TLR4 mediates inflammation and hepatic fibrosis induced by chronic intermittent hypoxia in rats

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Received December 18, 2018; Accepted March 5, 2020

DOI: 10.3892/mmr.2020.11134

Abstract. Obstructive sleep apnea syndrome (OSAS) is a common and complex disorder that is associated with liver injury. Moreover, previous studies have revealed that chronic intermittent hypoxia (CIH) is associated with the development of non-alcoholic fatty liver disease and hepatic fibrosis. However, the underlying molecular mechanisms remain largely unknown. The present study aimed to investigate whether chronic intermittent hypoxia induced hepatic fibrosis, in addition to determining its underlying mechanisms, in CIH model rats using immunohistochemistry, western blotting and reverse transcription-quantitative PCR. The present results suggested that CIH caused hepatic fibrosis and increased the expression levels of interleukin (IL)-1β, IL-8, monocyte chemotactic-1, tumor necrosis factor-α, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in the liver; these conditions could be reversed by Toll-like receptor 4 (TLR4) short hairpin RNA lentivirus treatment. Moreover, immunohistochemistry and western blotting results indicated that TLR4 and NF-κB expression levels were significantly increased in the CIH and CIH-TLR4 empty vector lentivirus group. However, protein expression levels of TLR4, NF-κB, inhibitor of NF-κB and phosphorylated-mitogen-activated protein kinase (MAPK)-1 in the hypoxia/reoxygenation group were significantly higher compared with the control group ($P<0.05$), and these results were reversed by the MAPK inhibitor U0126 *in vitro*. Collectively, the present preliminary results suggested that inflammation and the TLR4/NF-κB/MAPK signaling pathway may be involved in CIH-induced liver fibrosis.

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Key words: obstructive sleep apnea syndrome, chronic intermittent hypoxia, inflammation, liver fibrosis, Toll-like receptor 4

Introduction

Obstructive sleep apnea syndrome (OSAS) is a common and complex disorder consisting of complete or partial upper airway obstruction and sleep fragmentation, affecting >4% of the world's general population and 30-45% of patients with obesity (1-3). As a result of upper airway collapse, OSAS causes repeated nocturnal hypoxia and alternate episodes of normoxia, such chronic intermittent hypoxia (CIH) during sleep, resembling the pathophysiologic mechanisms in ischemia/reperfusion multi-organ injury, which can trigger cardiovascular morbidity, lung injury and chronic kidney disease (4-7). Moreover, previous studies have reported that CIH contributes to the pathogenesis of non-alcoholic fatty liver disease and exacerbates liver fibrogenesis (2-8). However, the underlying mechanisms of hepatic fibrosis in patients with OSAS are not fully understood.

Epidemiological studies and clinical investigations have shown that OSAS is a type of chronic and mild systemic inflammatory response disease (8,9). For example, inflammatory factors such as interleukin (IL)-β, IL-6, IL-18, tumor necrosis factor-α (TNF-α), interferon-γ and C-reactive protein (CRP) can be detected at high levels in the blood of patients with OSAS (10,11). Furthermore, Ryan and McNicholas (11) revealed a significant positive association between serum TNF-α levels and OSAS severity, with higher serum levels of TNF-α in patients with OSAS compared with non-OSAS subjects. In addition, Drager *et al* (12) reported that the expression of CRP declined in adult patients with OSAS after effective treatment with continuous positive airway pressure. Therefore, these findings suggest that CIH may be associated with inflammation and may contribute to the increased incidence of liver fibrogenesis in patients with OSAS. However, although several studies have closely linked CIH to systemic inflammation, the mechanism of this association has not been fully established.

Recent studies have shown that inflammation is associated with innate immune activation, including that involving Toll-like receptors (TLRs) and its underlying signal pathway (13,14). TLRs belong to a class of receptors with well-known pattern recognition, and can accurately perceive pathogens and bacterial-derived molecules (15). Moreover, activation of TLRs can lead to inflammatory responses, thus

increasing the production of proinflammatory cytokines (16). TLR4, a typical representative of TLRs, mediates both innate and adaptive immune responses and plays an essential role in promoting inflammation activation (14,17). It has also been reported that TLR4 can trigger the main adaptor protein, myeloid differentiation factor 88 (MyD88)-dependent pathway, leading to rapid activation of the classical NF-κB and mitogen-activated protein kinase (MAPK) signaling pathway, which upregulates the transcription and translation of proinflammatory genes, such as IL-1β, IL-6, IL-8, IL-18 and TNF-α (18,19). Previous studies have revealed that CIH can increase mRNA and protein expression levels of TLR4 in the heart and hypothalamus of rats, and can induce myocardial remodeling and neuronal cell damage in hippocampus, which may be involved in CIH-induced inflammation (20,21). Furthermore, it has been shown that TLR4 expression in the serum of patients with OSAS is significantly increased compared with healthy individuals (22). However, the relationship between abnormal expression of TLR4 and the severity of liver injury in patients with OSAS has not been fully elucidated, and there is limited information on the downstream changes of TLR signal transduction after CIH exposure.

The aims of the present study were as follows: i) To investigate whether CIH exposure can induce similar liver fibrosis pathological changes in rats as in patients with OSAS; ii) to examine whether CIH affects proinflammatory cytokine production in the liver; and iii) to identify the underlying molecular mechanisms and signaling pathways that may be involved in CIH-induced liver fibrosis.

Materials and methods

Animals. A total of 24 adult male Sprague-Dawley rats (age, 9 weeks; weight, 200±10 g) were purchased from Shanghai Xipuer-Bikai Laboratory Animal Co., Ltd. Rats were fed and housed in a standard pathogen-free environment with a 12 h light/12 h dark cycle (7:00 a.m. lights on and 7:00 p.m. lights off automatically). The rats were provided with special compound diet and sterilization water *ad libitum*. Room temperature and humidity were controlled at ~23±2˚C and 50±10%, respectively. All animal procedures were approved by the Ethical Committee of Experimental Animals of Fujian Medical University and The Second Affiliated Hospital of Fujian Medical University. Animal experiments were carried out in accordance with animal welfare requirements and the Guidelines for the Care and Use of Laboratory Animals published by the P.R. China Ministry of Health (January 25, 1998) (23).

CIH animal model construction and experimental design. Rats were divided into the control (Con) group (n=6), CIH group (n=6), CIH + TLR4 short hairpin (sh)RNA lentivirus group (CIH-TLR4) (n=6) and CIH + TLR4 empty vector lentivirus group (CIH-Vector) (n=6). Firstly, 10 μl ($2x10^6$ TU/μl) TLR4 shRNA lentivirus (5'-GATCCGCACTCTT GATTGCAGTTTCATTCAAGAGATGAAACTGCAATCA GAGTGCTTTTTTG-3'), scrambled TLR4 shRNA (5'-GTA GCTAAATGATGAACAACATGGCCTTATATCTCCTAGT AATCTTCGTGGCTTCGTAGTGAT-3') and TLR4 shRNA empty vector lentivirus (Shanghai GenePharma Co., Ltd.) were separately injected into the rats of the CIH-TLR4 group, Con group or CIH-Vector group, respectively, via the tail vein. After 1 week of recovery, each rat was placed in a transparent plastic container (length, 40 cm; width, 30 cm; height, 18 cm), which was connected to a device for controlling O_2 concentration and pressure (Puhe Biotechnology). For consistency with the rat sleep cycle, the experimental time was set from 8:00 to 20:00 every day. Rats in the Con group were supplied with air, whereas those in the CIH, CIH-TLR4 and CIH-Vector group were subjected to intermittent hypoxia treatment, by injecting 95% N_2 for 20 sec to rapidly reduce O_2 concentration to 7.4-7.8%. Container pressure was reduced to 600 mmHg. The set-up was held for 12 sec, and then injected with air for 28 sec to recover O_2 concentration to 21% and achieve normal air pressure. The entire experiment lasted for 4 weeks.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). After CIH modeling, all rats were anesthetized (sodium pentobarbitone, 40 mg/kg) and sacrificed by exsanguination. A small piece of liver in each group was quickly cut and removed. RNA was extracted using a RNeasy Mini kit (Qiagen GmbH) following the manufacturer's protocol and stored at -80˚C for subsequent use. Total RNA was reverse transcribed into cDNA at 42˚C for 30 min using a QuantiTect Reverse Transcription kit (Qiagen GmbH), according to the manufacturer's protocol. qPCR reactions were performed using iTaq SYBR Green kits (Toyobo Life Science), according to the manufacturer's protocol. All reactions were carried out in duplicate and the cycles were run on a Bio-Rad CFX96 RT system (Bio-Rad Laboratories, Inc.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 30 sec; followed by 35 cycles of annellation at 58˚C for 40 sec and elongation at 72˚C for 30 sec. The primers used were as follows: IL-1β forward, 5'-AGGAGAGACAAGCAA CGACAA-3' and reverse, 5'-GTTTGGGATCCACACTCT CCA-3'; IL-8 forward, 5'-ATGGCTGCTGAACCAGTAGA-3' and reverse, 5'-CTAGTCTTCGTTTTGAACAG-3'; monocyte chemotactic-1 (MCP-1) forward, 5'-TCCACCACTATGCAG GTCTC-3' and reverse, 5'-TGGACCCATTCCTTATTGGG-3'; TNF-α forward, 5'-AGAACTCCAGCCGGTGTCTGTG-3' and reverse, 5'-GTGGCAAATCGGCTGACGGTGT-3'; intercellular adhesion molecule-1 (ICAM-1) forward, 5'-GGC GTCCATTTACACCTATTA-3' and reverse, 5'-TTCCTTTTC TTCTCTTGCTTG-3'; vascular cell adhesion molecule-1 (VCAM-1) forward, 5'-AACTGCACGGTCCCTAAT-3' and reverse, 5'-AGATGGTGGGTTCTTTCG-3'; and β-actin forward, 5'-AGCCATGTACGTAGCCATCC-3' and reverse, 5'-ACCCTCATAGATGGGCACAG-3'. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (24).

Tissue immunohistochemistry. After CIH modeling, all rats were anesthetized (sodium pentobarbitone, 40 mg/kg) and sacrificed by exsanguination. A small piece of liver in each group was quickly cut and fixed with 10% formalin for 48 h at 4˚C. Samples were then embedded in paraffin and cut into tissue sections (3-μm) using a tissue microtome. The deparaffinized tissue sections were subjected to hematoxylin and eosin (H&E) staining, both for 10 min at room temperature, and observed under a light microscope (magnification, x400) to obtain accurate pathological diagnosis.

Different sections in each group were stained via standard Sirius red staining at room temperature for 30 min to observe and determine reactive fibrosis, along with its distribution as described previously (25). Liver tissues were blocked with 5% BSA (Toyobo Life Science) for 1 h at room temperature followed by incubation with IL-1β (Abcam; cat. no. ab9787), IL-8 (Santa Cruz Biotechnology, Inc.; cat. no. sc-376750), MCP-1 (Abcam; cat. no. ab25124), TNF-α (Abcam; cat. no. ab9755), ICAM-1 (Abcam; cat. no. ab171123), VCAM-1 (Abcam; cat. no. ab78712), TLR4 (Abcam; cat. no. ab95562) and NF-κB antibodies (Abcam; cat. no. ab16502) diluted at 1:100 in PBS with 5% BSA (Toyobo Life Science) at 4˚C overnight. After washing with PBS three times, all tissue sections were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Abcam; cat. nos. ab7090 and ab97040) diluted at 1:200 at room temperature for 30 min to assess liver inflammation in rats. The sections were then stained with 3'3-diaminobenzidine at room temperature for 15 min and the immune reaction results were observed by light microscopy (magnification, x400). The statistical results of these immunohistochemical images were analyzed by ImageJ version 1.52t software (National Institutes of Health).

Western blot analysis. Total protein of each group was isolated from the liver tissue by ultrasonic homogenization (20 kHZ; 15 sec; 4˚C) in pre-cooled cell lysis solution (Sangon Biotech Co., Ltd.) containing protease and phosphatase inhibitors to inhibit protein degradation (Sangon Biotech Co., Ltd.). Then, cell lysate products were centrifuged at 10,000 x g for 30 min at 4˚C in a refrigerated centrifuge. Total protein was quantified using the bicinchoninic acid assay method. Proteins (30 μg/μl/lane) were separated by 10% SDS-PAGE in Tris-glycine-SDS buffer by vertical electrophoresis for 90 min. After separation via electrophoresis, proteins were immediately transferred to prepared nitrocellulose (NC) membranes using a trans-blot transfer system (Sangon Biotech Co., Ltd.). NC membranes were blocked with 5% skim milk diluted by 2% PBS (Sangon Biotech Co., Ltd.) for 60 min at room temperature and then incubated with primary anti-TLR4 (Abcam; cat. no. ab95562; 1:1,000), anti-inhibitor of NF-κB (IκB; Abcam; cat. no. ab32518; 1:500), anti-MAPK-1 (Abcam; cat. no. ab32081; 1:500), anti-phosphorylated (p)-MAPK-1 (Abcam; cat. no. ab223500; 1:500), anti-NF-κB p65 (Abcam; cat. no. ab16502; 1:500) and anti-β-actin (Abcam; cat. no. ab8226; 1:3,000) antibodies overnight at 4˚C. Then, the antibodies were diluted with 5% BSA (Sangon Biotech Co., Ltd.) in TBS-0.1% Tween-20. After washing with PBS three times, NC membranes were incubated with 5% skim milk diluted HRP-conjugated secondary antibodies (cat. nos. D110261 and D110273, 1:1,000; Sangon Biotech Co., Ltd.) for 60 min at room temperature. The membrane blots were then visualized using a chemiluminescence instrument and enhanced chemiluminescence liquid kit (Pierce; Thermo Fisher Scientific, Inc.). Optical density value of each blot was determined using Image Lab 3.0 software (Bio-Rad Laboratories, Inc.) for the chemiluminescence instrument and ImageJ 1.52t software (National Institutes of Health).

Hepatic stellate cell (HSC) culture. HSC lines were purchased from Sangon Biotech Co., Ltd. and cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin at 37˚C in a cell incubator with 5% CO_2. HSCs ($3x10^5/cm^2$) in the hypoxia/reoxygenation (HR) group were seeded on a 6-well plate and incubated at 37˚C for 24 h. After treatment with or without 10 μM MAPK inhibitor U0126 (MedChemExpress) at room temperature for 5 min, cells were placed in 94% N_2, 5% CO_2 and 1% O_2 humidified culture incubator for 6 h at 37˚C, followed by reoxygenation with 5% CO_2 and 95% air for 12 h until harvest. Cells in the Con group were cultured in a cell incubator with 5% CO_2 (normoxic conditions) and harvested at the same time as the experimental group (18 h).

Statistical analysis. Data are presented as the mean ± SEM (n=6/group). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test using SPSS 18.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CIH induces liver fibrosis in rats. To investigate the effects of CIH exposure in rats, Sirius red staining was used to evaluate liver histological structure. It was identified that 4 weeks of CIH exposure induced a substantial amount of collagen fibers around the macrovesicular and microvesicular structures, while collagen fibers were rarely detected in the Con group (Fig. 1A and B). When the gene expression of TLR4 was knocked down by TLR4 shRNA lentivirus, liver fibrosis was alleviated (Fig. 1B and D; CIH-TLR4 group vs. CIH group). Furthermore, the results indicated that there was no difference between the CIH-Vector group and CIH group (Fig. 1B and C). Therefore, it was speculated that 4 weeks of CIH can induce liver fibrosis in rats and this condition may be associated with TLR4 protein expression.

CIH induces liver inflammation in rats. Previous studies have reported that systemic inflammation is a primary cause of myocardium fibrosis (11). Therefore, the present study examined whether CIH-induced liver fibrosis in rats was associated with inflammation. RT-qPCR and immunohistochemistry were used to detect the expression levels of inflammatory cytokines in the liver of the model animals. RT-qPCR (Fig. 2), immunohistochemical (Fig. 3) and immunohistochemical image analysis (Fig. 4) demonstrated that, compared with Con group, the livers of the CIH and CIH-Vector groups had significantly higher expression levels of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1 ($P<0.01$ vs. Con group). Moreover, it was found that these effects were reversed by TLR4 shRNA lentivirus treatment ($P<0.05$ or $P<0.01$ vs. CIH group). However, no significant differences were found between the CIH-Vector group and CIH group. Thus, the results suggested that inflammation may play an important role in CIH exposure-induced liver fibrosis.

TLR4/NF-κB/MAPK signaling pathway is involved in CIH-induced liver fibrosis. To determine whether the TLR4/NF-κB/MAPK signaling pathway was involved in CIH-induced liver fibrosis, TLR4 and NF-κB expression

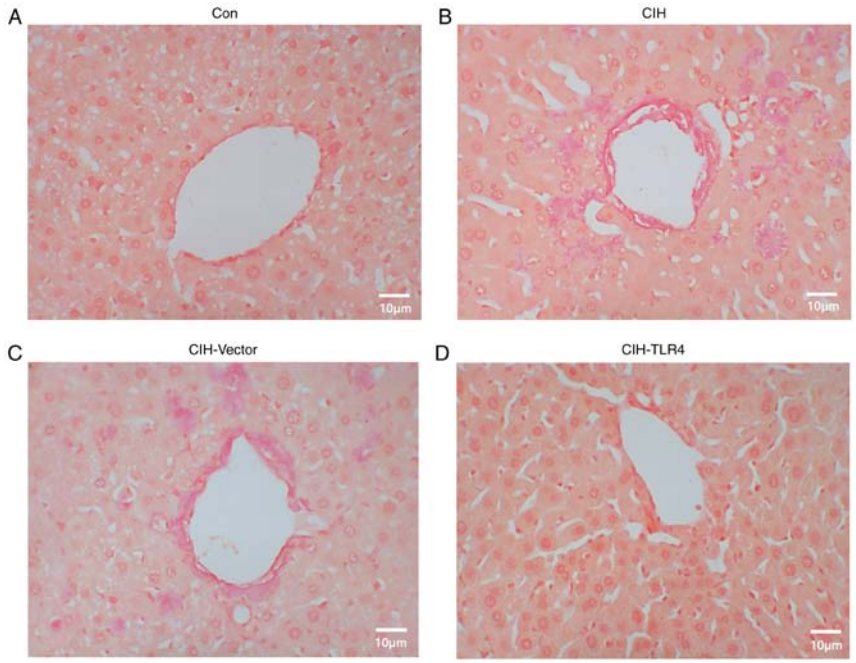

Figure 1. CIH-induced liver fibrosis. After exposing rats to CIH for 4 weeks, liver tissues were harvested and subjected to Sirius red staining. (A) Con group, (B) CIH group, (C) CIH-Vector and (D) CIH-TLR4 group. Representative images of immunohistochemistry. Magnification, x400. Con, control; CIH, chronic intermittent hypoxia; CIH-Vector group, CIH + empty vector lentivirus; CIH-TLR4 group, CIH + TLR4 shRNA lentivirus; shRNA, short hairpin RNA; TLR4, Toll-like receptor 4.

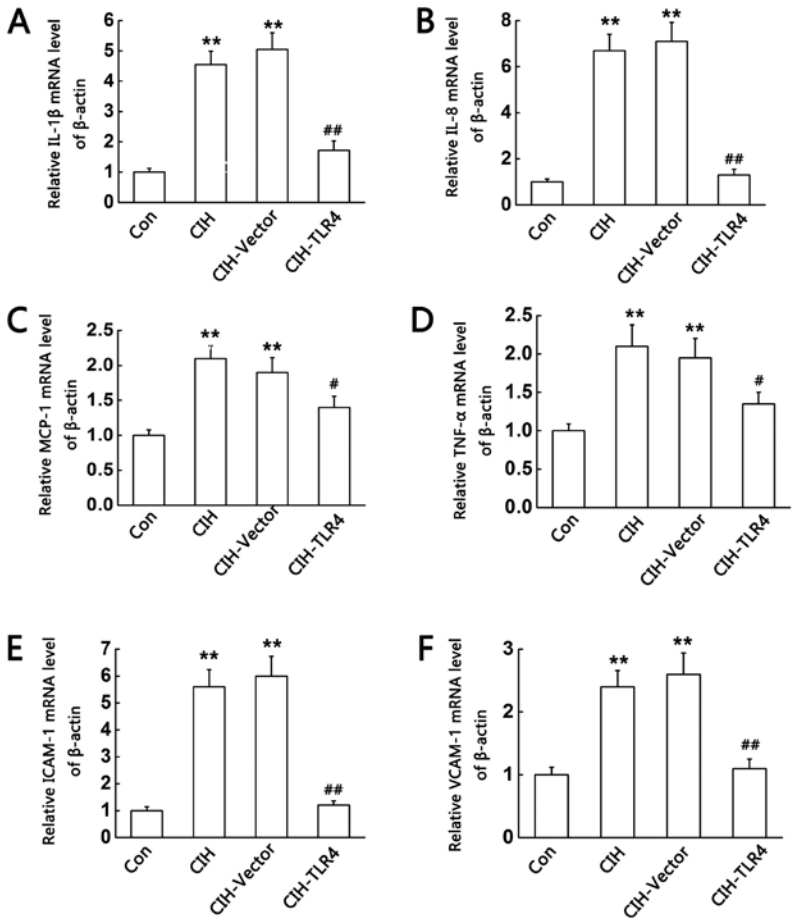

Figure 2. CIH-induced liver mRNA expression levels of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1. After exposing rats to CIH for 4 weeks, liver tissues were harvested and subjected to reverse transcription-quantitative PCR. (A) IL-1β, (B) IL-8, (C) MCP-1, (D) TNF-α, (E) ICAM-1 and (F) VCAM-1 expression levels. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CIH. Con, control; CIH, chronic intermittent hypoxia; CIH-Vector group, CIH + empty vector lentivirus; CIH-TLR4 group, CIH + TLR4 shRNA lentivirus; shRNA, short hairpin RNA; TLR4, Toll-like receptor 4; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

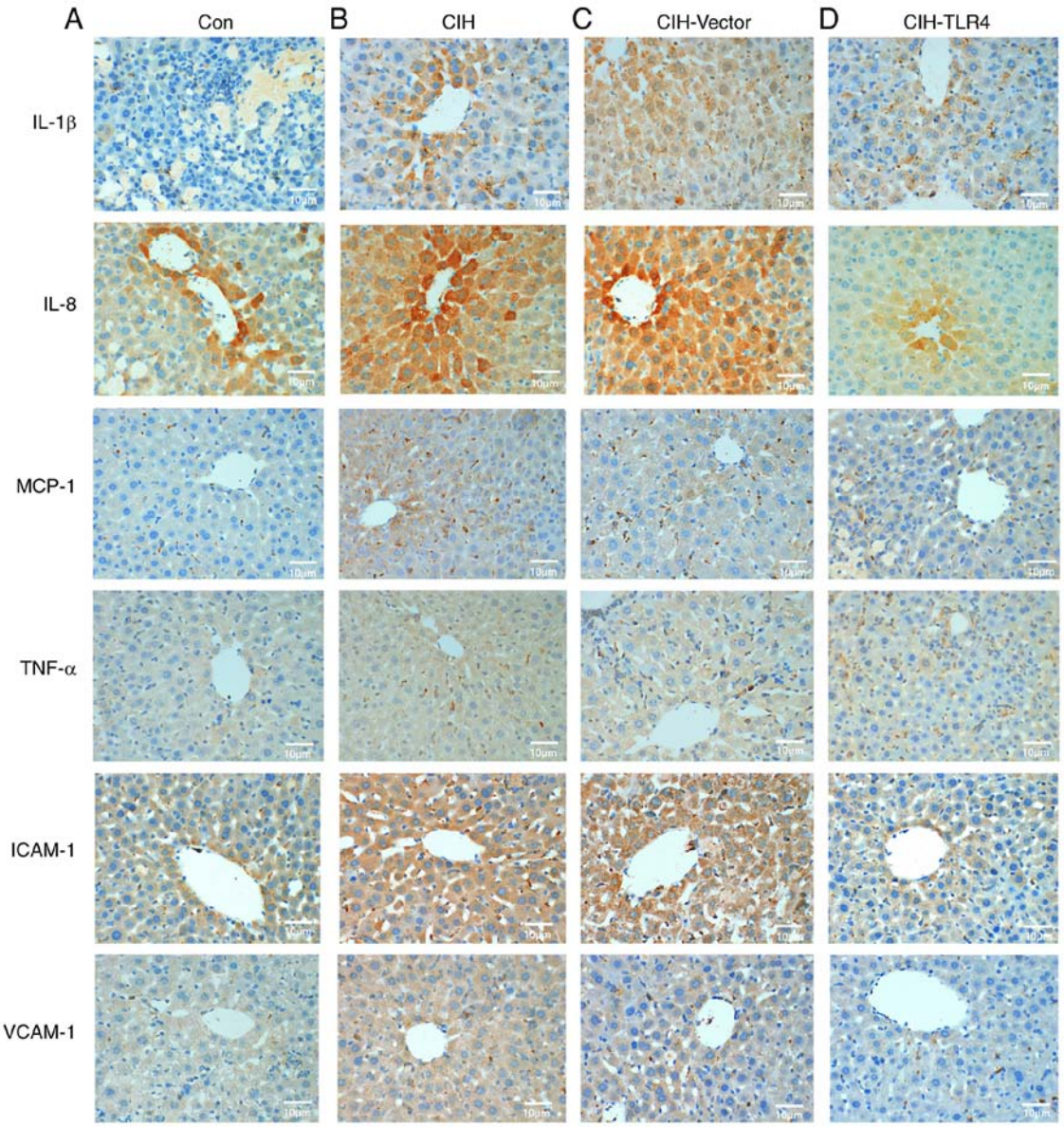

Figure 3. CIH-induced liver inflammation. After exposing rats to CIH for 4 weeks, liver tissues were harvested and subjected to immunohistochemistry staining with antibodies of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1. (A) Con group, (B) CIH group, (C) CIH-Vector group and (D) CIH-TLR4 group. Representative images of immunohistochemistry assay. Magnification, x400. Con, control; CIH, chronic intermittent hypoxia; CIH-Vector group, CIH + empty vector lentivirus; CIH-TLR4 group, CIH + TLR4 shRNA lentivirus; shRNA, short hairpin RNA; TLR4, Toll-like receptor 4; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

levels were measured by immunohistochemistry and western blot analysis in rats. It was identified that TLR4 and NF-κB expression levels were significantly increased in the CIH and CIH-Vector groups (Fig. 5; $P<0.01$ vs. Con group), while TLR4 shRNA lentivirus treatment decreased the expression levels of these proteins (Fig. 5; $P<0.01$ vs. CIH group).

The induction of TLR4, NF-κB, IκB and p-MAPK-1 was assessed by western blot analysis of HSCs. It was demonstrated that the protein expression levels of TLR4, NF-κB, IκB and p-MAPK-1 in the hypoxia/reoxygenation (HR) group were significantly higher compared with the Con group ($P<0.01$; Fig. 6). Furthermore, these effects were significantly reversed by application of U0126, a type of MAPK inhibitor. Collectively, the results indicated the involvement of the TLR4/NF-κB/MAPK signaling pathway in CIH-induced liver fibrosis.

Discussion

Numerous animal models used in the study of hypoxia have been developed over the past years, of which the most widely used is the CIH model, which simulates the intermittent hypoxia factor of OSAS (3,8). There are several different methods to verify the successful establishment of the OSAS model, such as monitoring the electroencephalogram, oronasal air flow, dynamic blood oxygen for 2 h, mean blood oxygen saturation, minimum blood oxygen saturation and sleep apnea index (6). In the present study, a CIH model was established and used to study the underlying mechanisms of liver fibrosis in OSAS. It was found that 4 weeks of CIH exposure induced distinct hepatic fibrosis around the macrovesicular and microvesicular structures. Moreover, it was demonstrated that knockdown of TLR4 using TLR4 shRNA lentivirus resulted in alleviated

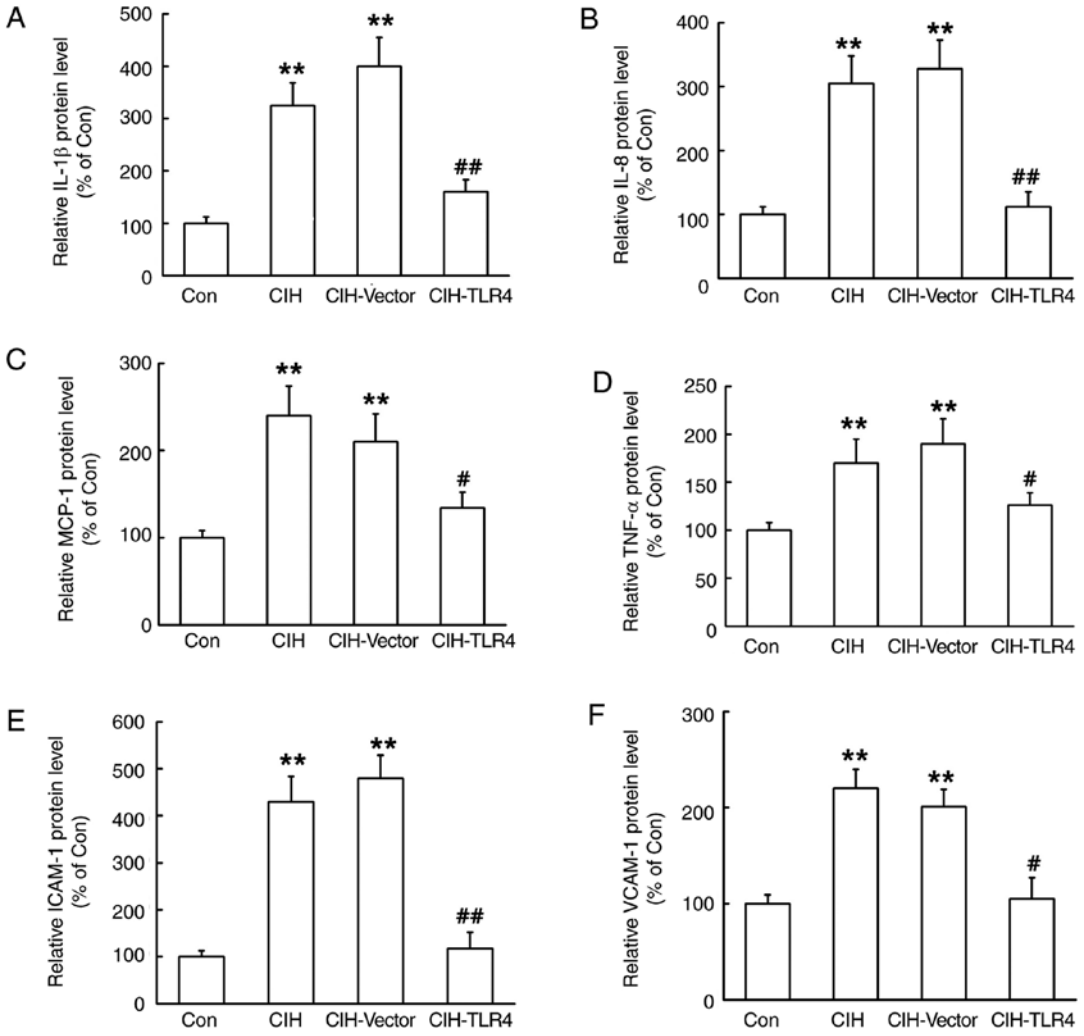

Figure 4. Statistical results of immunohistochemical images. After exposing rats to CIH for 4 weeks, liver tissues were harvested and subjected to immunohistochemistry staining with antibodies of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1. (A) IL-1β, (B) IL-8, (C) MCP-1, (D) TNF-α, (E) ICAM-1 and (F) VCAM-1 expression levels. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. CIH. Con, control; CIH, chronic intermittent hypoxia; CIH-Vector group, CIH + empty vector lentivirus; CIH-TLR4 group, CIH + TLR4 shRNA lentivirus; shRNA, short hairpin RNA; TLR4, Toll-like receptor 4; IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

liver fibrosis. Immunohistochemical results also identified that liver samples of the CIH and CIH-Vector groups presented an inflammatory state with increased expression levels of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1, which could be reversed by TLR4 shRNA lentivirus treatment. These results were consistent with previous findings reporting that patients with OSAS have increased systemic inflammation (2), which contributes to liver fibrosis and may be alleviated by inhibiting TLR4 expression.

Previous studies have shown that OSAS is an inflammatory state characterized by increases in the levels of circulating biomarkers of inflammation (2,26). While the inflammatory mechanism underlying OSAS has not been fully elucidated, it has been observed that the increased inflammation is partly mediated by HR (26,27). Savransky *et al* (28) revealed that CIH is a potent effective proinflammatory cytokine that not only induces hyperglycemia and liver lipid peroxidation, but also enhances the activity of NF-κB, which is the main regulator of the inflammatory response. In addition, it has been observed that patients with OSAS exhibit significantly increased serum NF-κB activities (2). Furthermore, serum levels of multiple NF-κB-dependent proinflammatory cytokine and adhesion molecules, such as IL-1β, IL-6, IL-8, TNF-α, MCP-1 and VCAM-1, are also elevated in patients with OSAS (29,30). Moreover, Aron-Wisnewsky *et al* (8) reported that CIH is strongly associated with increased systemic inflammatory responses, as well as with more serious fibrosis or inflammatory liver injuries. The present results suggested that 4 weeks of CIH exposure induced liver fibrosis in the CIH and CIH-Vector groups, while the CIH-Vector group presented an inflammatory state with increased expression of IL-1β, IL-8, MCP-1, TNF-α, ICAM-1 and VCAM-1. NF-κB is considered to be an oxidant-sensitive transcription factor, and activation of NF-κB promotes inflammation and multiple tissue injury in response to CIH and in liver disease conditions (31,32). Furthermore, the present results indicated that 4 weeks of CIH exposure increased NF-κB and TLR4 expression levels in the liver. In addition, it was found that protein expression levels of TLR4, NF-κB, IκB and p-MAPK-1 in the HR group were significantly higher compared with the Con group, and these effects could be reversed by application of the MAPK inhibitor U0126. However, a limitation of the present study

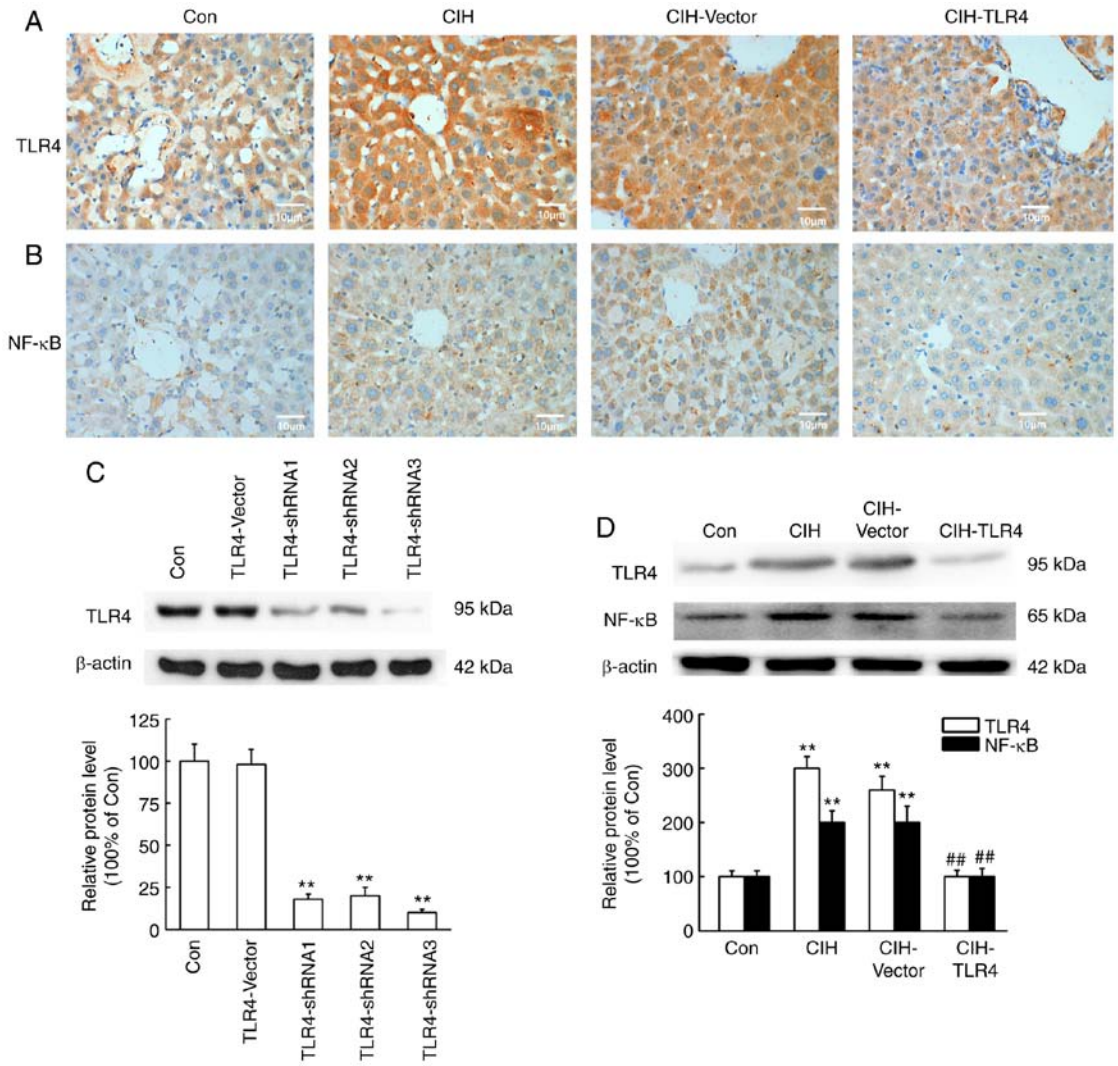

Figure 5. TLR4 shRNA lentivirus treatment decreases the expression levels of TLR4 and NF-κB. After exposing rats to CIH for 4 weeks, liver tissues were harvested and subjected to immunohistochemistry staining and western blotting. Immunohistochemistry results of (A) TLR4 and (B) NF-κB. Protein expression levels of (C) TLR4 and (D) NF-κB. Data are presented as the mean ± SEM, n=6 in each group. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. CIH. Con, control; CIH, chronic intermittent hypoxia; CIH-Vector group, CIH + empty vector lentivirus; CIH-TLR4 group, CIH + TLR4 shRNA lentivirus; shRNA, short hairpin RNA; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor.

was that p-NF-κB was not detected; p-NF-κB is the activated state of NF-κB, thus it would be beneficial to detect the effects of CIH and CIH-TLR4 on p-NF-κB.

CIH can induce an unbalanced production of large amounts of reactive oxygen species (ROS) and endogenous antioxidant defense mechanisms, which enhance oxidative stress (33,34). ROS can also activate nuclear transcriptional factors, including NF-κB and hypoxia-inducible factors (HIFs), which in turn promote the production of inflammatory cytokines, such as IL-1β and IL-8, as observed in a CIH model (35,36). HIFs are the main transcriptional regulators of the hypoxia response, and belong to a family of heterodimeric transcription factors (37). Furthermore, in almost all tissues and cells, HIFs act as the main regulator of the body to maintain homeostasis in response to hypoxia (38). Activated HIFs can be rapidly transferred to the nucleus, where they bind to hypoxia response elements of the target gene promoter region to regulate gene transcription (37,39). Previous studies have revealed that activation of HIF1α plays a key role in downstream signaling transduction of lipopolysaccharide (LPS) stimulation via the pattern recognition receptor TLR4. For example, LPS can upregulate HIF1α expression in rat liver, thus upregulating the expression of aldolase, a type of HIF1α target gene (40,41). In addition, it has been shown that the activity of LPS-induced HIF1α mainly depends on NF-κB, the inflammatory master regulator of a group of proteins, which is predominantly regulated by inhibiting the transcriptional action of IκB (38,42).

Clinical and animal studies have reported that the expression of TLR4 is closely related to activation of inflammatory response in patients with OSAS (20,22). Moreover, TLR4 is a typical pattern recognition receptor located upstream of NF-κB, and is closely related to the activation of inflammatory responses mainly via MyD88- and TIR-domain-containing adapter-inducing interferon-β-dependent pathways (43). It has also been revealed that activation of the TLR4/MyD88 signaling pathway in HR is positively correlated with myocardial injury in animal models (44). Shimamoto *et al* (45) observed that myocardial ischemia activates the TLR4/MyD88-dependent signal pathway and increases the activation of NF-κB, which ultimately leads to the release of

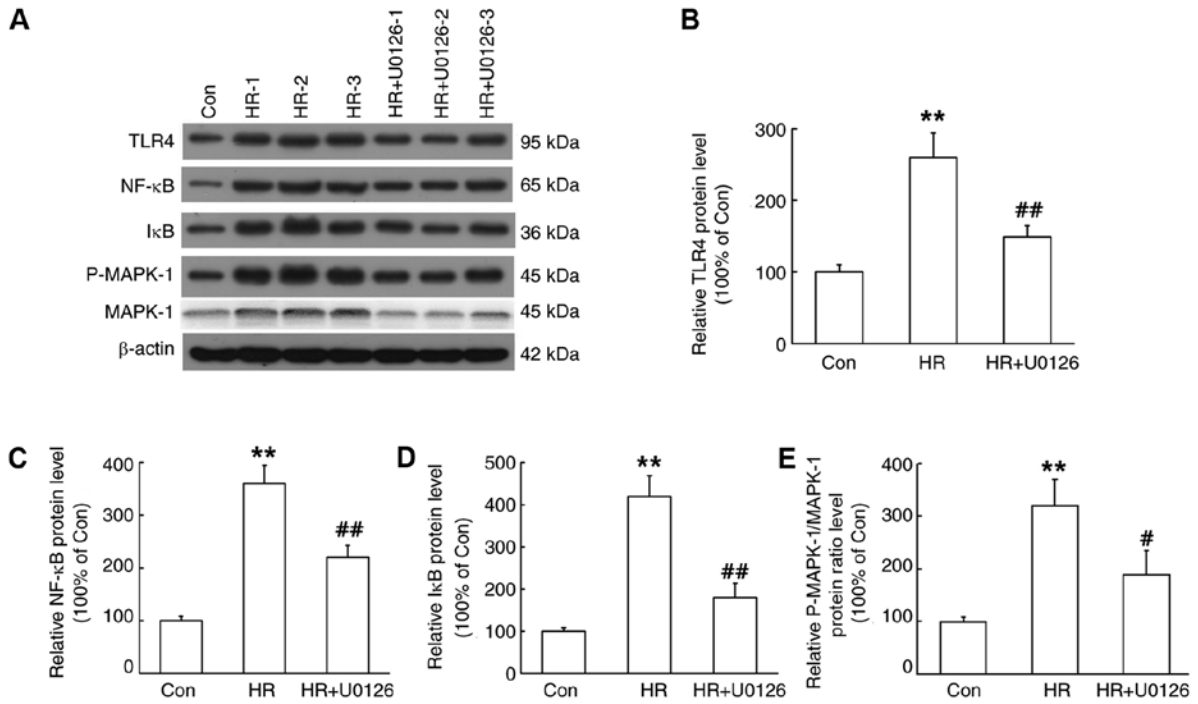

Figure 6. Effects of HR and MAPK inhibitor U0126 on the expression levels of TLR4, NF-κB, IκB and p-MAPK-1 in HSCs. After treating hepatic stellate cells with or without MAPK inhibitor U0126 (10 μM), cells were placed in a 94% N_2, 5% CO_2 and 1% O_2 humidified culture incubator for 6 h, followed by reoxygenation with 5% CO_2 and 95% air for 12 h. Protein expression was determined by western blot analysis. (A) Representative protein bands. Protein expression levels of (B) TLR4, (C) NF-κB, (D) IκB and (E) p-MAPK-1. Data are presented as the mean ± SEM, n=6 in each group. **P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. HR. Con, control; p, phosphorylated; IκB, inhibitor of NF-κB; TLR4, Toll-like receptor 4; MAPK, mitogen-activated protein kinase; HR, hypoxia/reoxygenation.

innate cytokines in the heart. Furthermore, Takahashi *et al* (46) showed that healthy mice and TSK mouse fibrosis models exhibited notable fibrosis after bleomycin treatment, while TLR4 knockout mice were protected from fibrosis, thus indicating that TLR4 plays an important role in the fiberization process. Moreover, non-functional TLR4 mutations or TLR4 knockout can effectively protect mice against the development of renal tissue dysfunction, inflammatory damage and fibrosis in a model of chronic kidney injury (15,16). It was found that similar injury also occurred in the rat liver in the present study, and this condition could be significantly alleviated by knocking down TLR4. Therefore, it was speculated that TLR4 and NF-κB mediated CIH-induced inflammation and liver fibrosis.

In conclusion, the preliminary results indicated that CIH could induce liver fibrosis in rats, and this effect was positively associated with inflammation and the TLR4/NF-κB/MAPK signaling pathway. Furthermore, it was demonstrated that the expression of TLR4 was associated with the pathogenesis of liver fibrosis in CIH; thus, the development of a novel method that inhibits TLR4 expression may be a viable strategy for clinical prevention of liver fibrosis in patients with OSAS.

Acknowledgements

Not applicable.

Funding

This work was supported by Natural Science Foundation of Fujian Province (grant nos. 2018J0105, 2018Y0032 and 2016J01441) and Young and Middle-aged Backbone Key Research Project of Fujian Province (grant no. 2016ZQN55).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZPL, XPY, YJZ and SYC conducted the experiments and analyzed the data. HLL conceived and supervised the project, contributed to the design of the experiments, discussed the data and wrote the manuscript with contributions from ZPL, XPY, YJZ and SYC. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Ethical Committee of Experimental Animals of Fujian Medical University and The Second Affiliated Hospital of Fujian Medical University. Animal experiments were carried out in accordance with animal welfare requirements and the Guidelines for the Care and Use of Laboratory Animals published by the P.R. China Ministry of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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