver fibrosis and hepatic sinusoidal remodeling.

Research methods

In this study, a mouse model of liver fibrosis was constructed, and adeno-associated viral vector technology, pathological staining, multifactor analysis, multicolor immunofluorescence staining, transmission electron microscopy, TUNEL apoptosis

assays, western blot analysis and other experimental methods were used to comprehensively explore the relationship and mechanism among ACE2, autophagy and liver fibrosis.

Research results

In vivo experiments showed that rAAV2/8-ACE2 treatment could inhibit HSC activation and angiogenesis, induce HSC apoptosis, and alleviate HSC proliferation and liver fibrosis by inhibiting HSC autophagy. This study also demonstrated that ACE2 overexpression could inhibit HSC autophagy in mouse liver tissues through the AMPK/mTOR pathway. The completion of this study provides new ideas for the prevention and targeted treatment of liver fibrosis and portal hypertension.

Research conclusions

The study demonstrates that autophagy plays a crucial role in HSC activation and liver fibrosis. ACE2 overexpression can inhibit HSC activation and promote apoptosis by regulating HSC autophagy through the AMPK/mTOR pathway, thereby alleviating liver fibrosis and hepatic sinusoidal remodeling.

Research perspectives

The pathogenesis of liver fibrosis and cirrhosis is a complex process involving the interaction of various growth factors, cytokines, and vasoactive substances. We need further clinical research to improve patient treatment outcomes through advanced technologies such as drug carrier-targeted HSC-specific therapies.

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