Activation of autophagy through calcium-dependent AMPK/mTOR and PKCθ pathway causes activation of rat hepatic stellate cells under hypoxic stress

Yuepeng Jin1,*, Yongyu Bai2,*, Haizhen Ni1, Li Qiang2, Lechi Ye3, Yunfeng Shan1 and Mengtao Zhou1

1 Department of Surgery, The First Affiliated Hospital, Wenzhou Medical University, China
2 Wenzhou Medical University, China
3 Department of Oncological Surgery, The First Affiliated Hospital, Wenzhou Medical University, China

Correspondence
M. Zhou, Department of Surgery, First Affiliated Hospital, Wenzhou Medical University, 2 Fuxue Lane, Wenzhou, Zhejiang, China
Fax: +86 577 55578033
Tel: +86 577 55578033
E-mail: zmt0417@hotmail.com

*Equal contributors and co-first authors.

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Hepatic fibrosis remains the major cause of morbidity and mortality worldwide and probably affects more than 100 million people per year. Chronic injury to the liver due to pathogen infection, drug usage, metabolic disorders, or autoimmune imbalance leads to fibrosis [1–4]. Long-term liver injury results in hepatic fibrosis accompanied by expansion of fibrotic septa, which ultimately accelerates portal hypertension, cirrhosis, and hepatocellular carcinoma [5,6]. However, the underlying precise mechanism remains elusive, and there are no effective clinical therapies apart from diet control and physical exercise.

Hepatic fibrosis is characterized by an excessive deposition of the extracellular matrix (ECM), particularly fibrillar type I and III collagens [7]. Hepatic stellate cells (HSCs) are the major sources of ECM proteins in the liver; thus, the proliferation and expansion of HSCs is a key step in the fibrogenic process [8]. During liver injury, HSCs transform into the myofibroblast-like phenotype, characterized by decreased intracellular vitamin A content, increased proliferation and migration, enhanced secretion of ECM proteins, and production of matrix metalloproteinases [9]. HSCs may be activated under multiple stressful conditions including hypoxia.

Hypoxia is a common environmental stress occurring in numerous pathophysiological conditions. Emerging evidence has suggested a role for hypoxia in the

**Abbreviations**
AMPK, 5′-adenosine monophosphate-activated protein kinase; HSCs, hepatic stellate cells; mTOR, mammalian target of rapamycin; PKCθ, protein kinase C-theta; PLC, phospholipase C; α-SMA, alpha-smooth muscle actin.
development of hepatic fibrosis. Hypoxia is a hallmark of liver fibrosis and activates HSCs, thereby leading to ECM deposition. It has been reported that hypoxic stress results in activation of the HSC cell line LX-2 along with an increase in alpha-smooth muscle actin (α-SMA) and collagen I protein expression [10]. However, the molecular mechanisms of fibrogenesis mediated by hypoxic stress in HSCs are not completely understood.

Autophagy, a metabolic process in which eukaryotic cells degrade long-lived proteins and damaged organelles, plays a crucial role in the development, differentiation, and homeostasis of cells [11,12]. Recently, autophagy has been shown to be an important regulator of liver homeostasis during hepatic fibrosis [13,14]. Numerous studies have suggested that autophagy may play an important role in hepatic fibrosis by regulating the activation of HSCs [15,16]. However, the role of autophagy in hypoxic stress-induced activation of HSCs remains to be determined.

In the present study, we explored the stimulatory role of hypoxic stress on autophagy and the underlying mechanisms related to the intracellular calcium concentration ([Ca^{2+}]) and the Ca^{2+}-dependent signaling pathways during rat HSC activation.

Materials and methods

Ethics statement

The Institutional Animal Committee of Wenzhou Medical University (Wenzhou, China) approved the protocol for the animal experiment (Permit Number: wydw2015-0082). All animals received care in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ as stated in the Helsinki Declaration. At the end of the experiment, the animals were euthanized using pentobarbital sodium.

HSC isolation and culture

Primary HSCs were routinely prepared by collagenase/protease digestion of the livers of 8-week-old Sprague-Dawley rats using a perfusion system as described previously [17]. Cells were maintained using Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU·mL^{-1} penicillin, and 100 IU·mL^{-1} streptomycin. All of the cells were cultured at 37 °C and 5% CO_{2}.

Hypoxic treatment

Hypoxic conditions were achieved by incubating the cells in a humidified modular incubation chamber. Before the hypoxic treatment, the cells were cultured in DMEM containing 1% FBS for 12 h. Then, the cells were transferred to a combined workbench/incubator 20 with a humidified atmosphere of 1%, 10%, or 21% oxygen and 5% CO_{2} balanced with N_{2} and incubated at 37 °C for 6, 12, 18, or 24 h. For each oxygen concentration, the experiments were performed on two separate occasions.

Immunofluorescence

Microtubule-associated protein light chain 3 (LC3) expression in HSCs was detected by a laser scanning confocal microscope. HSCs were cultured in covered glass-bottom dishes. After treatment, the cells were fixed with 4% paraformaldehyde for 15 min and treated with ice-cold (−20 °C) methanol for 15 min. The cells were washed in phosphate-buffered saline (PBS) containing 5% bovine serum albumin. The HSCs were incubated with the primary antibody anti-LC3 (Cell Signaling Technology, Boston, MA, USA) and then incubated with FITC-conjugated secondary antibodies (Sigma, St. Louis, MO, USA). After washing with PBS, the HSCs were labeled with DAPI for 10 min. Finally, these cells were viewed under an inverted confocal microscope (Olympus, Tokyo, Japan).

Western blot

Cells were lysed in RIPA buffer supplemented with a protease inhibitor mixture (Sigma), followed by measurement of the protein concentration using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL, USA). An aliquot (30 μg) was resolved by denaturing 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were probed with primary antibodies overnight at 4 °C, followed by incubation with a secondary antibody for 1 h. The blots were developed using the Western Blue® stabilized substrate for alkaline phosphatase. LC3B antibody was purchased from Cell Signaling Technology. The antibodies against GAPDH, Beclin-1, P62, 5'-adenosine monophosphate-activated protein kinase (AMPK), p-AMPK, mammalian target of rapamycin (mTOR), p-mTOR, extracellular signal-regulated kinase (ERK), calmodulin-dependent kinase β (CaMKKβ), death-associated protein kinase (DAPK), α-SMA, and collagen type I were purchased from Abcam (Cambridge, UK). The protein kinase C-theta (PKCθ) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Small interfering RNA

The cells were transfected with small interfering RNA (siRNA) oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Beclin-1-specific siRNAs corresponding to distinct regions of Beclin-1 were synthesized by GenePharma (Shanghai, China) and are shown in...
Table S1. A scrambled control siRNA was purchased from Genepharma. Transfection of HSCs with Beclin-1 siRNA at 100 nM was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. After transfection for 72 h, Beclin-1 protein and mRNA levels were analyzed by western blotting and real-time polymerase chain reaction (PCR), respectively.

**Real-time PCR**
The total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. The cDNA was synthesized using the SuperScript cDNA Synthesis Kit (Invitrogen). The real-time PCR used 0.1 μL of cDNA with SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7500 Sequence Detection System. The relative mRNA expression levels were normalized to GAPDH and calculated using the comparative cycle threshold calculation method. The primers used are listed in Table S2.

**Ca2+ imaging**
The measurement of [Ca2+]i was performed as described previously [18]. In brief, HSCs were plated onto glass coverslips and treated as described above. The cells were induced with 4 μM fura-2 and 0.02% acetoxymethyl (Biotium, Hayward, CA, USA) at 37 °C in the dark for 40 min. The coverslips were washed and placed in a chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE-2000-S, Burlingame, CA, USA). Fura-2 was excited alternately at 340 and 380 nm, allowing ratiometric measurements of changes in cytosolic Ca2+ levels, and emission was measured at 510 nm. The data were analyzed by averaging the pixel ratio values in circumscribed regions of cells within the field of view. The values were exported from AXON IMAGING WORKBENCH software to SIGMAPLOT (Systat Software, Inc., San Jose, CA, USA) for further analysis and plotting.

**Statistical analyses**
Data are expressed as the mean ± standard error of the mean. Before presenting the data, the normality of the distributions was tested. The statistical analysis was performed using one-way analysis of variance followed by the least significant difference post hoc test and the unpaired Student’s t-test, using statistical software spss 17.0 (Chicago, IL, USA). *P* < 0.05 was considered significant.

**Results**

**Hypoxic stress causes autophagy in rat HSCs**
To examine whether autophagy is increased in rat quiescent HSCs, HSCS after 2 days in culture were exposed to hypoxic stress and the localization of LC3 was analyzed by immunofluorescence using confocal microscopy. As the oxygen concentration decreased from 21% to 10% and then to 1% for 12 h, the green fluorescence density and LC3 puncta formation dramatically was increased. In contrast, fewer cells with LC3-positive puncta were observed in the HSCs treated with 21% O2 (Fig. 1A). These results indicate a potent induction of autophagy by hypoxia in rat HSCs.

To further validate this conclusion, rat HSCs were incubated with 21%, 10%, or 1% oxygen for 6 or 12 h, and immunoblotting was performed to assess the protein levels of LC3, Beclin-1, and p62, all of which are markers of autophagy. In comparison to the level at normoxic conditions, the LC3-II protein level was significantly increased only in the HSCs exposed to 1% O2 for 6 h (*P* < 0.01, Fig. 1B). However, when the treatment time was extended to 12 h, the LC3-II protein level was increased in a dose-dependent manner (Fig. 1C). Similarly, the Beclin-1 level was increased at 1% O2 but not at 10% O2 for 6 h, whereas it was significantly increased at both 1% O2 and 10% O2 for 12 h. P62 is selectively incorporated into autophagosomes through direct binding to LC3 and is degraded by autophagy. Exposure of HSCs to hypoxia led to the significant downregulation of p62 in a dose- and time-dependent manner (Fig. 1B,C). As exposure of HSCs to 1% O2 resulted in significant upregulation of LC3-II and Beclin-1 along with downregulation of p62, HSCs were further treated with 1% O2 for 6, 12, 18, and 24 h. The induction of LC3-II reached a peak at 12 h and declined by 18 h after hypoxic stimulation (Fig. 1D). Similar trends were observed in terms of the Beclin-1 protein level. The p62 level was decreased at 12 h, but it recovered at 18 h. As the level of p62 inversely correlates with autophagic activity, these results demonstrate that the early phase of hypoxic stress might causes autophagy in rat HSCs.

To further support that the induction of LC3-II was caused by autophagy induction, hypoxic stress-induced autophagic flux was assessed in the presence or absence of autophagosome–lysosome fusion inhibitor chloroquine. As expected, pretreatment with 5 μM chloroquine alone resulted in increased LC3B-II level, whereas cotreatment with chloroquine and hypoxic stimulation caused further enhanced turnover of LC3-II (Fig. 1E), indicating that the accumulation of LC3-II with hypoxia is caused by autophagy induction rather than reduced autophagy flux.
Hypoxic stress-induced \([\text{Ca}^{2+}]_i\) increase contributes to autophagy in rat HSCs

It is now well-established that \([\text{Ca}^{2+}]_i\) is one of the regulators of autophagy in cells [19]. We examined whether hypoxic stress-induced autophagy of rat HSCs is associated with changes in \([\text{Ca}^{2+}]_i\). We treated rat HSCs with 1% \(\text{O}_2\) and measured the changes in cytosolic \(\text{Ca}^{2+}\) levels with a ratiometric assay. As shown by representative traces in Fig. 2A, exposure of rat HSCs to 1% \(\text{O}_2\) induced a transient elevation in \([\text{Ca}^{2+}]_i\) at about 100 s.

To further elucidate the pathways involved in the elevation of \([\text{Ca}^{2+}]_i\) in rat HSCs, the cell-permeable cytosolic \(\text{Ca}^{2+}\) chelator 1,2-bis(2-aminophenoxy) ethane-N,N',N'',N'''-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) and the extracellular \(\text{Ca}^{2+}\) chelator ethylene glycol tetraacetic acid (EGTA) were added to cells prior to hypoxic stimulation with 1% \(\text{O}_2\). We found that BAPTA-AM, but not EGTA, abolished the intracellular \(\text{Ca}^{2+}\) increase elicited by hypoxic stress (Fig. 2A). \(\text{Ca}^{2+}\) release from intracellular stores is a well-established process involving phospholipase C (PLC)-mediated synthesis of inositol 1,4,5-trisphosphate. Similar to the effects of BAPTA-AM, the increase in \([\text{Ca}^{2+}]_i\) stimulated by hypoxic stress was prevented by the PLC-specific inhibitor U73122 (Fig. 2A). These results indicated that the increases in \([\text{Ca}^{2+}]_i\)
elicited by hypoxic stress might be attributed to Ca$^{2+}$ release from intracellular stores in rat HSCs. Intriguingly, the hypoxic stress-induced increase in LC3-II protein levels was significantly reversed by BAPTA-AM (Fig. 2B,C). To investigate whether the hypoxic stress-induced [Ca$^{2+}$] increase correlates with the induction of autophagy in rat HSCs, BAPTA-AM was added at a time point before or after the calcium peak under hypoxic stress. As shown in Fig. 2D, the decrease in p62 level was abolished in the treatment with BAPTA-AM before calcium peak. In contrast, after calcium peak, BAPTA-AM had no effect of the decreased p62 level under hypoxic stress, suggesting that there is an association of [Ca$^{2+}$], elevation with autophagy activation in rat HSCs exposed to hypoxic stress.

**Hypoxic stress induces autophagy through the Ca$^{2+}$–AMPK–mTOR pathway in rat HSCs**

The AMPK–mTOR signaling pathway is a downstream target of Ca$^{2+}$ signaling and plays an important role in the regulation of autophagy in response to different stresses [20,21]. To evaluate the role of the AMPK–mTOR pathway in autophagy activation in rat HSCs exposed to hypoxic stress, p-AMPK, AMPK, p-mTOR, and mTOR protein levels were assessed by immunoblotting. Notably, when compared with 21% O$_2$ (normoxia), the phosphorylation of AMPK significantly increased in the cells exposed to 1% O$_2$ hypoxic stimulation for 12 h (Fig. 3A). Conversely, phosphorylation of mTOR, downstream of AMPK, significantly decreased in response to 1% O$_2$ hypoxia compared with the normoxia control (Fig. 3A,B), suggesting activation of the AMPK pathway and inhibition of the mTOR pathway during hypoxic stress.

To gain insight into the mechanism by which the Ca$^{2+}$–AMPK–mTOR axis positively regulates HSC autophagy, we treated cells in the presence or absence of BAPTA-AM under 1% O$_2$ hypoxic conditions for 12 h, and the expression levels of p-mTOR and mTOR were detected by western blotting analysis. As
shown in Fig. 3C,D, BAPTA-AM treatment significantly prevented the decrease of the phosphorylation of mTOR by 1% O2 hypoxic stimulation in rat HSCs.

Hypoxic stress induces autophagy through the Ca\(^{2+}\)-dependent PKC\(\theta\) pathway in rat HSCs

It has been established that Ca\(^{2+}\)-induced autophagy is mediated by PKC\(\theta\) [22]. Next, we asked whether activation of PKC\(\theta\) contributes to hypoxia-induced autophagy by assessing the phosphorylation of PKC\(\theta\) via immunoblotting. As shown in Fig. 4A,B, exposure of rat HSCs to either 10% O2 or 1% O2 for 12 h resulted in significant phosphorylation of PKC\(\theta\), with a much stronger effect by 1% O2, demonstrating activation of the PKC\(\theta\) pathway during hypoxic stress. Moreover, we found that BAPTA-AM suppressed the hypoxia-induced phosphorylation of PKC\(\theta\), indicating that the activation of PKC\(\theta\) pathway in rat HSCs under hypoxic stress (Fig. 4C,D).

It is well known that Ca\(^{2+}\)-dependent ERK [23], CaMKK\(\beta\) [24], and DAPK [25] play a role in autophagy; however, our western blotting analysis showed that hypoxic stress did not lead to significant phosphorylation of ERK, CaMKK\(\beta\), or DAPK (Fig. S1).

These results indicate that these pathways are unlikely to have a major function in the regulation of autophagy during hypoxic stress in rat HSCs.

Blocking autophagy attenuates hypoxic stress-induced activation of HSCs

To examine the activation of HSCs by hypoxic stress, the expression levels of \(\alpha\)-SMA and collagen type I, indicators of activated HSCs, were detected by western blotting and real-time PCR. Either 10% O2 or 1% O2 for 12 h led to a significant increase in \(\alpha\)-SMA and collagen I protein levels, with a much stronger effect by 1% O2 (Fig. 5A,B). Similar upregulation of the mRNA levels of both \(\alpha\)-SMA and collagen I was observed as assessed by real-time PCR (Fig. 5C). These results showed that HSCs were activated under hypoxic stress.

To investigate the potential role of autophagy in the activation of rat HSCs in response to hypoxic stress, we knocked down Beclin-1 by siRNA. When compared with control siRNA, Beclin-1-specific siRNA significantly reduced the Beclin-1 protein (Fig. S2A) and mRNA (Fig. S2B) levels in rat HSCs as assessed by western blotting and real-time PCR. These results
showed that silencing of Beclin-1 attenuated the increase of LC3-II expression following treatment with 1% O2 for 12 h (Fig. 5D), indicating a functional inhibition of autophagy.

We then assessed the protein level of α-SMA in rat HSCs in the presence or absence of Beclin-1 siRNA transfection under 1% O2 hypoxic stimulation for 12 h. Transfection of rat HSCs with Beclin-1 siRNA significantly abolished the upregulation of α-SMA by hypoxia (Fig. 5E,F). Moreover, treatment with the autophagy inhibitor Bafilomycin A1 also significantly attenuated the induction of α-SMA expression by hypoxic stress (Fig. 5E,F). These results imply that blocking autophagy attenuates hypoxic stress-induced activation of HSCs.

**Discussion**

Hypoxic stress is a well-known microenvironmental factor associated with hepatic fibrosis. In the present study, we demonstrated that hypoxic stress induces autophagy in rat HSCs through Ca²⁺-, AMPK-, mTOR-, and PKCθ-mediated signaling pathways. In addition, inhibition of autophagy by the autophagy inhibitor Bafilomycin A1 and targeted silencing of Beclin-1 inhibited hypoxic stress-induced activation of HSCs. Our results indicate that autophagy may have an important role in the activation of HSCs under hypoxic conditions, suggesting that targeting autophagy is a promising strategy for the treatment of hepatic fibrosis.

Autophagy is a catabolic pathway that controls homeostasis in cells through self-digestion by lysosomal enzymes. Increasing evidence has shown a protective role of autophagy during stress [26]. It is well known that HSCs are major fibrogenic cells in liver. During hepatic fibrosis, autophagy has been suggested as a mechanism responsible for HSC activation, although both negative and positive effects of autophagy on hepatic fibrosis have been reported [5,27]. Moreover, recent data demonstrate that, in other liver cell types, autophagy decreases profibrogenic signals by protecting hepatocytes from apoptosis [28] and exerting anti-inflammatory effects in Kupffer cells [29]. However, some study found that autophagy promotes HSC activation and causes hepatic fibrosis [15,16]. Thus, the effect of autophagy on hepatic fibrosis is very complex. In our study, hypoxic stress, implicated in many liver diseases including hepatic fibrosis, increased the autophagy level in rat HSCs as analyzed using several methods, including the localization of LC3 and western blotting analysis for LC3, Beclin-1,
and P62. The autophagic flux has been widely used to reflect the dynamic process, including autophagosome synthesis, maturation, autophagosome lysosome fusion, macromolecule digestion, and lysosome recycling, and is a more reliable indicator of autophagic activity [30,31]. We found an increase in autophagic flux as demonstrated by the increase in LC-3 II level after cotreatment with chloroquine and hypoxic stress. The dynamic activation of autophagy indicated that the early phase of hypoxic stress might play a significant role to activate autophagy in rat HSCs.

HSC activation plays a fatal role in hepatic fibrosis. Several studies have indicated that the elevation of [Ca^{2+}]_{i} induced by stress is associated with the activation of autophagy [32]. The role of intracellular Ca^{2+} in the regulation of autophagy has been known since 1993 [33], and the intracellular Ca^{2+} level can be enhanced through Ca^{2+} influx from the extracellular space or the intracellular stores. It has been proposed that [Ca^{2+}]_{i}, which is transiently released by both intracellular Ca^{2+} release and extracellular Ca^{2+} influx, contributes to autophagy activation in cells exposed to hyperosmotic stress [34]. In the present study, we found that BAPTA-AM, but not EGTA, suppressed hypoxic stress-induced increases in [Ca^{2+}]_{i} in rat HSCs, indicating that Ca^{2+} released from intracellular stores is involved in the increase of [Ca^{2+}]_{i} in HSCs exposed to hypoxic stress. Moreover, we found that the PLC-specific inhibitor U73122 inhibited hypoxic stress-induced increases in [Ca^{2+}]_{i}, implying that Ca^{2+} release from intracellular stores in rat HSCs is PLC-dependent. When HSCs were exposed to hypoxic stress, hypoxia stress evoked transient increase in cytosolic calcium for about 100 s and correlated with the induction of autophagy in rat HSCs. Moreover, autophagy was activated as early as 6 h after exposure to 1% O_{2}, with the highest level at 12 h and a gradual reduction during longer exposure. These results demonstrated that the early phase of hypoxic stress might causes autophagy in rat HSCs. This long-lasting autophagy elevation by hypoxia can be explained that once a transient increases in [Ca^{2+}]_{i} by hypoxia activates some continuous proautophagic effects, and more studies are needed to elucidate the mechanism of lasting action.
AMPK, a metabolic and stress sensor, is composed of three subunits, namely α, β, and γ, and it is pivotal for the regulation of cell responses to various forms of metabolic stress [35]. The activation of AMPK results in the downregulation of mTOR activity, causing autophagy activation. Our results clearly illustrated hypoxic stress-induced autophagy via AMPK activation and mTOR inhibition. BAPTA-AM inhibited the decrease in phosphorylation of mTOR, indicating that calcium signaling is responsible for activation of the AMPK–mTOR pathway in rat HSCs.

The endoplasmic reticulum (ER) is an important intracellular calcium storage organelle in the cell. Members of the PKC family are activated by Ca2+ released from the ER [36]. Recently, a study has indicated that Ca2+-dependent PKCθ activation is essential for autophagy during ER stress [22]. Consistent with these previous studies, we found that phosphorylation of PKCθ was critical for hypoxic stress-induced autophagy in HSCs. Treatment with BAPTA-AM decreased PKCθ phosphorylation, demonstrating that Ca2+ is essential for PKCθ phosphorylation in rat HSCs. Moreover, several studies have shown that Ca2+-induced autophagy can be mediated by several pathways, including ERK, CaMKKβ, and DAPK [23–25]. However, in our study, we did not find apparent activation of these signaling pathways by hypoxic stress in rat HSCs, suggesting that hypoxic stress can induce HSC autophagy in an ERK-, CaMKKβ-, and DAPK-independent manner.

During the process of hepatic fibrosis, hypoxia occurs in the liver due to microvasculature damage, normal hepatic blood flow disruption, and excessive deposition of ECM in the sinusoidal space [37,38]. Several studies have found that a hypoxic microenvironment has a key role in the pathogenesis of hepatic fibrosis and the activation of HSCs [39,40]. Cells in response to various stresses may result in autophagy, which is an important catalytic process. Interestingly, hypoxia can induce the activation of autophagy to promote cancer cell survival [41]. In the present study, we detected the expression of HSC activation markers in rat HSCs. An increase in α-SMA and collagen type I expression in response to the hypoxic microenvironment in HSCs confirmed our hypothesis that hypoxic stress induces autophagy as well as the activation of HSCs. To reveal the relationship between autophagy and HSC activation, autophagy was inhibited by knocking down Beclin-1 using RNAi technology and using the autophagy inhibitor Bafilomycin A1. Beclin-1-deficient cells and Bafilomycin A1-treated cells had decreased α-SMA expression, suggesting that autophagy contributes to HSC activation during hypoxic stress.

Hypoxic stress induces a series of changes in HSCs [42]. Several previous studies have investigated the impact of hypoxia on gene expression in activated HSCs, with a particular focus on hypoxia-inducible factor (HIF)-1α, and found that HIF-1α is a hypoxia sensor responsible for the effect of hypoxic stress in HSCs [43,44]. Importantly, knocking out HIF-1α abolished the increase of autophagy during hypoxic stress [45], suggesting that regulation of autophagy by HIF-1α might be an important step for HSC activation. Therefore, further studies are required to explore whether HIF-1α mediates autophagy activation during hypoxic stress in HSCs through intercellular Ca2+ and the Ca2+-dependent signaling pathway, which might be therapeutic targets to treat hepatic fibrosis.

In conclusion, our results demonstrated that hypoxic stress could induce the activation of HSCs through autophagy. Elevation of [Ca2+]i from intracellular stores induced by hypoxic stress in HSCs may trigger Ca2+-AMPK–mTOR and PKCθ activation, which leads to enhanced HSC autophagy and ultimately causes HSC activation (Fig. 6). Because HSCs in hypoxic stress contribute to the development of hepatic fibrosis, it is reasonable to infer that hypoxia-
induced autophagy activation in vivo might cause the progress of hepatic fibrosis, which will be investigated in our future research.

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Author contributions

YJ contributed to the conception and design of the study, the acquisition of data, and the analysis and interpretation of the data. All authors participated in drafting or revising the manuscript, and all authors approved the final version of the manuscript for submission.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Table S1. The sequences of small interfering RNAs (siRNAs) of Beclin-1 and a negative control.

Table S2. The PCR primers used in this study.

Fig. S1. Hypoxia did not alter the phosphorylation of ERK, CaMKKβ, or DAPK in rat HSCs.

Fig. S2. Beclin-1 expression was efficiently reduced by Beclin-1 siRNA.