Aldosterone induces hepatic stellate cells activation and liver fibrosis through Cav-1 dependent pathway

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Abstract

Hepatic stellate cell (HSC) activation is a pivotal event in liver fibrosis. Aldosterone (Aldo) is upregulated in liver fibrosis, but its role in HSC activation remains unclear. Caveolin-1 (Cav-1) is a key structural protein of caveolae that facilitates the actions of Aldo in various cellular processes. However, the expression and function of Cav-1 in HSCs during HSC activation and liver fibrosis are controversial. Our findings demonstrate that the intracellular expression of Aldo and Cav-1 is upregulated during HSC activation. Administration of spironolactone or knockdown of Cav-1 in HSCs resulted in ameliorated liver fibrosis induced by Aldo infusion or bile duct ligation (BDL) in rats. Mechanistically, Aldo recruited proteins such as mineralocorticoid receptor (MR), nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), and NADPH oxidase 4 (NOX4) into HSC caveolae via Cav-1, thereby elevating intracellular levels of interleukin-1β (IL-1β) and reactive oxygen species (ROS), consequently enhancing HSC contraction and migration abilities. Our study reveals a novel mechanism through which Aldo regulates HSC activation and promotes liver fibrosis via the Cav-1 dependent pathway, highlighting the therapeutic potential of blocking the Aldo receptor or specifically targeting intracellular Cav-1 to ameliorate cholestatic liver fibrosis.

1. Introduction

Liver fibrosis is a pathological condition characterized by the excessive accumulation of fibrous scar tissue in the liver, resulting from the abnormal deposition of extracellular matrix(1). Several diseases are associated with liver fibrosis, including viral hepatitis (HBV/HCV), alcoholic or non-alcoholic fatty liver disease, primary (and secondary) biliary cholangitis, primary sclerosing cholangitis, and biliary atresia(2). These diseases can be classified into two categories based on their etiology: hepatotoxic injury and cholestatic injury. Chronic injurious factors stimulate the proliferation of myofibroblasts within the liver, leading to an increase in intrahepatic pressure and subsequent collagen deposition(3). The cell fate mapping and deep phenotypic analysis have revealed that activated HSCs serve as the primary source of myofibroblast(4). Therefore, the targeted intervention of activated HSCs is a promising antifibrotic strategy in the process of liver fibrosis(5).

The renin-angiotensin-aldosterone system (RAAS) is well known for its crucial role in the physiological regulation of blood pressure, blood volume, and sodium homeostasis(6). In addition to the systemic RAAS, extensive studies have revealed the presence of an active tissue-level RAAS that work locally in various organs(7). The pro-inflammatory and pro-fibrotic effects of Angiotensin II have been extensively studied(8–11). As for Aldo, a downstream component of the RAAS, has been reported to be elevated in patients with cirrhosis(12). Our previous works have demonstrated the contributory role of Aldo in liver fibrosis(13) and its ability to induce the activation of HSCs(14). However, the therapeutic potential of targeting Aldo to ameliorate liver fibrosis has been largely underestimated. Aldo's involvement in liver fibrosis is primarily attributable to an incomplete understanding of the underlying regulatory mechanisms.
Caveolin-1 (Cav-1) is a structural protein component of caveolae, which are invaginations of the plasma membrane involved in various cellular processes(15). Caveolae are found to varying degrees in most differentiated cell types(16), however, organ-specific variations in Cav-1 expression persist(17). Studies have demonstrated that the liver is among the tissues and organs with the lowest relative level of expression of Cav-1 protein, while Cav-1 levels are reported to be significantly different in various hepatic cell types. In the liver, Cav-1 is mainly distributed in non-parenchymal cells such as HSCs and liver sinusoidal endothelial cells(18). Interestingly, the role of Cav-1 in regulating hepatic fibrosis and HSC activation is the subject of significant disputes in current research. Some studies suggest mutual inhibition between Cav-1 and the TGF-β signaling pathway(19) and therefore propose that Cav-1 has inhibitory functions in HSC activation(20). Conversely, Mariana et al. propose that Cav-1 may facilitate HSC activation and demonstrate that upregulation of Cav-1 levels specifically in HSCs alone is sufficient to induce their activation(21). Even in animal models, there are two distinctly different views on the evaluation of Cav-1 expression levels in liver fibrosis(20, 22). Therefore, although the mechanisms by which Cav-1 regulates HSC activation remain incompletely understood, it is evident that Cav-1 plays an important role in HSC activation and the formation of liver fibrosis.

Currently, there is limited research on the association between Cav-1 and HSC activation, and the role of Cav-1 in regulating HSC function remains controversial. However, it has been reported that Aldo is closely involved in the pathological and physiological regulation of Cav-1 in various organs such as the heart, lungs, and kidneys(23–25). In this study, we found that Aldo can promote HSC activation and liver fibrosis in Cav-1 related pathway, and specific knockdown of Cav-1 in HSCs with AAV8 alleviated Aldo- or BDL-induced liver fibrosis in rats. Our study aims to investigate the regulatory mechanisms of Aldo on HSC activation and liver fibrosis through the Cav-1-dependent pathway.

2. Result

Upregulation of Cav-1 Protein Expression in HSCs of Liver Fibrosis Patients

Liver tissue from patients undergoing hepatic lobectomy for intrahepatic bile duct stones was categorized as the fibrosis group, while surgical specimens from patients with hepatic hemangioma served as the non-fibrosis group. Pathological staining and hydroxyproline detection indicated a significant increase in collagen area content in the fibrosis group compared to the non-fibrosis group (Fig. 1A, B). Immunohistochemistry staining and Western blot analysis revealed an upregulation of Cav-1 expression in fibrotic liver tissue (Fig. 1A, D). Immunohistochemistry staining indicated that the upregulated Cav-1 in fibrotic liver tissue was predominantly distributed in the portal vein area and around thickened bile ducts. Therefore, immunofluorescent co-staining of Cav-1 and α-SMA was performed, and the results demonstrated upregulation of Cav-1 and α-SMA expression in fibrotic liver tissue with co-localization (Fig. 1C). These findings suggest that the upregulation of Cav-1 primarily occurs in activated HSCs within the fibrotic liver tissues.

Upregulation of Cav-1 in primary rat HSCs and promotes its activation
Primary HSCs were isolated and cultured from rats (Fig. 2A). Immunofluorescent staining revealed a significant increase in α-SMA and Cav-1 protein expression in primary HSCs on day 14 of in vitro culture compared to day 3 (Fig. 2B), suggesting spontaneous activation of HSCs in vitro accompanied by upregulation of Cav-1 protein expression. Transmission electron microscopy showed the presence of characteristic small vesicular structures in activated HSCs (Fig. 2C). Knocking down Cav-1 expression in primary rat HSCs resulted in a significant decrease in the extent of cell contraction and migration abilities on day 14 (Fig. 2D-E). The expression of α-SMA and collagen I was also downregulated (Fig. 2F), indicating that Cav-1 knockout can inhibit the spontaneous activation of HSCs in vitro. These results suggested that intracellular upregulation of Cav-1 expression might be involved in the regulation of HSC activation.

**Specific knockdown of Cav-1 in HSCs can inhibit BDL-induced liver fibrosis in rats**

To determine the in vivo role of Cav-1 in HSC activation, an HSC-specific promoter-adeno-associated virus (AAV) targeting Cav-1 was generated and delivered to rat via the portal vein (Fig. 3A). Liver fibrosis was assessed in each group of rats after 4 weeks of BDL, and results showed that liver fibrosis was significantly attenuated in the BDL group with Cav-1 knockdown compared to the BDL group and the BDL group injected with the negative control virus (Fig. 3A-E). Immunohistochemistry staining demonstrated a significant reduction in the number of α-SMA positive cells in the liver of the BDL + Cav-1 KD group compared to the BDL and BDL + NC groups, indicating a decrease in activated HSCs (Fig. 3A). These findings indicated that Cav-1 facilitates HSC activation in rats with BDL-induced liver fibrosis.

**Cav-1 recruits signaling proteins in caveolae to mediating Aldo-induced HSC activation**

We extracted caveolae from primary rat HSCs and performed Western blot analysis, which revealed the enrichment of MR in caveolae of activated HSCs (Fig. 4A). Subsequently, we evaluated the expression and secretion of Aldo and MR ligands in primary rat HSCs. The results showed that with prolonged in vitro culture, the expression of Aldo synthase increased in HSCs, accompanied by elevated levels of intracellular and supernatant Aldo (Fig. 4B-D). Meanwhile, exogenous Aldo induced HSC activation, enhancing the extent of cell contraction, migration ability, and collagen synthesis (Fig. 4E-H). To investigate the regulatory mechanism of Aldo on HSCs, we further examined the proteins c-Src, NLRP3, and NOX4 in caveolae. The results showed that Aldo induced the enrichment of these proteins in caveolae of HSCs (Fig. 4I). Immunofluorescent co-staining and immunoprecipitation confirmed the direct interaction between Cav-1 and MR, c-Src, NLRP3, and NOX4 induced by Aldo (Fig. 4J, K; Figure S1A-I). After treatment with exogenous Aldo, the co-localization and interaction of NLRP3 inflammasome components increased in HSCs (Figure S1J), suggesting that Aldo may induce assembly of the NLRP3 inflammasome. Interestingly, we failed to detect ASC or Caspase-1 proteins in the caveolae (Fig. 4L), which may indicate that after the assembly, the NLRP3 inflammasome dissociates from the caveolae and enters the cytoplasm. To determine the binding site between Cav-1 and NLRP3, we constructed three subunits of NLRP3 and confirmed that the NACHT domain interacts with Cav-1 upon Aldo stimulation (Fig. 4M). These results suggested that intracellular Aldo expression was upregulated during HSC
activation and is involved in HSC activation. Furthermore, the upregulated Aldo induced the enrichment of MR, c-Src, NLRP3, NOX4, and other proteins in caveolae by interacting with Cav-1, which may be closely related to HSC activation (Fig. 4N).

Aldo-induced HSC activation depends on Caveolae

We disrupted and reconstituted caveolae by depleting cholesterol using methyl-β-cyclodextrin (MCD) in HSCs (Figure S5A). When caveolae were disrupted or c-Src was knocked down, the effects of Aldo on promoting HSC contraction and migration were suppressed (Fig. 5A, B). Western blot analysis demonstrated that the expression and phosphorylation of Cav-1 protein in HSCs were upregulated with prolonged Aldo stimulation (Fig. 5C). Interestingly, although disruption of caveolae inhibited the phosphorylation of Cav-1 and c-Src proteins induced by Aldo, it had no effect on the total protein expression of c-Src (Fig. 5D, E). Moreover, caveolae disruption suppressed the expression of α-SMA and collagen I induced by Aldo in HSCs (Fig. 5F). Similarly, the MR blocker eplerenone (EPL) inhibited the phosphorylation of Cav-1 and c-Src proteins induced by Aldo but did not significantly affect c-Src expression (Fig. 5G, H). The c-Src inhibitor PP2 inhibited the phosphorylation of Cav-1 protein induced by Aldo, suggesting that the phosphorylation of c-Src may mediate the regulation of Cav-1 function by Aldo (Fig. 5I, J). Blocking the phosphorylation of Cav-1 or c-Src proteins inhibited Aldo-mediated HSC activation (Fig. 5K-M). In summary, these results suggested that the phosphorylation of Cav-1 and c-Src proteins induced by Aldo may be involved in regulating the recruitment and signal transduction of relevant proteins in caveolae, resulting in the HSCs activation.

Aldo recruits NLRP3 through Caveolae to induce HSC inflammation and activation

Figure 4I indicated that Aldo could induce the enrichment of NLRP3 in HSC caveolae, while our previous work also showed that downregulation of NLRP3 expression and interventions such as the application of the NLRP3 inhibitor VX765 or neutralization with IL-1β antibodies could inhibit Aldo's promoting effect on rat HSC contraction and migration ability (Figure S2 B&C). Therefore, we hypothesized that caveolae might mediate the effect of Aldo on promoting inflammation and cellular activation in HSCs through the NLRP3-related signaling pathway. To test this hypothesis, we used antagonists such as EPL, MCD, and PP2 to intervene on primary rat HSCs treated with Aldo, and the results showed that the expression of NLRP3 inflammasome components induced by Aldo was inhibited (Figure S2 D). Furthermore, downregulation of NLRP3 protein, application of NLRP3 inhibitor, or neutralization with IL-1β antibodies could inhibit the expression of mlc, moe, and Smad3 proteins induced by Aldo (Figure S2 E&F). These findings suggested that Aldo can upregulate NLRP3 expression in rat HSCs, induced IL-1β production, and promoted cellular activation, and these effects of Aldo are closely associated with caveolae and proteins such as Cav-1 and c-Src.

Aldo recruits NOX4 through caveolae to induce increased ROS generation and cellular activation in HSCs

Figure 4I further demonstrated that Aldo could recruit NOX4 protein within caveolae. Western blot analysis showed that Aldo upregulated the expression of NOX4 protein in HSCs, and this effect of Aldo
could be inhibited by EPLE treatment, downregulation of Cav-1, or disruption of Caveolae (Fig. 6a). Furthermore, downregulation of NOX4 or the use of the inhibitor Vas2870 attenuated the effect of Aldo on HSC contraction and migration ability. These results suggest that Aldo may induce HSC activation by regulating NOX4. Since ROS occupies a central position in HSC inflammation and cellular activation, we detected ROS in HSCs and the result showed that Aldo increased intracellular ROS and H$_2$O$_2$ levels which was blocked by Aldo inhibition, disruption of caveolar structure, or administration of NOX4 inhibitor (Fig. 6D, E). Moreover, downregulation of NOX4 or the use of the NOX4 inhibitor Vas2870 inhibited Aldo-induced ROS and H$_2$O$_2$ generation in HSCs, as well as the expression of α-SMA and type I collagen proteins (Fig. 6F-H). These findings suggest that Aldo may recruit NOX4 through caveolae, elevating intracellular oxidative stress levels in HSCs, thereby inducing cellular activation. The results of immunoprecipitation did not show the interaction between NLRP3 and NOX4 proteins (Figure S3G, H), however, it was observed that downregulation of NLRP3 inhibited Aldo-induced expression of NOX4 protein in HSCs (Figure S3A). Similarly, inhibition of NLRP3 function and neutralization of IL-1β also reduced the level of NOX4 protein in HSCs (Figure S3B). DCFH-DA detection indicated that inhibition of NLRP3 or IL-1β antagonism reduced Aldo-induced intracellular ROS levels in HSCs. Conversely, inhibition of NOX4 or antagonism of intracellular ROS also downregulated the expression of NLRP3 inflammasome-related proteins and IL-1β induced by Aldo in HSCs. Therefore, even though there was no direct binding between NLRP3 and NOX4 proteins recruited by Aldo within caveolae, the intracellular inflammation and oxidative stress products caused by both can still influence each other.

**Aldo rapidly alters inflammation and oxidative stress levels in HSCs via a caveolae-dependent non-genomic pathway**

According to the result of Fig. 5C, we noticed that a 5min of Aldo stimulation could result in an increase in phosphorylation of Cav-1 levels within HSC, which suggested that Aldo-induced phosphorylation of Cav-1 and enrichment of proteins within caveolae can occur within a short period of time. Therefore, we examined the changes in protein levels shortly after Aldo induction. The results showed that Aldo significantly increased the phosphorylation levels of Cav-1 and c-Src in HSCs after 5 minutes of stimulation, and this effect of Aldo was inhibited by disruption of caveolar structure (Fig. 7A, B). Similarly, IL-1β showed a transient increase after 5 minutes of Aldo stimulation, which could be inhibited by MCD but was unaffected by transcription or translation inhibitors (Fig. 7C, D). In parallel, short-term Aldo stimulation increased the levels of mature caspase-1 and IL-1β in HSCs without altering NLRP3 and ASC levels. This effect of Aldo was inhibited by EPLE but unaffected by transcription or translation inhibitors (Fig. 7E, F). These results suggested that the assembly of Aldo-induced NLRP3 inflammasomes and the maturation of caspase-1 and IL-1β may be regulated through non-genomic mechanisms. Similarly, we observed a transient increase in ROS levels in HSCs after 5 minutes of Aldo stimulation, and this effect of Aldo was still caveolae-dependent and unaffected by transcription or translation inhibitors (Fig. 7G, J). Furthermore, we found that Aldo could also upregulate RhoA activity in HSCs within a short period of time, and this effect could be inhibited by interventions targeting MR, Cav-1, NLRP3, or NOX4 (Fig. 7K-N).
These results suggested that Aldo may rapidly promote inflammation and oxidative stress in HSCs through non-genomic mechanisms, leading to HSCs activation.

**Aldo contributes to BDL-induced activation of HSCs and liver fibrosis in rats**

A rat model of fibrosis was established by BDL (Fig. 8A-C). Compared to the control group, rats in the BDL group showed increased levels of \( \text{H}_2\text{O}_2 \) and serum IL-1\( \beta \) in the liver (Fig. 8D, E). Measurement of Aldo levels revealed an upregulation of Aldo in both the serum and liver of BDL rats compared to the control group (Fig. 8F). Interestingly, although the liver Aldo levels in the spironolactone-treated group were not significantly different from those in the BDL group, liver fibrosis was improved compared to the BDL group (Fig. 8F). This suggested that BDL-induced liver fibrosis in rats may be associated with the upregulation of Aldo in the liver. Similar to the in vitro studies, immunofluorescence staining showed increased co-localization of \( \alpha \)-SMA, NLRP3, NOX4, and Cav-1 proteins in the liver of BDL rats (Fig. 8G, H), indicating the accumulation of NLRP3 and NOX4 in HSC caveolae during BDL-induced liver fibrosis. Analysis of caveolar and total protein levels in primary HSCs from different groups showed that the levels of MR, NLRP3, and NOX4 proteins were upregulated in the caveolae and cellular compartments of BDL rats compared to the control group, while spironolactone treatment reduced the expression of these proteins (Fig. 8J-M). These results suggested that BDL-induced activation of HSCs may be associated with the elevation of liver Aldo levels and the accumulation of oxidative and inflammatory-related proteins in HSC caveolae. We established an Aldo rat model by continuous intraperitoneal pumping of Aldo using a micro-pump. To avoid the renal effects of Aldo, the rats were not provided with high sodium drinking water. The results showed that rats in the Aldo group developed liver fibrosis, with increased levels of \( \text{H}_2\text{O}_2 \) and serum IL-1\( \beta \), as well as significant increases in Cav-1 and \( \alpha \)-SMA-positive cells. Treatment with spironolactone inhibited the effects of Aldo (Figure S4 A-F, I). Similar to the BDL model, co-localization of NLRP3 and NOX4 in HSC caveolae significantly increased in the Aldo infusion group (Figure S4 G, H). Analysis of caveolae and total protein levels in HSCs showed that the levels of MR, NLRP3, and NOX4 proteins were upregulated in the caveolae and cells of the Aldo group, compared to the control group and the spironolactone treatment group, suggesting that the promotion of these protein accumulation and expression by Aldo may be caveolae-dependent.

**CAV-1 knockdown attenuates liver fibrosis induced by BDL or Aldo in rats**

Rats were intravenously injected with HSC-specific AAV targeting Cav-1 knockdown, followed by the induction of liver fibrosis through either BDL or Aldo infusion. The results showed that compared to the negative control AAV group, rats with knockdown Cav-1 in HSCs exhibited reduced HSC activation and liver fibrosis after BDL or Aldo treatment (Figure S5A, Figure S6A-C, E, F), and the levels of \( \text{H}_2\text{O}_2 \) and IL-1\( \beta \) in the liver were also decreased (Figure S5C, D, Figure S6G, H). However, knocking down Cav-1 in HSCs did not affect the local levels of Aldo induced by BDL or Aldo infusion (Figure S5B, Figure S6D). Consistent with the in vitro experiments, the expression of NLRP3 inflammasome-related components and NOX4 protein in HSCs was downregulated in the liver fibrosis model group after Cav-1 knockdown compared to the negative control AAV group (Figure S5E-G, Figure S6I, J). These results suggested that
Cav-1 may regulate BDL- or Aldo-induced activation of HSCs and liver fibrosis through the modulation of NLRP3 inflammasome and NOX4.

**Serum Aldo levels and MR expression in HSCs are upregulated in patients with liver fibrosis**

We measured Aldo levels in the serum and liver tissue of patients with liver fibrosis. The results showed that the serum Aldo levels in liver fibrosis patients did not significantly differ from those in patients without fibrosis, but the intrahepatic Aldo levels were significantly elevated in fibrosis patients (Fig. 9A). The expression of CYP11B2, an enzyme involved in Aldo synthesis, was also upregulated in the liver tissue (Fig. 9B). This suggested that the elevation of intrahepatic Aldo levels may precede the occurrence of hyperaldosteronism, indicating that Aldo may exert biological effects beyond regulating water and sodium metabolism during the progression from liver fibrosis to cirrhosis. Consistent with the results of animal experimental, liver fibrosis patients exhibited increased levels of H$_2$O$_2$ in the liver and elevated serum IL-1β levels compared to patients without fibrosis (Fig. 9C, D). There was also an increased enrichment of NLRP3 and NOX4 proteins in HSC caveolae (Fig. 9F, G). Similarly, compared to patients without fibrosis, liver fibrosis patients showed upregulated expression of MR, NLRP3 inflammasome-related proteins, and NOX4 in HSCs (Fig. 9H, I).

**3. Discussion**

This study elucidated the mechanisms by which Aldo regulated HSC activation and liver fibrosis through a Cav-1-dependent pathway. The main findings of this study are as follows: (i) Aldo induces HSC activation and liver fibrosis, and blockade of its receptor MR could inhibit Aldo-induced HSC activation and improves liver fibrosis in BDL-induced rats; (ii) Cav-1 mediates the upregulation of inflammatory factors and ROS in HSCs by recruiting signaling proteins such as NLRP3 and NOX4 into caveolae, thereby promoting HSC activation and liver fibrosis in response to Aldo; (iii) Aldo exerts non-genomic effects through caveolae, rapidly promoting the assembly of the NLRP3 inflammasome and ROS generation within cells, leading to inflammatory activation of HSCs; (iv) Blocking MR or Cav-1 knockdown in HSCs with AAV can inhibit Aldo-induced HSC activation and improve liver fibrosis in BDL-induced rats. This study provides the first evidence of the Cav-1-dependent genomic and non-genomic pathways by which Aldo induces inflammatory activation of HSCs during liver fibrosis, highlighting the importance of targeting Aldo receptors or Cav-1 for therapeutic interventions based on the underlying etiology of liver fibrosis.

Aldo is a mineralocorticoid secreted by the adrenal cortex glomerulosa zone, involved in regulating the metabolism of sodium, potassium, and water in organisms(26). In addition to maintaining sodium and potassium balance, the activation of Aldo has been reported be associated with increases in inflammation, oxidative stress, endothelial dysfunction, insulin resistance, and fibrosis(27). Research indicated that Aldo exhibits pro-fibrotic effects in multiple organs like the heart, kidney, and lungs, and anti-Aldo therapy has been shown to improve fibrotic lesions in these organs in laboratory and clinical settings(28–31). Previous studies have found elevated levels of Aldo in plasma and urine of patients with
liver cirrhosis, which may be associated with the increased secretion of Aldo and decreased degradation by the liver(32). High Aldo levels have also been considered as an important factor in the development of portal hypertension during liver fibrosis(33). Queisser et al. found that Aldo infusion may induce liver fibrosis independent of blood pressure elevation by upregulating oxidative stress levels(34). Consistent with their findings, our previous studies also demonstrated that MR antagonists promote the regression of liver fibrosis(35). At the cellular level, Rombouts et al. found that Aldo promotes collagen synthesis in HSCs, although its ability to activate HSCs is milder compared to TGF-β(36). In this study, we found that intracellular Aldo is upregulated during HSC activation. This upregulation induces the assembly of NLRP3 inflammasomes and promotes ROS generation, leading to increased inflammation and oxidative stress levels within HSCs. These effects enhance the contraction and migration abilities of HSCs, contributing to a positive feedback loop that promotes HSC activation.

Caveolae is a flask-like plasma membrane organelle, measuring approximately 50-100nm, which plays a crucial role in cellular processes such as macromolecular transport and mechanical signal transduction (16). Cav-1 is a fatty acid- and cholesterol-binding protein that constitutes the major structural protein of caveolae(17, 37). Cav-1 is involved in various cellular processes, such as endocytosis, signal transduction and mechanosensation(17). In liver, Cav-1 is mainly localized in non-parenchymal cells, including HSCs and liver sinusoidal endothelial cells(17, 38). In liver diseases, Cav-1 has been shown to participate in processes such as energy metabolism regulation, bile acid signaling, and oxidative stress(39–41). It is also involved in liver regeneration, alcoholic liver disease, and the progression of liver cancer(42–44). Interestingly, the expression and role of Cav-1 in liver fibrosis remain controversial(21, 45–49).

The specific mechanisms of Cav-1 in the activation of HSCs during liver fibrosis remain unclear. Contradictory opinions exist regarding the expression levels and functions of Cav-1 in liver tissue and HSCs during liver fibrosis, which is one of the reasons why we conducted this study. Consistent with the findings of Thabut(47), our study confirmed that the expression of Cav-1 protein is increased in HSCs of rats with BDL-induced liver fibrosis. Furthermore, we found that BDL-induced liver fibrosis was alleviated when we specifically inhibited Cav-1 expression in rat HSCs using AAV8. Based on our findings and literatures, we believe that the expression of Cav-1 in HSCs may be closely associated with the progression of liver fibrosis. In the context of toxic liver injury (CCL4-induced liver fibrosis), TGF-β is the main factor inducing HSC activation. While in BDL-induced liver fibrosis, in addition to bile acids(50), the upregulation of Cav-1 may also be one of the keys activating factor for HSCs. Our in vitro experimental evidence supports the above viewpoints. We observed that primary rat HSCs spontaneously activated under culture conditions exhibited increased intracellular expression of Cav-1, which further promoted HSC activation through positive feedback. However, Chen et al. obtained different conclusions in their study on the animal model of CCL4-induced liver fibrosis(48). Since HSC activation through the TGF-β pathway is the primary mechanism in the CCL4-induced animal model of liver fibrosis, it is worth considering the role of Cav-1 in regulating HSC activation in CCL4 or TGF-β-induced liver fibrosis. After all, Cav-1 is expressed at relatively low levels in quiescent HSCs, and there are even studies suggesting that quiescent HSCs do not express Cav-1 protein(51). The inhibitory effect of TGF-β on Cav-1 protein, which is already low or not expressed in quiescent HSCs, may not be crucial for HSC activation. Therefore, we
believe that the regulation of Cav-1 on HSC activation may be more significant in cholestasis-related liver fibrosis.

Currently, there is no research reported on the interaction between Aldo and Cav-1 in HSC. Nevertheless their interaction in inducing contraction and migration of vascular smooth muscle cells implies a potential role in HSC activation(52). In our study, we demonstrated that intracellular Cav-1 and Aldo are upregulated during HSC activation and induce an upregulation of protein expressions such as NLRP3 and NOX4, thereby enhancing intracellular inflammation and oxidative stress. Furthermore, we found that during HSC activation, Aldo can rapidly recruit various signaling proteins, including MR, NLRP3, and NOX4, through a Cav-1-dependent pathway, leading to a rapid increase in intracellular IL-1β and ROS levels and enhancing HSC contractility and migration in a short period of time. This finding also supports the hypothesis proposed by Rombouts et al. regarding the non-genomic regulation of HSCs by Aldo(36).

It is worth noting that the regulation of Cav-1-associated non-genomic signaling pathways by Aldo, as investigated in this study, may have particular significance during the early stages of liver fibrosis when intrahepatic pressure increases. Philipp et al. have demonstrated distinct differences in intrahepatic pressure at different stages of liver fibrotic lesions induced by various etiologies(3). In BDL-induced liver fibrosis, intrahepatic resistance significantly increases during the early phase of fibrosis, whereas it increases gradually in CCl4-induced liver fibrosis. BDL-associated liver fibrosis can cause narrowing of the hepatic sinusoidal capillaries due to HSC migration and contractile wrapping before static intrahepatic pressure increases (resulting from excessive extracellular matrix production and deposition), leading to elevated dynamic intrahepatic pressure. Interestingly, Cav-1 plays a crucial role in cellular sensing and transmission of mechanical pressure signals(53, 54). Therefore, Cav-1 protein may be significant in inducing HSC activation during the process of rapid elevation of intrahepatic pressure in the early stages of biliary obstruction-related liver fibrotic lesions. Although our current research data are insufficient to establish a definitive regulatory relationship among the relationship among intrahepatic pressure, Cav-1, and HSC activation, these findings suggest that the interaction between these factors may mediate the rapid increase in dynamic intrahepatic pressure during the early stages of obstructive liver fibrosis. Unfortunately, our study lacked sufficient clinical samples to analyze the expression and localization of Cav-1 in tissues from all types of liver fibrosis patients. In addition, although animal experiments provide evidence that anti-Aldo therapy can improve liver fibrosis(55), existing clinical studies suggest that the efficacy of Aldo antagonists in the treatment of liver cirrhosis and its related complications is uncertain(56, 57). The lack of Aldo antagonists based on liver fibrosis etiological classification may be one of the contributing factors to the uncertainty in their therapeutic efficacy. In fact, we found that the pathological characteristics of high Aldo-induced rat liver fibrosis were similar to those of BDL-induced rat liver fibrosis (Fig. 8A, Fig S8-1A). Therefore, conducting clinical cohort studies on Aldo antagonistic therapy combined with the etiology of liver fibrosis may be a meaningful exploration.

The NLRP3 inflammasome is a large multiprotein complex that senses intracellular danger signals through NOD-like receptors(58). The activation of the NLRP3 inflammasome can further lead to the
cleavage of pro-IL-1β and pro-IL-18 into their mature forms and emerging evidence suggests that NLRP3 inflammasome activation plays a crucial role in various acute and chronic liver diseases(59). However, the mechanisms by which NLRP3 contributes to HSC activation remain unclear. In our previous studies, we found that Aldo can upregulate the levels of NLRP3 in HSCs and induce HSC activation(14). In this study, we further confirmed that Aldo can recruit NOX4 to caveolae which represents an important pathway for NLRP3 activation, thereby increasing ROS levels in HSCs. Additionally, our results suggest the possibility of Aldo directly inducing the assembly and activation of the NLRP3 inflammasome through caveolae-dependent mechanisms. However, further research is needed to determine whether the nuclear receptor MR can exert similar effects to TLR receptors. In this study, we detected the presence of NLRP3 within the caveolae of activated HSCs, which may be closely related to the caveolae-mediated endocytic process of HSC activation mediated by Cav-1. Interestingly, Gaul et al. recently found that HSCs can activate themselves through the internalization of exogenous NLRP3 inflammasomes(60). Therefore, further investigation is needed to determine whether Cav-1 is also involved in mediating the uptake and internalization of exogenous NLRP3 inflammasomes by HSCs. Since we did not detect the presence of ASC and Caspase-1 proteins within the caveolae, it is possible that the assembly of the Cav-1-mediated NLRP3 inflammasome does not occur within the caveolae. We observed an upregulation of intracellular NLRP3 expression during HSC activation in vitro. Therefore, the potential sources of NLRP3 detected in the vesicles may vary. MR is a nuclear transcription factor(61) and through JASPAR database, we predicted that MR could bind to the promoter region of IL-1β, suggesting that after Aldo-induced enrichment of MR in caveolae, MR signaling may transduce into the nucleus, promoting IL-1β expression (results not shown). Therefore, Aldo may play a promoting role in both steps of NLRP3 inflammasome activation. These results highlight the contribution of Aldo and Cav-1 to NLRP3 inflammasome activation in liver fibrosis and have important translational implications for the accurate design of approaches to treat liver disease through molecular manipulation.

Due to the low immunogenicity and low carcinogenicity, AAV is considered a safe and effective gene delivery instrument in clinical trials(62). Rezvani and Chen successfully treated liver fibrosis in mice by targeting and modifying relevant genes within HSCs using AAV vectors(48, 63). Since Cav-1 is expressed minimally in normal hepatic parenchymal cells but shows increased specific expression in HSCs after liver injury(64), this characteristic provides a promising application for manipulating Cav-1 to regulate HSC activation in liver fibrosis. Both in vitro and in vivo results of our study demonstrate that targeted knockdown of Cav-1 expression within HSCs can reduce HSC activation during liver fibrosis and ameliorate BDL-induced liver fibrosis. Additionally, our research elucidates the mechanism by which local Aldo recruit inflammation and oxidative stress related proteins within HSC caveolae through a Cav-1-dependent pathway, inducing HSC activation. This provides new evidence for the clinical anti-Aldo treatment of liver fibrosis and patients with high hepatic pressure. Notably, we discovered that knocking down Cav-1 within rat HSCs had a more promising effect in alleviating BDL-induced rat liver fibrosis compared to Aldo antagonist treatment. Similarly, Oberti et al. reported limitations in anti-Aldo treatment for liver fibrosis(65). These also suggests that the induction of HSC activation by Cav-1 may involve other regulatory pathways beyond Aldo/MR. In another study of ours, we extracted caveolae from primary
mouse HSCs and found that Cav-1-mediated HSC activation regulation may be closely associated with glucose metabolism(66). In conclusion, we found that Cav-1 and Aldo regulate NLRP3 inflammasome and oxidative stress-mediated activation of HSCs during cholestasis-induced liver fibrosis. AAV-specific knockdown of Cav-1 expression within HSCs or anti-Aldo treatment can ameliorated rat liver fibrosis. Further exploration of other therapeutic methods targeting Cav-1 holds significant value and may provide new insights for the development of anti-liver fibrosis treatments.

4. Methods

Animal and hepatic fibrosis model

Sprague Dawley rats (4 weeks old, male, 150–180 g) were provided by the Guangdong Medical Laboratory Animal Center. The rats were housed in the Laboratory Animal Centre of South China Agricultural University and provided ad libitum access to food and water. For the BDL procedure, a laparotomy was performed, and the bile duct was doubly ligated using sutures and then transected between the ligatures while the sham-operated mice underwent the same surgical procedure without the actual ligation of the bile duct. For the osmotic micro-pump(Alzet, 2004) implantation procedure, rats were treated with Aldosterone(0.6µg/g/day) or saline for 4 weeks. For the gastric perfusion of Spironolactone, rats were orally administered Spironolactone at a dose of 10mg/kg/day and continued once daily for 4 weeks. Rats were injected with AAV-Cav-1 KD-GFAP (expressing GFAP promoter-driven shCav1) or AAV-Cav-1 NC-GFAP (1.5 × 10^{12} viral genome copies/rat) by intravenous injection. All animal experiments were conducted in accordance with the guidelines and regulations approved by the Ethics Committee of the Laboratory Animals of Southern Medical University.

Patient specimens

Fibrotic liver biopsy specimens (fibrosis stage: F3-4) were obtained from 20 patients with liver fibrosis caused by choledocholithiasis. Additionally, normal liver specimens were obtained from 20 patients who underwent partial liver resection for hepatic hemangiomas. All specimens were obtained from Nanfang Hospital of Southern Medical University. All participants gave their informed consent, and the use of samples was approved by the local hospital's ethics committee.

Quantification of Aminotransferase

Blood samples were centrifuged at 3000rpm for 15 minutes to obtain plasma. Subsequently, plasma alanine aminotransferase (ALT) was detected using the commercial kits (C009-3-1, Jiancheng Bioengineering Inc., Nanjing, China) following manufacturers' instructions.

Hydroxyproline assay of liver tissues

Liver tissues were homogenized in phosphate-buffered saline. The hydroxyproline content was measured using a hydroxyproline assay kit. Tissue homogenates were processed according to the manufacturer's
instructions, including incubation with chloramine-T reagent, addition of perchloric acid solution, and reaction with Ehrlich's reagent. Absorbance was measured at 550 nm, and hydroxyproline concentration was determined using a standard curve.

Isolation of primary rat HSCs

Primary rat HSCs were isolated from rat liver as previously reported (67). Briefly, primary rat HSCs were isolated from cell suspensions using a retrograde perfusion system with 0.05% collagenase type IV (Sigma, St. Louis, MO, USA) and density gradient centrifugation. Isolated HSCs were cultured in RPMI 1640 medium containing 20% fetal bovine serum (FBS).

Masson Staining

Livers were fixed in 10% buffered formalin and paraffin embedded. Liver sections with a thickness of 4µm were stained using Masson staining (MST-8004, Maixin Biotech Co., Ltd, Fuzhou, China) to evaluate collagen in liver fibrosis and non-fibrosis samples, with collagen appearing blue. Stained sections were observed and photographed using the Olympus IX63 microscope (Olympus, Tokyo, Japan). The ratio of the blue collagen-stained area within the field of view to the total area was calculated and averaged.

Cav1 knockdown using small interfering RNA

HSCs were cultured in 6-well plates until they reached 60–80% confluency. Transfection of small interference RNA (siRNA) was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The Lipofectamine 3000 or si-Cav1 was diluted with serum-free medium and added to the cells along with fresh RPMI1640 containing 20% FBS. The cells were then cultured at 37°C with 5% CO2 for 48 hours, after which cellular proteins were extracted.

MCD and MCDC

Cells were seeded in a 6-well plate and incubated. Afterward, the cells were treated with 1mM MCD (M812850, Macklin, Shanghai Macklin Biochemical Co., Ltd, China), MCDC (cholesterol, C6213, Macklin, Shanghai Macklin Biochemical Co., Ltd, China) for saturation of MCD, or PBS for approximately 30 minutes. Following this, the medium was replaced with a serum-free medium and the cells were incubated for 24 hours. The cells were then harvested and subjected to extraction.

Reverse transcription and qPCR

Liver tissue was subjected to RNA extraction using TRIzol reagent (15596026, Invitrogen, Carlsbad, CA, USA), while total cellular RNA was obtained using the RNA extraction kit (B0004DP, EZBioscience, Roseville, CA, USA). The concentration and purity of the extracted RNA were assessed using a nucleic acid protein analyzer (NanoDropND-2000 micrometer, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the reverse transcription kit (RR036A, TaKaRa Bio Inc, Tokyo, Japan) following the manufacturer's instructions. RT-PCR was conducted using the LightCycler 480 Real-
Time PCR System (4887352001-p96, Roche, Basel, CH). The ratio of gene expression levels was determined by comparing them with β-actin, which served as the endogenous housekeeping gene.

**Western Blot**

Cell samples or liver tissues were lysed using RIPA buffer (P0013B, Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (GRF10, EpiZyme Biomedical Technology Co., Ltd, Shanghai, China). The lysates were subjected to ultrasonication and centrifuged at 12,000 g for 15 minutes at 4°C. The protein content in the resulting supernatant was determined using the protein assay dye reagent (P0012, Beyotime Biotechnology, Shanghai, China). Proteins were separated by SDS-PAGE (P0015L, Beyotime Biotechnology, Shanghai, China) and incubated with primary antibodies including α-SMA (Proteintech, 14395-1-AP), Collagen I (Proteintech, 14695-1-AP), β-actin (Proteintech, 66009-1-lg), Cav-1 (Proteintech, 16447-1-AP), p-CAV-1(Abcam, ab38468), Desmin(Boster, BM4101), MR(Abcam, ab41912), NLRP3(Proteintech, 19771-1-AP), NOX4(Abcam, ab133303), c-SRC(Proteintech, 60315-1-lg), p-c-SRC(Abcam, ab4816), mlc(CST, 3672), moe(CST, 3146), smad3(Abcam, ab40853), ASC(Proteintech, 10500-1-AP), RhoA(Abcam, ab219371), IL-1β(Abcam, ab9722), caspase-1(Abcam, ab1872), Mouse IgG(Abcam, ab188776), Rabbit IgG(Abcam, ab172730). Following incubation with secondary antibodies, the proteins were detected and analyzed using the Odyssey system (Odyssey CLx, Lincoln, Nebraska, USA).

**Immunohistochemistry and Immunofluorescence**

The liver paraffin sections underwent dewaxing and hydration, following the previously described method. Antigen retrieval was performed in citrate buffer at an elevated temperature for 30 minutes. Subsequently, the sections were blocked with 10% lamb serum at room temperature for 1 hour. Hematoxylin, eosin, and primary antibodies were used for staining, followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibody (GK500705, Gene Tech Co., Ltd, Shanghai, China) for 1 hour. The protein staining reaction was visualized using the DAB chromogen. For immunofluorescence, antibodies against Cav1, α-SMA, and PFKL were applied to the antigen, followed by the corresponding secondary antibody. DAPI was used to stain the nucleus. Images were captured using an Olympus BX51/IX63 microscope (Olympus, Tokyo, Japan).

**Transwell assay**

The transwell invasion assay was conducted with 24 transwell chambers (353097, BD Biosciences, San Jose, CA, USA). After 12 hours of migration induction, the migrated cells were quantified, and representative images of crystal violet-stained cells were captured. The images were obtained using a fluorescence microscope (Olympus, Tokyo, Japan).

**Collagen gel contraction assay**

Collagen lattice contraction models were constructed following the manufacturer's instructions (C8062-10mg, Solarbio Science & Technology Co., Ltd, Beijing, China). Cells were loaded into each collagen gel,
and after 12 hours, the contraction of the collagen gels was observed and recorded.

**Immunoprecipitation**

Co-IP (co-immunoprecipitation) and IP (immunoprecipitation) assays were conducted using protein A/G magnetic beads (C0914, Biosynthesis Biotechnology Co., Ltd., Beijing, China). The beads were rinsed twice with lysis buffer and incubated with the primary antibody at 4°C overnight. After three washes with lysis buffer, the beads were incubated with 1 mg of total protein overnight at 4°C. Following another three washes with lysis buffer, the antigen-bound proteins were eluted with 1X loading buffer and analyzed by western blot.

**Plasmid construction and transfection**

For transfection, expression plasmid constructs were utilized, including pcDNA3.1(+)-Flag-NACHT-domain, pcDNA3.1(+)-Flag-LRR-domain deletion, and pcDNA3.1(+)-Flag-PYD-domain. Primary rat HSCs were seeded in a 6-well plate at a density of 3 x 10^5 cells per well. After 24 hours, the primary rat HSCs were transfected with 2.5 mg of plasmid, 5 ml of Lipo 3000, and 5 ml of P3000. To maintain equal amounts of transfected plasmids, an empty pcDNA3.1(+) vector was added to each well.

**Transmission Electron Microscopy (TEM)**

HSCs were fixed with 2.5% glutaraldehyde solution at 4°C for 24 hours. Subsequently, they were treated with a 2% uranyl acetate solution for 2 hours. After dehydration with escalating concentrations of acetone (50%, 70%, 90%, and 100%), the cells were embedded to create ultrathin sections. These sections were then observed under an electron microscope (Hitachi H-7500).

**Statistical analysis**

Error bars represent the ± standard deviation from triplicate experiments. The Student’s t-test was utilized to compare 2 groups of data. One-way analysis of variance was used to compare data with 3 or more groups. Results with p values < 0.05 were considered statistically significant.

**Declarations**

Conflict of interest statement: The authors have no conflicts of interest to declare. Financial support statement: This work was supported by the National Natural Science Foundation of China (grant numbers 81670556 and 82170641 to Xu Li), The Youth Fund of National Natural Science Foundation of China (grant number 82100660 to Yang Li), and China Postdoctoral Science Foundation (grant number 2021M691458 to Yang Li). Authors contributions: XL and YM designed and supervised the study. XL and YL acquired funding. YL, TC and YZ performed the experiments. YL, TC, QX, YZ, and YL analyzed the data. JG, QY and ZC collected samples and clinical information. YL and TC drafted the manuscript. XL and YM reviewed the manuscript.
References


Figures

Figure 1

The expression of Cav-1 is up-regulated in the HSCs of patients with liver fibrosis.
(A) Liver sections from patients with or without liver fibrosis were stained with H&E, Masson, and Immunohistochemistry (IHC) for Cav-1 expression. (B) Liver hydroxyproline levels were detected in patients with or without liver fibrosis. (C) Representative images of Cav-1 (green) and α-SMA (red) immunofluorescence (IF) in non-fibrotic and fibrotic liver sections; scale bars, 100 μm. (D) Immunoblot for Cav-1, α-SMA and type I collagen expression in HSCs in patients with or without liver fibrosis. Data in B is reported as the mean ± SD with an indicated significance (*p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant; Student’s t test).

Figure 2

Cav-1 is up-regulated and promotes HSCs activation in vitro.

(A) Primary rat HSCs were isolated and identified by desmin staining. (B) Representative images of Cav-1 (red) and α-SMA (green) IF of primary rat HSCs cultured in vitro after 3 days and 14 days; scale bars, 10 μm. (C) The caveolae of HSCs were observed by transmission electron microscope after 14 days culture in vitro. (D) Gel contract assay of primary rat HSCs. (E) Transwell culture system was used to
detect migration ability of primary rats HSCs. (F) Immunoblot for α-SMA and type I collagen expression in primary rat HSCs. Data in F is reported as the mean ± SD with an indicated significance (*p <0.05, **p <0.01, ***p <0.001, n.s.: not significant; One-way ANOVA).

Figure 3

Knockdown Cav-1 in HSCs inhibits BDL-induced rat liver fibrosis.

(A) Rat liver sections from each group were stained with H&E, Masson and for α-SMA and Cav-1 expression; scale bars, 50μm. (B) ISHAK score of liver fibrosis. (C) Collagen area of Masson staining. (D) Testing of serum ALT. (E) Hydroxyproline assay of liver tissues. Data in (B, C, D, E) are reported as the mean ± SD with an indicated significance (*p <0.05, **p <0.01, ***p <0.001, n.s.: not significant; One-way ANOVA).
Figure 4

Enhanced Aldo expression during HSC activation promotes recruitment of MR and other proteins in caveolae to facilitate positive activation of HSCs.

(A) HSC caveolae were extracted using a discontinuous sucrose density gradient method. Immunoblot for Cav-1 revealed a concentrated presence of caveolae in the 3rd and 4th fractions, with detection of MR.
protein as well. (B) Detection of cytoplasmic Aldo in primary rat HSCs. (C) QRT-PCR detection of CYP11B2 mRNA expression in HSCs. (D) Detection of Aldo in cell culture supernatant of primary rat HSCs. (E) IF detection of colocalization among Cav-1 (green) and α-SMA (red) in HSCs (scale bar: 10 μm). (F) Gel contract assay of primary rat HSCs. (G) Cell migration experiment of primary rat HSCs (scale bar: 50 μm). (H) Immunoblot for α-SMA and type I collagen expression in primary rat HSCs. (I) Immunoblot for c-Src, nlrp3 and NOX4 in the caveolae of primary rat HSCs. (J) IF detection of colocalization among Cav-1 (red), NLRP3 (green) and NOX4 (violet) in primary rat HSCs (white triangles, scale bar: 10 μm). (K) Co-IP testing of interactions among protein Cav-1, MR, c-Src, nlrp3 and NOX4 in primary rat HSCs. (L) Immunoblot for caspase-1 and ASC in the caveolae of primary rat HSCs. (M) Immunoblot test for the interaction of NACHT, LRR and PYD with Cav-1. (N) Schematic diagram of Aldo’s induction of signaling protein enrichment in the caveolae. Data in (B, C, D, H, M) are reported as the mean ± SD with an indicated significance (*p<0.05, **p<0.01, ***p<0.001, n.s.: not significant; (H,M) Student’s t test; (B-D) One-way ANOVA).

**Figure 5**

Aldo induces HSCs activation by promoting phosphorylation of Cav-1 and c-Src.

(A) Gel contract assay of primary rat HSCs. (B) Cell migration experiment of primary rat HSCs (scale bar: 50 μm). (C-M) Immunoblot for relative protein expression in primary rat HSCs.
Figure 6

Aldo promotes HSCs activation by up-regulating NOX4 expression and increasing ROS production.

(A) Immunoblot for NOX4 expression in primary rat HSCs. (B) Gel contract assay of primary rat HSCs. (C) Cell migration experiment of primary rat HSCs (scale bar: 50 μm). (D,E) DCFH-DA was used to detect ROS levels in primary rat HSCs. (E,G) Hydrogen peroxide detection of primary rat HSCs. (H) Immunoblot
for α-SMA and type I collagen expression in primary rat HSCs. (I) Schematic diagram of Aldo induces NLRP3 and NOX4 enrichment in caveolae and promotes inflammasome assembly and IL-1β and ROS production. Data in (A, D, E, F, G) are reported as the mean ± SD with an indicated significance (*p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant; One-way ANOVA).

Figure 7

Aldo induce the rapid production of IL-1β and ROS in HSCs via non-genomic pathways.
(A-F) Immunoblot for relative protein expression in primary rat HSCs. (G,I) DCFH-DA was used to detect ROS levels in primary rat HSCs. (H,J) Hydrogen peroxide detection of primary rat HSCs. (K-N) Immunoblot for active RhoA and total RhoA expression in primary rat HSCs. Data in (G, H, I, J) are reported as the mean ± SD with an indicated significance (*p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant; One-way ANOVA).

**Figure 8**

Spironolactone reduces the expression of NLRP3 and NOX4 in HSCs and improves BDL-induced rats liver fibrosis.

(A) Rat liver sections from each group were stained with H&E and Masson; scale bars, 100µm. (B) ISHAK score of liver fibrosis. (C) Liver hydrogen peroxide assay of each group. (D) Liver hydroxyproline levels were detected in each group. (E) Testing of serum IL-1β. (F) Serum and liver Aldo were detected. (G,H) Representative images of α-SMA/Cav-1/NLRP3 and α-SMA/Cav-1/NOX4 immunofluorescence of each group; scale bars, 50µm. (I) Cav-1 and α-SMA in each group were detected by IHC. (J) Immunoblot for relative protein expression in caveolae of primary rat HSCs. (K-M) Immunoblot for relative protein
expression in primary rat HSCs. Data in (B, D, E, C, F) are reported as the mean ± SD with an indicated significance (*p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant; One-way ANOVA).

Figure 9

Upregulated expression of Aldo, NLRP3 inflammasomes, and NOX4 protein in HSCs in fibrotic liver patients

(A) Serum and liver Aldo were detected in patients with or without liver fibrosis. (B) QRT-PCR detection of CYP11B2 mRNA in liver of patients with or without liver fibrosis. (C) Liver hydrogen peroxide detection of patients. (D) Testing of serum IL-1β. (E) IHC staining of smad3 and type I collagen in liver sections (scale bar: 50 μm). (F-G) Representative images of α-SMA/Cav-1/NLRP3 and α-SMA/Cav-1/NOX4 IF of each group (scale bars, 50μm). (H-I) Immunoblot for relative protein expression in HSCs of patients with or without liver fibrosis. Data in (A-D) are reported as the mean ± SD with an indicated significance (*p<0.05, **p<0.01, ***p<0.001, n.s.: not significant; Student’s t test).

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