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Hypoxia-inducible factor-1alpha regulates autophagy to activate hepatic stellate cells

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ABSTRACT

The role of autophagy in Hif-1α modulated activation of hepatic stellate cells was illustrated in current work. Autophagy markers were determined in livers of *Schistosoma japonicum* infected mice and hypoxia or LPS treated human hepatic stellate cell, LX-2 cells. The action of Hif-1 to autophagy was defined as increase of autophagy markers was significantly suppressed in Hif-1α siRNA transfected cells upon hypoxia or LPS stimulation. The function of autophagy in activation of LX-2 cells was assessed as increase of activation markers was blocked using autophagy inhibitors under hypoxia and LPS stimulation. Conclusively, Hif-1α regulates activation of hepatic stellate cell by modulating autophagy.

1. Introduction

Liver fibrosis is a reversible wound-healing pathological process, which follows acute or chronic liver injuries, including viral hepatitis, alcoholic hepatitis, non-alcoholic steatohepatitis, parasitic infections like schistosomiasis and autoimmune diseases [1–2]. It is characterized by excessive deposition of extracellular matrix (ECM), especially collagen in the liver [3]. The activation of hepatic stellate cell (HSC) is the key event of liver fibrosis. Quiescent HSCs are lipid droplets storing cells. While during chronic liver injury, HSCs transform into myofibroblast-like cells (MFC) which results in expression of vimentin, α-smooth-muscle actin (α-SMA) [4], synthesizing plenty of ECM, and loss of lipid droplets. Therefore, to study the mechanism of HSC activation will provide novel targets for prevention of hepatic fibrosis.

Autophagy is an evolutionary conserved process by which cells metabolize and degrade components like proteins, cellular organelles and invading microbes [5]. It is usually activated by hypoxia or starvation, and is also closely related with human pathophysiological processes such as growth, differentiation, inflammation, infection and tumor. The process of autophagy is involved in formation of double-membrane autophagosome in which cellular contents are engulfed, and then autophagosome fuses with lysosome to form autolysosome where cellular substances are degraded by lysosomal enzymes [6–8]. Recent studies have shown that autophagy can exacerbate liver diseases [9]. Moreover, some researchers have discovered that inhibition of autophagy leads to accumulation of lipid droplets in HSC, resulting in decreased fibrogenesis [10], indicating that autophagy plays an important role in HSC activation and lipid metabolism.

Hypoxia inducible factor-1 (Hif-1) is an important transcriptional factor that mediates cellular responses to hypoxia and stressors like infection and inflammation. It is a heterodimer consisting of an α-subunit Hif-1α and β-subunit Hif-1β. Hif-1α is highly regulated by micro-environmental oxygenation, whereas Hif-1β is constitutively expressed [11–12]. Our previous researches have revealed that Hif-1α is involved in activation of HSC [13]. In this paper, we want to explore whether autophagy participates in Hif-1α modulated activation of HSC, thus, understanding liver fibrosis in depth.

In this study, we firstly detected autophagy markers in liver tissues of *Schistosoma japonicum* infected mice, to observe whether autophagy occurs in liver infection accompanying with HSC activation. A human hepatic stellate cell line LX-2 was further used to evaluate the effect of Hif-1α to autophagy and HSC activation

Abbreviations: HSC, hepatic stellate cell; Hif-1α, hypoxia-inducible factor-1α; α-SMA, α-smooth muscle actin; LC3B, microtubule-associated protein light chain3B; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; ECM, extracellular matrix; MFC, myofibroblast-like cell; ATG, autophagy-related gene.

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under hypoxia or LPS treatment, to further investigate HSC activation mechanism.

2. Materials and methods

2.1. Animals

BALB/c female mice, 6–8 weeks old, were obtained from the Wuhan Institute of Biological Products, Wuhan, China. The experiment was approved by the Committee on Animal Research of Tongji Medical College, Huazhong University of Science and Technology. Mice were randomly divided into two groups: the infected group and the control group. Oncomelania snails infected with *S. japonicum* were purchased from Hunan Province Institute of Parasitosis Control and Prevention, Yueyang, China. *S. japonicum* cercariae were shed from the snails. Each anaesthetized mouse in the infected group was percutaneously infected with 25 cercariae through the shaved abdomen. The mice were sacrificed at 6 or 8 weeks post-infection and samples of liver were collected [14].

2.2. Immunohistochemistry

The formalin-fixed and paraffin-embedded liver tissues were cut into 4-μm sections and then deparaffinized routinely [15]. The slides were incubated with antibodies (vimentin, 2707-1, Epitomics, USA; VEGF, sc-7269, Santa-Cruz, USA; α-SMA, 1184-1, Epitomics, USA; LC3B, 12741, Cell signaling, USA), then washed with PBS and incubated with Envision™ (DAKO, Shanghai, China; polyperoxidase-anti-mouse/rabbit IgG). After washing, the slides were colored with 3,3-diaminobenzidine and counterstained with haematoxylin [15].

2.3. Cell culture, stimulation and chemical regents

Human hepatic stellate cell line, LX-2, was used as cell model [16]. LX-2 was cultured at 37 °C in room air (HF151, Heal Force, China) or in 1% oxygen in incubator (HF100, Heal Force, China) in Dulbecco's modified Eagle medium with 5% CO_2 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells seeded in 6-well plate were stimulated by 2 μg/ml LPS (Sigma, L-2880, USA). Rapamycin(S1039, Selleckchem, USA) was used as a positive control to induce autophagy and chemical inhibitors (LY294002, S1737, Beyotime, China; chloroquine, C6628, Sigma, USA) were utilized to prevent autophagy process.

2.4. siRNA transfection

LX-2 cells were plated at a density of 2×10^5 cells per well in 6-well plates. Eighteen hours post-seeding, cells were transfected with 50nM specific siRNA (Hif-1α, sc-35561, Santa-Cruz, USA) or nonspecific (NS) siRNA using Lipofectamine 2000 (Invitrogen, USA) following manual instructions.

2.5. Western blot

Cells were collected at indicated time and protein samples were obtained as described [13]. Western blotting was performed to determine the expression of Hif-1α (2015-1, Epitomics, USA), vimentin, α-SMA, LC3B, GAPDH (KC-5G5, Kangchen, China), Beclin-1 (3495, Cell signaling, USA), Atg5 (12994, Cell signaling, USA), and β-actin (sc-1616, Santa-Cruz, USA).

2.6. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and counterstained with antibodies against LC3B and vimentin. The coverslips were mounted onto microscope slides in Antifade Mounting Medium (P0126, Beyotime, China) and fluorescent images were visualized and captured using Olympus BX51 upright fluorescent microscope (Olympus, Japan).

2.7. Statistical analysis

Statistical significance was assessed by 1-way ANOVA using GraphPad Prism 5, $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Molecular markers of autophagy and HSC activation apparently increased in liver of S. japonicum infected mice

We have previously demonstrated that Hif-1 regulated cascade plays an important role in HSC activation as livers were injured by infections such as *S. japonicum*. In order to detect whether autophagy is involved in Hif-1 regulated HSC activation, mouse model of *S. japonicum* infection was used to test occurrence of autophagy and HSC activation. Cell infiltration and granulomatous inflammation surrounding eggs of *S. japonicum* were formed in livers of infected mice (Fig. 1A), as described elsewhere [15].

LC3 (microtubule-associated protein light chain 3), a mammalian homolog of yeast Atg8, is a widely recognized protein for monitoring autophagic activity. There are three isoforms of LC3 in mammalian cells including LC3A, LC3B and LC3C, among which, LC3B is a widely used autophagy marker [17]. After synthesis, LC3 is cleaved to generate cytosolic LC3-I and lipidated LC3-II. Since lipidated LC3-II is the actual form involved in formation of autophagosome and autolysosome [18–19], therefore, in this study, we exploit LC3B-II or LC3B-II/LC3B-I to detect autophagy activity.

We firstly detected LC3B, vimentin, VEGF and α-SMA in livers of *S. japonicum* infected mice compared with non-infected mice (Fig. 1B and C), which indicated that LC3B obviously increased in liver tissues as HSCs were activated. Positive LC3B expressing cells are morphologically alike with vimentin, VEGF or α-SMA positive cells, accordingly, non-parenchymal liver cells, or mesenchymal cells. However, in tissue sections, autophagy-processing LC3B-II form is not able to be recognized.

Quantitative expression of HSC activation markers, vimentin, VEGF, α-SMA, and expression ratio of LC3B-II (14 kDa) to LC3B-I (16 kDa) was evaluated with Western blot using proteins extracted from liver tissues of mice (Fig. 1D). The results revealed that vimentin, VEGF and α-SMA apparently increased in liver extract of infected mice compared with non-infected mice, with the duration of infection from 6 weeks to 8 weeks, as well as the ratio of LC3B-II/LC3B-I (Fig. 1D).

Histological evidence from immunohistochemistry and quantitative analysis from biochemistry presented above pointed out that autophagy might be involved in HSC activation and start-up of hepatic fibrosis.

3.2. 1% O_2 and LPS induced formation of autophagic vacuoles and increased expression of autophagy molecular markers

LPS, known as endotoxin, the pathogenic component of Gram-negative bacteria, was used to be another stimulating factor to mimic pro-inflammatory condition, aiming to explore Hif-1 regulating mechanism in broader physiological pathology scope. To test

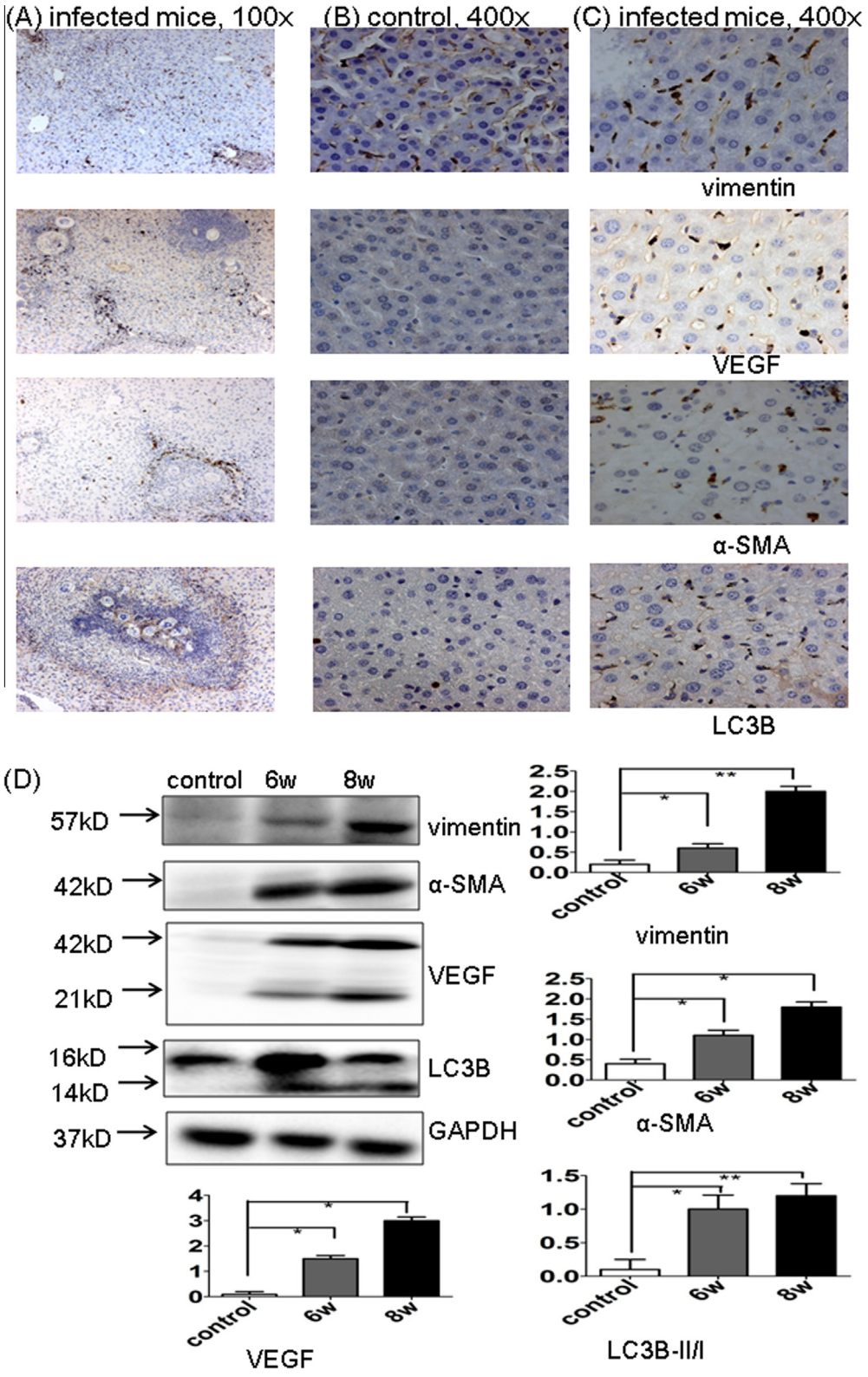

Fig. 1. Molecular markers of autophagy and HSC activation apparently increased in liver of *s. japonicum* infected mice. BALB/c female mice, 6–8 weeks old, were percutaneously infected with 25 cercariae of *Schistosoma japonicum* through the shaved abdomen, sacrificed at 6 or 8 weeks post-infection and samples of liver were collected. The expression of vimentin, VEGF, α-SMA and LC3B in *Schistosoma japonicum* infected ($n = 3$) and non-infected ($n = 3$) mice liver was detected with immunohistochemistry and Western blot. (A) Acute inflammatory cell infiltration and granulomatous inflammation, surrounding eggs of *Schistosoma japonicum*. (B) Expression of vimentin, VEGF, α-SMA and LC3B in non-infected mice liver. (C) Expression of vimentin, VEGF, α-SMA and LC3B in mice liver 6 weeks post-infection. (D) Western blot was applied to detect vimentin, α-SMA, VEGF, LC3B and GAPDH from protein extracted from the liver of non-infected mice, infected mice 6 weeks post-infection and infected mice 8 weeks post-infection ($n = 3$). Densitometric analysis was performed and data are mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$.

the accumulation of autophagic puncta, human hepatic stellate cell line LX-2 was cultured under 1% O_2 or 2 μg/ml LPS for 24 h, 100 nM rapamycin-treated cells using as positive control, and immunocytochemistry was performed to detect LC3B. It was found that

autophagic vesicles formed clearly in both hypoxic and LPS treated cells and also in rapamycin-treated cells, however, not in control cells (Fig. 2A). Molecular markers of HSC activation and autophagy were further detected from cell lysates treated at different time (Fig. 2B). The results demonstrated that, accompanied by the induction of Hif-1α, expression of α-SMA, LC3B-II, Beclin-1 and Atg5, was also up-regulated, illustrating that hypoxia and LPS led to occurrence of autophagy in HSC.

3.3. Knockdown of Hif-1α inhibited increased expression of autophagy molecular markers

In order to determine whether Hif-1α regulates autophagy in HSC upon hypoxia and LPS stimulation, specific siRNA targeting *hif-1α* was used to block synthesis of Hif-1α in LX-2 cells (Fig. 3A and D). Expression of LC3B and Beclin-1 was then assessed when cells were treated with 1% O_2 and 2 μg/ml LPS. It was shown that inhibition of Hif-1α expression significantly suppressed increase of LC3B-II and Beclin-1 in LX-2 cells upon hypoxia, revealing that Hif-1α regulates HSC activation through autophagy (Fig. 3A, B and C). In LPS treated cells, increase of LC3B-II was also suppressed by Hif-1α knockdown (Fig. 3D).

3.4. Blocking of autophagy inhibited increased expression of HSC activation molecular markers

To explore whether autophagy is the mechanism involved in HSC activation, chemical autophagy inhibitors LY294002 (PI3K inhibitor) [20] and chloroquine (lysosome inhibitor) [21] were used to prevent the process of autophagy in HSC prior to hypoxia or LPS treatment. HSC activation markers α-SMA, vimentin were further detected. It was found that cells pretreated with 30 μM LY294002 or 50 μM chloroquine for 3 h inhibited increased expression of α-SMA at 15 min and 1 h (Fig. 4A) upon hypoxia treatment. However, at 24 h of hypoxic treatment, α-SMA expression was recovered (Fig. 4A). Likewise, when cells were pretreated with chloroquine for 3 h, the increased expression of α-SMA in LPS-treated cells was abolished (Fig. 4A), indicating that inhibition of autophagy suppressed HSC activation under inflammatory conditions.

Another HSC activation marker, vimentin was detected with immunofluorescence staining (Fig. 4B). Cells were pretreated with 30 μM LY294002 and 50 μM chloroquine for 3 h, and then exposed to 1% O_2 for 1 h. Similarly with α-SMA, enhanced level of vimentin triggered by hypoxia was suppressed when autophagy activity was inhibited by LY294002 and chloroquine.

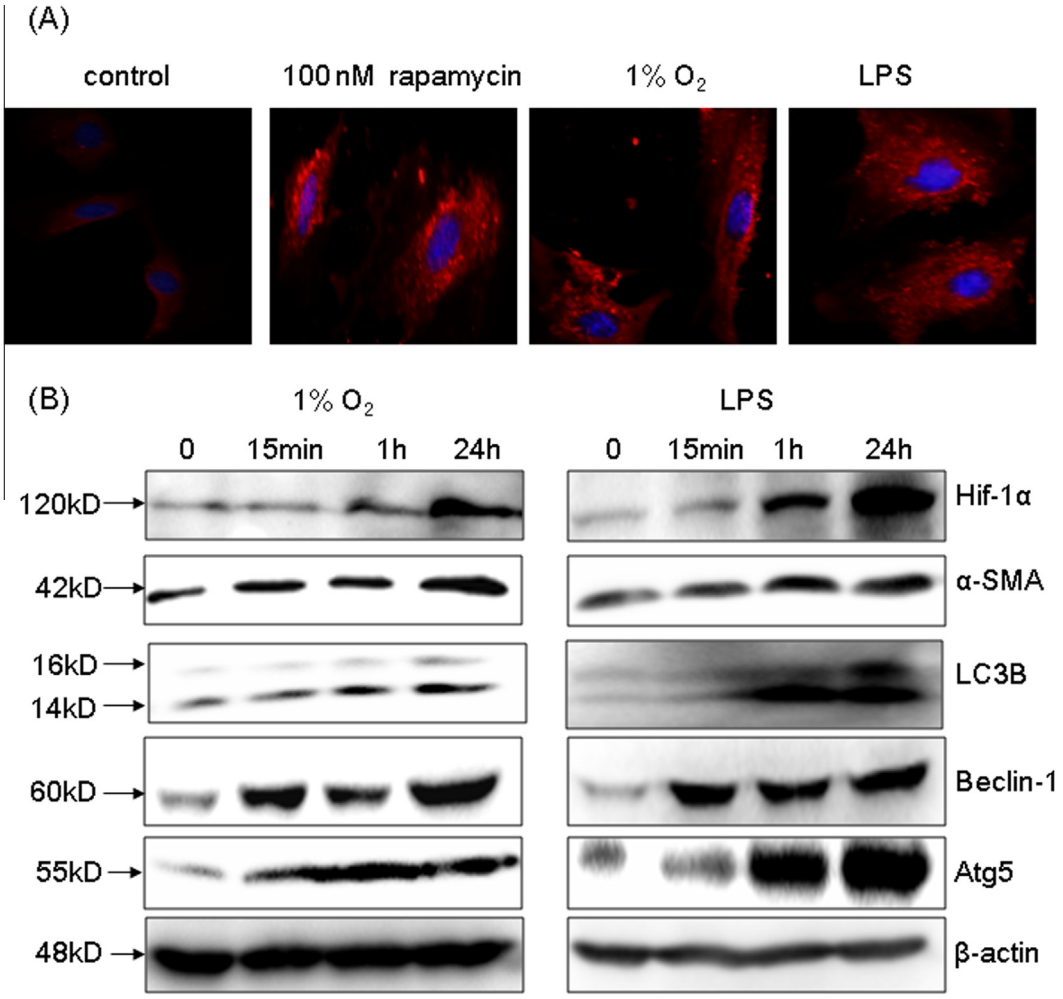

Fig. 2. 1% O_2 and LPS induced autophagic activity of hepatic stellate cells. (A) Cells were treated with 100nM rapamycin, 1% O_2 and 2 μg/ml LPS for 24 h, and immunofluorescence assay was performed to detect LC3B (red) which reflects the formation of autophagic vesicles. Rapamycin treatment was used as a positive control and DAPI (blue) was used as nuclear staining (magnification is 400×). (B) cells were collected at indicated time and cell lysates were subjected to detect Hif-1α, α-SMA, LC3B, Beclin-1, Atg5 and β-actin with Western blot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

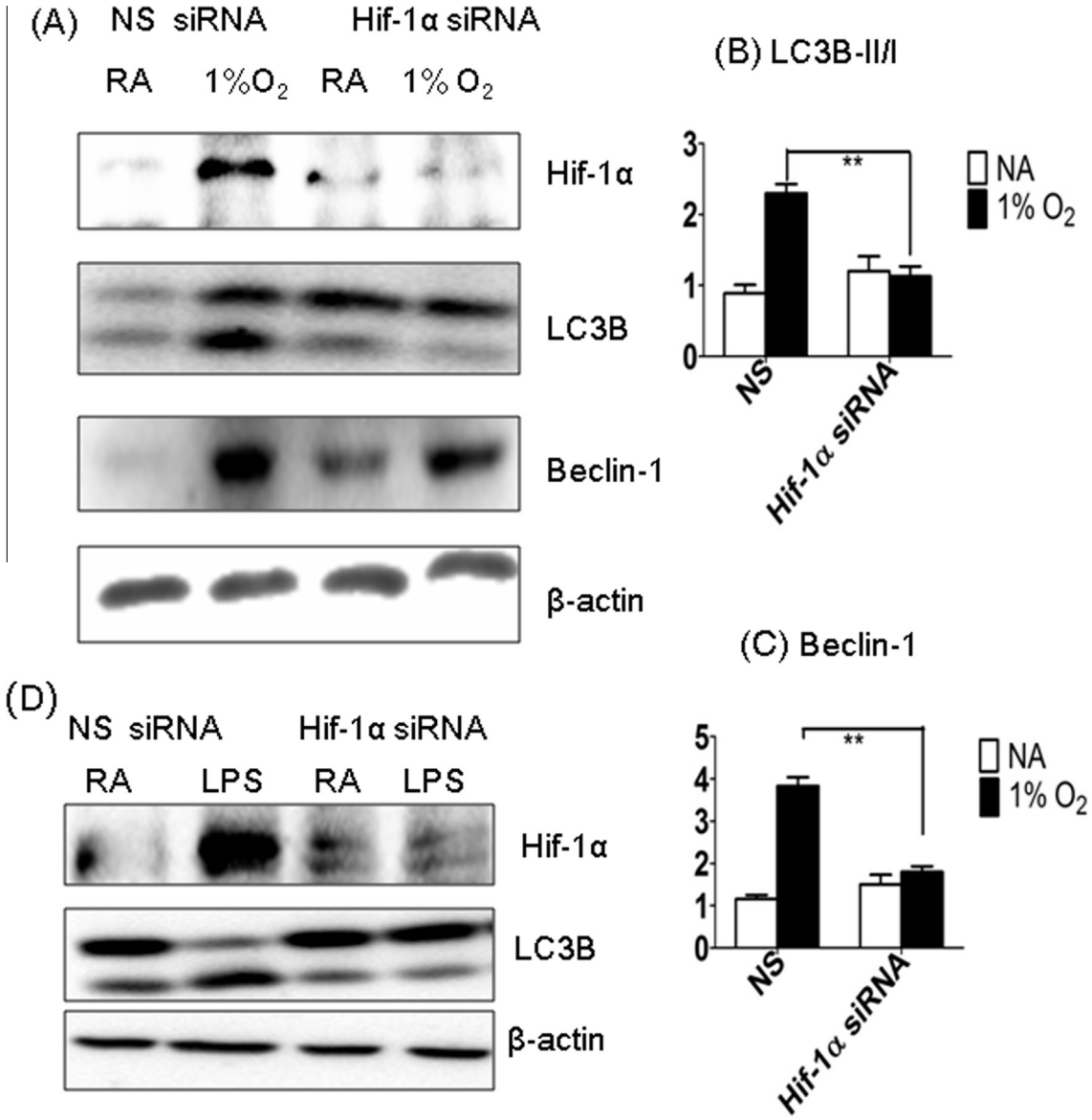

Fig. 3. Knockdown of Hif-1α inhibited increased expression of autophagy molecular markers. LX-2 cells in 6-well plates were transfected with either 50 nM Hif-1α siRNA or nonspecific (NS) siRNA for 48 h and then cultured in room air or in 1% oxygen or 2 μg/ml LPS for 24 h. (A, D) Cells were collected and cell lysates were subjected to detect Hif-1α, LC3B, Beclin-1 and β-actin with Western blot; Densitometric analysis was performed using pooled data from three such experiments. Data are mean ± SD. (B) LC3B-II/I; (C) Beclin-1, $^{*}P < 0.05$, $^{**}P < 0.01$ (RA: room air).

4. Discussion

The progression of liver fibrosis is involved in excessive deposition of fibrotic collagen and other extracellular matrix (ECM) proteins which distorts the normal hepatic architecture and causes portal hypertension. Liver fibrosis is reversible in its early stage, while chronic and persistent fibrogenesis will lead to irreversible cirrhosis, hepatocellular carcinoma and ultimately liver failure [22]. Therefore, studying the pathogenesis of hepatic fibrosis is helpful to discover new targets to inhibit fibrogenesis.

It is known that activated hepatic stellate cells (HSCs) are the main source of ECM, and inhibition of HSC activation is an effective method to prevent the progression of liver fibrosis. Our previous researches have demonstrated that local inflammatory and hypoxic micro-environment plays an important role in the pathogenesis of liver fibrosis and activation of HSCs. Moreover, Hif-1α acts as a key regulator in modulating HSCs activation. Whereas, the exact mechanism of this process is still rarely known, thus, further investigations are needed to clarify how Hif-1α participates in the activation of HSCs.

Cells exposed to various stressors may activate autophagy, an important catalytic process. Recent studies have revealed that hypoxia can activate autophagy, regulating cancer cell survival [23], and some reports have revealed that HSCs can utilize autophagy to degrade intracellular lipid droplets to fuel HSC activation [24,25], indicating that autophagy may be a crucial signaling pathway regulating HSC activation and fibrogenesis.

In present study, first of all, we detected the expression of autophagy and HSC activation markers in liver tissues of *S. japonicum* infected mouse. Increase of vimentin, α-SMA and autophagic marker LC3B in the liver of *S. japonicum* infected mouse, confirmed our hypothesis that autophagy occurred during liver injuries, as well as the activation of HSCs. Although morphologically positive LC3B expressing cells seem alike with vimentin, VEGF or α-SMA positive-indicated cells, mainly non-parenchymal liver cells, however, whether autophagy arises in activated HSCs due to *S.*

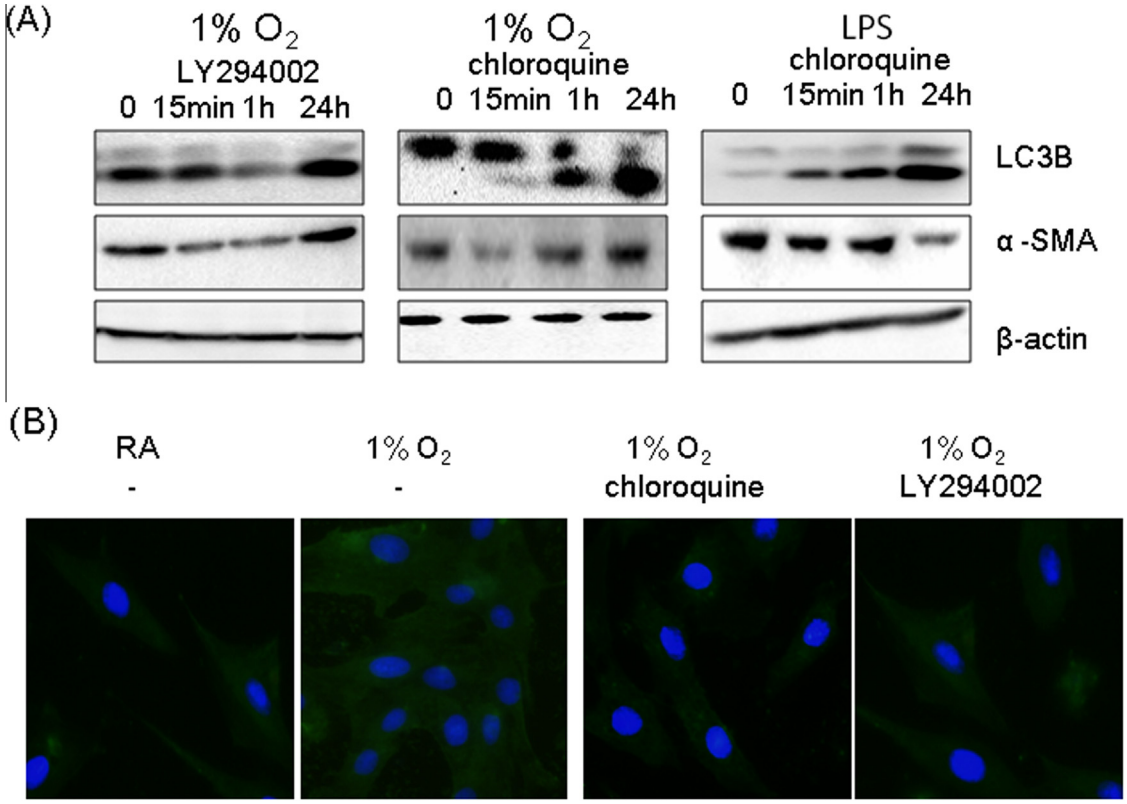

Fig. 4. Blocking of autophagy inhibited increased expression of HSC activation molecular markers. LX-2 cells in 6-well plates were pretreated with 50 μM chloroquine or 30 μM LY294002 for 3 h and then cultured in room air or 1% oxygen or 2 μg/ml LPS for 24 h. (A) Cells were collected and cell lysates were subjected to detect LC3B, α-SMA and β-actin with Western blot. (B) Immunofluorescence assay was performed to detect the expression of vimentin (green) exposed to hypoxia for 1 h, DAPI (blue) was used as nuclear staining, and the magnification is 400× (RA: room air). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

japonicum infection, still needs further investigation. Moreover, whether autophagy observed in livers from *S. japonicum* infected mice is regulated by Hif-1, which is also increased in tissue section in infected mice presented in our previous work, is worthy to research.

Then, in order to further verify our hypothesis, a human cell line LX-2 was exposed to 1% O_2. The results demonstrated that the number of LC3B punctate structures was apparently enhanced, with the increase of Beclin-1, Hif-1α, α-SMA and LC3B-II/LC3B-I, displaying that autophagy participates in activation of HSCs and Hif-1α might act as a regulator in this process. When *hif-1α* was silenced by siRNA, the increase of LC3B-II and Beclin-1 was inhibited upon hypoxic treatment, which reminds that Hif-1 might target some ATGs to modulate autophagic activity in HSCs upon hypoxia. Apart from these, another marker of autophagy, Atg5, was also tested. When *hif-α* was silenced by siRNA, Atg5 expression level did not exhibit obvious alteration in LX-2 under hypoxic circumstance (data not shown), implying that Atg5 may not be directly regulated by Hif-1α. Whether *lc-3* and *beclin-1* are target ATG genes transcriptionally or through indirect mechanism regulated by Hif-1, remains further investigation.

We further want to study whether autophagy mediates HSC activation regulated by Hif-1, hence, chemical inhibitors LY294002 and chloroquine were used to suppress autophagic activity before hypoxia stimulation, followed by evaluation of HSC activation. It was found that increase of activation markers α-SMA and vimentin was markedly suppressed in HSCs upon hypoxic exposure as pretreated with autophagic inhibitors, which indicated that inhibition of autophagy blocks the activation of HSCs upon hypoxic exposure. Nevertheless, the suppression of HSC activation upon hypoxia by autophagic inhibitors was recovered when the hypoxic time extended to 24 h. It was supposed that the limited blocking effect of autophagy inhibitors to HSC activation upon hypoxia might be related with active duration of chemical inhibitors of autophagy.

Besides hypoxia, inflammatory micro-environment is also involved in the progression of liver fibrosis. Thus, LPS, the pathogenic component of Gram-negative bacteria, was used to mimic pro-inflammatory condition. LPS is enriched within the intestinal lumen and portal circulation, and can arrive at liver through portal blood under some conditions, leading to liver damages related to fibrogenic reactions. The results showed that autophagic activity, HSC activation as well as enhanced accumulation of Hif-1α were induced upon LPS stimulation, demonstrating that autophagy and Hif-1 regulated cascade are important mechanisms of HSCs activation in not only hypoxic but also inflammatory micro-environment. Different from hypoxia treatment, the expression of HSC activation markers α-SMA did not recover when stimulated with LPS for 24 h, which might be caused by the different reactive extent of cells to these two factors.

In conclusion, our studies demonstrate that autophagy plays a crucial role in HSCs activation. Moreover, Hif-1α modulates autophagy and acts as an important regulator for autophagy in hepatic stellate cells. On the whole, our researches may provide new understanding of HSC activation and pathogenesis of liver fibrosis, but further studies are still needed to clarify the exact mechanism of Hif-α targeted autophagy.

Acknowledgments

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