Carvedilol improves liver cirrhosis in rats by inhibiting hepatic stellate cell activation, proliferation, invasion and collagen synthesis

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Abstract. Portal hypertension (PHT) is one of the most severe consequences of liver cirrhosis. Carvedilol is a first-line pharmacological treatment of PHT. However, the antifibrogenic effects of carvedilol on liver cirrhosis and the intrinsic mechanisms underlying these effects have not been thoroughly investigated. The present study aimed to investigate the antifibrogenic effects of carvedilol on liver cirrhosis in vivo and in vitro. Liver cirrhosis was induced in rats by carbon tetrachloride (CCL₄) administration for 9 weeks; carvedilol was administered simultaneously in the experimental group. Blood samples were collected for serum biochemistry. Liver tissues were used for fibrosis evaluation, histological examination, immunohistochemistry and western blot analysis. The human hepatic stellate cell (HSC) line LX-2 was used for in vitro studies. The effects of carvedilol on LX-2 cell proliferation and invasion were evaluated by Cell Counting Kit-8 assay and Transwell invasion assays, respectively. The effect of carvedilol on transforming growth factor β (TGFβ)-induced collagen synthesis in LX-2 cells and the molecular mechanisms were examined by western blot analysis. The results demonstrated that carvedilol improved CCL₄-induced structural distortion and fibrosis in the liver. Carvedilol inhibited HSC activation, proliferation and invasion. Carvedilol inhibited HSC collagen synthesis through the TGFβ/SMAD pathway. In conclusion, carvedilol may alleviate liver cirrhosis in rats by inhibiting HSC activation, proliferation, invasion and collagen synthesis. Carvedilol may be a potential treatment of early-stage liver cirrhosis.

Introduction

Portal hypertension (PHT) is one of the most severe clinical consequences of patients with liver cirrhosis, which results in life-threatening complications including variceal bleeding and hepatic encephalopathy (1). Previous studies have indicated that increased intrahepatic vascular resistance (IHVR) is the initial and determinant factor of PHT (1-3). Various structural and functional factors are responsible for the IHVR increase (2,4-6). The formation of fibrous septa and regenerative nodules causes the distortion and compression of the venous system, which increases the resistance to portal venous blood flow (1,2,6). The progressive process of cirrhosis is characterized by the excessive deposition of extracellular matrix (ECM) proteins including type I collagen (Col I), Col III and fibronectin (FN). Hepatic stellate cells (HSCs) are the primary cells responsible for liver cirrhosis (7). Upon liver injury, quiescent HSCs acquire an activated phenotype, migrate to the damaged region, proliferate and produce ECM proteins (8-10). Transforming growth factor β (TGFβ), which is the most potent profibrogenic cytokine in the liver, promotes the synthesis of ECM proteins in HSCs (11,12). Multiple signaling pathways, including TGFβ/SMAD, PI3K/AKT and ERK, are involved in TGFβ signal transduction.

Carvedilol, a novel non-selective β-blocker (NSBB), is an antagonist of non-selective β- and selective α₁-adrenoceptors that effectively reduces portal pressure (13). Studies of the role of carvedilol in the reduction of portal pressure mainly focus on hemodynamics. Previous studies have reported that carvedilol improves myocardial and renal fibrosis (14,15). A small number of studies have examined the antifibrogenic effect of carvedilol on liver cirrhosis, and the underlying mechanisms are not well described (16). In chronic liver disease, portal pressure is mainly determined by the severity of the destruction of hepatic architecture (17). Therefore, it was hypothesized that the antifibrogenic effect of carvedilol may be involved in reducing the portal pressure. The present study aimed to investigate the antifibrogenic effect of carvedilol in vivo and in vitro. The rat model of liver cirrhosis induced by carbon tetrachloride was used to investigate the antifibrogenic effect of carvedilol in vivo. In vitro, the human line LX-2 was used to explore the mechanisms underlying carvedilol function.
Materials and methods

Materials. Carbon tetrachloride (CCl₄) was purchased from Sinopharm Chemical Reagent Co., Ltd. Carvedilol used in animal experiments was obtained from Qilu Pharmaceutical Co., Ltd. Carvedilol used in the cell-based experiments was purchased from PeproTech, Inc. Specific inhibitor of SMAD3 (SIS3) was purchased from Medchem Express.

Animals. A total of 40 male Wistar rats (weight, 180-200 g; age, 8 weeks) were purchased from the Central Animal Care Facility of Shandong University (Jinan, China). The rats were housed in the animal care facility under temperature- and humidity-controlled conditions (temperature, 22-24°C; humidity, 50±5) with a 12-h light-dark cycle and were provided free access to food and water. The mortality rate was 20%, and 13 rats were sacrificed for histological evaluation of liver cirrhosis and measurement of portal pressure. All rats were sacrificed under anesthesia induced by intraperitoneal injection of pentobarbital (30 mg/kg). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Animal Care and Use Committee of Shandong Provincial Hospital affiliated to Shandong University (approval no. 2018-005).

Induction of liver cirrhosis by CCl₄ and administration of carvedilol. Cirrhosis was induced by the intraperitoneal (i.p.) injection of CCl₄, as previously described (18). Rats were randomly divided into three groups: i) Control, which received an i.p. injection of olive oil (0.5 ml/kg body weight) twice weekly for 9 weeks; ii) CCl₄-intoxicated, which received an i.p. injection of CCl₄ (1 ml/kg; CCl₄ to olive oil v/v ratio, 1:1) twice weekly for 9 weeks; and iii) CCl₄+ carvedilol-treated, which received an i.p. injection of CCl₄ (1 ml/kg; CCl₄ to olive oil v/v ratio, 1:1) twice weekly for 9 weeks as well as concurrent treatment with carvedilol (10 mg/kg) via gavage daily for 9 weeks. Rats in the control and CCl₄-intoxicated groups received the vehicle (2 ml saline) by gavage daily for 9 weeks.

Serum assays. At the end of the experiment, rats were weighed and anesthetized. Laparotomy was performed to expose the inferior vena cava. In total, 3 ml venous blood was collected from each rat into procoagulant vacuum tubes from the inferior vena cava and centrifuged at 3,000 x g for 10 min at 4°C. Supernatant was collected and stored at -80°C until biochemical assays were performed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin (ALB) levels were measured using an AU1000 fully automatic biochemical analyzer (Olympus Corporation).

Histological examination. Following blood sample collection, rats were sacrificed and livers were harvested. Liver specimens were fixed in 4% paraformaldehyde for 24 h at room temperature, embedded in paraffin and cut into 4-µm-thick sections. Sections were stained with hematoxylin and eosin (H&E) for 5 min and for 30 sec, respectively, at room temperature for histopathological examination, and photographed under an Olympus BX63F light microscope (Olympus Corporation; magnification, x100). Sections were stained with sirius red (S-R) dye for 1 h at room temperature and with hematoxylin for 3 min at room temperature in order to visualize collagen deposition; the sections were photographed under a light microscope and under a Nikon Eclipse Ci-E polarized light microscope (Nikon Corporation; magnification, x200). The picrosirius-polarization method was used to evaluate the distribution of Col I (thick, strongly birefringent, yellow or red fibers) and Col III (thin, weakly birefringent, green fibers), as previously described (19). The collagen-positive area to total area ratio was quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Immunohistochemical analysis. Immunohistochemistry was performed using a Polink-2 Plus Polymer-Horseradish Peroxidase (HRP) Anti-Rabbit Immunoglobulin G (IgG) Detection System (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.), according to the manufacturer's protocol. Liver sections were deparaffinized and rehydrated in descending series of ethanol. After heat-mediated antigen retrieval with citrate buffer at 120°C for 3 min, the sections were incubated with 3% hydrogen peroxide for 10 min at room temperature to suppress endogenous peroxidase activity. The sections were incubated with polyclonal rabbit anti-rat α-smooth muscle actin (α-SMA; 1:300; cat. no. A03744; Boster Biological Technology) at 4°C overnight, and were subsequently warmed at 37°C for 30 min. Following incubation with the goat anti-rabbit IgG HRP-conjugated secondary antibody included in the kit at 37°C for 30 min, the sections were stained with diaminobenzidine solution, counterstained with hematoxylin and dehydrated through an increasing gradient of ethanol, according to the manufacturer's protocol. Images were captured under an Olympus BX63F light microscope. Sections incubated with PBS instead of the primary antibody were used as negative controls.

Western blotting. Liver tissue samples were stored in liquid nitrogen. Proteins were extracted from cells and liver tissues using the Tissue or Cell Total Protein Extraction kit (Sangon Biotech Co., Ltd.) according to the manufacturer's protocol. Protein concentrations were measured using a Bicinchoninic Acid Protein assay kit (Beijing Beyotime Institute of Biotechnology). Equal amounts (50 µg/well) of proteins were separated by 8% SDS-PAGE and transferred to PVDF membranes (EMD Milipore). Following blocking in 5% skimmed milk for 1 h at room temperature, the membranes were incubated with the primary antibodies at 4°C overnight, followed by incubation with the HRP-conjugated secondary antibodies goat anti-rabbit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; cat. no. ZB-5301; 1:5,000) or rabbit anti-goat (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; cat. no. ZB-2306; 1:5,000) for 2 h at room temperature. The signals of the target proteins were detected by enhanced chemiluminescence using Amersham Imager 600 (GE Healthcare). ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD, USA) was used to perform densitometric analysis. Band intensities were normalized to GAPDH. The primary antibodies used were as follows: Monoclonal rabbit anti-total AKT (cat. no. 4691; 1:1,000),...
monoclonal rabbit anti-phosphorylated (p)-AKT (cat. no. 4060; 1:1,000), polyclonal rabbit anti-total-p44/42 MAPK (t-ERK1/2; cat. no. 9102; 1:1,000), polyclonal rabbit anti-phospho-p44/42 MAPK (p-ERK1/2; cat. no. 9101; 1:1,000), monoclonal rabbit anti-total SMAD3 (cat. no. 9523; 1:1,000), monoclonal rabbit anti-p-SMAD2 (cat. no. 3108; 1:1,000) and monoclonal rabbit anti-GAPDH (cat. no. 5174; 1:1,000) purchased from Cell Signaling Technology, Inc.; polyclonal rabbit anti-FN (cat. no. ab2413; 1:1,000), monoclonal rabbit anti-α-SMA (cat. no. ab32575; 1:1,000), monoclonal rabbit anti-total SMAD2 (cat. no. ab40855; 1:2,000) and monoclonal rabbit anti-p-SMAD3 (cat. no. ab52903; 1:2,000) purchased from Abcam; and monoclonal goat anti-Col I (cat. no. 1310-01; 1:1,000) purchased from SouthernBiotech (Birmingham, AL, USA).

**Cell culture.** LX-2 is an activated human HSC cell line that is widely used as a model for hepatic fibrosis (9). LX-2 cells were a gift from Professor Weifen Xie (Shanghai Changzheng Hospital, The Second Military Medical University, Shanghai, China). Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO2.

**Cell Counting Kit-8 (CCK-8).** LX-2 cells were plated in 96-well culture plates (5x10^3 cells/well) in triplicate and incubated at 37°C overnight. Subsequently, the cells were incubated with 0, 1, 2, 5 or 10 µM carvedilol in a humidified incubator with 5% CO2 at 37°C for 24 h. Following the treatment, 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well. The plates were incubated in a humidified incubator with 5% CO2 at 37°C for 2 h. Cell viability was calculated according to the manufacturer’s protocol (Dojindo Molecular Technologies, Inc.). Optical density was measured at 450 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.). The experiment was repeated three times.

**Transwell invasion assay.** Serum-starved LX-2 cells (1x10^6/ml) treated with 0, 1, 2, 5 or 10 µM carvedilol in 100 µl serum-free culture medium were seeded into the upper chamber of a 24-well Transwell plate, and the membranes of the upper chamber were coated with Matrigel (BD Biosciences). Culture medium with 10% FBS was added to the lower chamber. Serum-free culture medium was used in the lower chamber as a non-induced control. Cells were incubated in a humidified incubator with 5% CO2 at 37°C for 24 h. Subsequently, the cell medium was discarded, and cells in the upper chamber were removed by a cotton swab. The cells on the lower surface of the chamber were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with hematoxylin for 10 min at room temperature. The migrated cells were assessed in six randomly selected fields under an Olympus BX63F light microscope.

**Statistical analysis.** All data are presented as the mean ± SD. SPSS Statistics 20.0 (IBM Corp.) was used for statistical analyses. Comparisons were performed using one-way ANOVA followed by Dunnett’s test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effects of carvedilol on body weight and biochemical parameters of CCl4-intoxicated rats.** The body weight of the CCl4-intoxicated group was significantly lower compared with the control group (P<0.01; Table I). The body weight of the CCl4 + carvedilol-treated group was lower compared with the CCl4-intoxicated group (P<0.05; Table I). Compared with the control group, CCl4 treatment significantly increased the serum levels of ALT and AST (P<0.01), whereas co-treatment with carvedilol decreased the elevated levels of ALT and AST (P>0.05 and P<0.01, respectively; Table I). Compared with the control group, CCl4 significantly decreased the serum level of ALB (P<0.01; Table I); however, not significant difference was identified in the levels of ALB between the CCl4-intoxicated and CCl4 + carvedilol-treated groups.

**Carvedilol improves CCl4-induced structural distortion and fibrosis in the liver.** Histological differences among the groups were demonstrated by H&E staining of liver sections. Liver sections of the control group exhibited normal lobular architecture, whereas liver sections of the CCl4-intoxicated group exhibited typical architectural distortions with regenerative nodules surrounded by proliferative connective tissue; co-treatment with carvedilol notably improved the architectural destruction induced by CCl4 (Fig. 1A). S-R staining revealed that, compared with control group rats, CCl4 treatment resulted in excessive collagen deposition in cirrhotic livers, which was notably reduced by co-treatment with carvedilol.

**Table I. Effects of CARV on body weight and biochemical parameters in CCl4-intoxicated cirrhotic rats.**

| Parameter | Control (n=7) | CCl4-intoxicated (n=6) | CCl4 + CARV (n=6) |
|-----------|--------------|------------------------|-------------------|
| Bodyweight (g) | 434.3±21.9 | 380.0±25.8^a | 341.8±40.5^b |
| ALT (U/l) | 50.98±12.76 | 99.04±14.04^a | 79.76±11.68^b |
| AST (U/l) | 90.45±12.87 | 170.45±42.50^a | 118.65±21.70^c |
| ALB (g/l) | 27.63±2.89 | 22.53±1.20^a | 21.20±1.84 |

^aP<0.01 vs. control. ^bP<0.05 vs. CCl4. ^cP<0.01 vs. CCl4. ALT, alanine aminotransferase; ALB, albumin; AST, aspartate aminotransferase; CARV, carvedilol; CCl4, carbon tetrachloride.
Carvedilol inhibits HSC activation in vivo. α-SMA is a marker of HSC activation (8). Immunohistochemical assay results demonstrated that the expression of α-SMA was notably higher in liver tissues of the CCl4-intoxicated group compared with the control group (P<0.01; Fig. 2A), whereas co-treatment with carvedilol suppressed the CCl4-induced increase of α-SMA (Fig. 2B). Compared with the control group, the protein expression of α-SMA was significantly upregulated by CCl4 (P<0.01; Fig. 2B), determined by western blotting, and co-treatment with carvedilol decreased the upregulated α-SMA protein expression levels (P<0.01). These results demonstrated that carvedilol may have an inhibitory effect on HSC activation in cirrhotic livers of rats.

Carvedilol inhibits HSC activation in vitro. LX-2 cells exhibit an activated HSC phenotype, which was assessed as previously described (9). LX-2 cells were treated with carvedilol at concentrations of 0, 1, 2, 5 and 10 μM for 24 h. α-SMA protein expression in LX-2 cells was significantly reduced by carvedilol at 5 and 10 μM compared with untreated control cells (P<0.01; Fig. 2C). This result further demonstrated that carvedilol may have an inhibitory effect on HSCs activation in vitro.

Carvedilol inhibits HSC proliferation. LX-2 cells were treated with carvedilol at concentrations ranging from 1 to 80 μM for 24 h. Carvedilol inhibited the proliferation of LX-2 cells in a dose-dependent manner (Fig. 3A). Carvedilol concentrations of ≤10 μM were selected for subsequent cell experiments as carvedilol did not significantly reduce cell viability at this concentration.

Carvedilol decreases the invasive ability of HSCs. The number of migrated FBS-induced LX-2 cells was significantly higher compared with the non-induced control group (P<0.01; Fig. 3B), whereas FBS-induced migration was significantly inhibited by carvedilol at concentrations of 5 and 10 μM (P<0.05 and P<0.01, respectively). This result demonstrated that carvedilol may inhibit the invasive ability of HSCs.
Carvedilol inhibits TGFβ1-induced collagen synthesis of HSCs. LX-2 cells were seeded in 6-well plates and incubated overnight. The cells were stimulated with TGFβ1 at concentrations of 10, 20, 30 and 50 ng/ml for 24 h. Compared with the control group, stimulation with TGFβ1 significantly increased the protein expression levels of Col I and FN (P<0.05 and P<0.01, respectively; Fig. 4A). TGFβ1 (20 ng/ml) was used to stimulate LX-2 cells for 24 h with or without co-treatment with carvedilol. The TGFβ1-induced upregulation of Col I and FN was downregulated by carvedilol at 5 and 10 µM (P<0.05 and P<0.01, respectively; Fig. 4B).

Carvedilol inhibits TGFβ1-induced HSC collagen synthesis via the TGFβ1/SMAD pathway. Signaling molecules...
downstream of TGFβ1 were screened in LX-2 cells treated with TGFβ1 (20 ng/ml) for 0.5, 1 and 2 h (Fig. 5). AKT and ERK phosphorylation levels did not change significantly (Fig. 5B and C, respectively), whereas the expression levels of p-Smad2 and p-Smad3 were upregulated in LX-2 cells stimulated with TGFβ1 compared with the untreated control (P<0.01; Fig. 5D and E, respectively).

TGFβ1 was used to stimulate LX-2 cells for 0.5 h with or without co-treatment with carvedilol. The phosphorylation of SMAD2 and SMAD3 induced by TGFβ1 was suppressed by pretreatment with carvedilol at concentrations of 5 and 10 µM (P<0.05 and P<0.01, respectively; Fig. 6A and B). SIS3 is a potent and selective inhibitor of SMAD3 (20). TGFβ1 was used to stimulate LX-2 cells for 24 h with or without co-treatment with 10 µM SIS3. Pretreatment with SIS3 significantly decreased the TGFβ1-induced upregulation of Col I and FN (P<0.01; Fig. 6C).

**Discussion**

Recent advances in our understanding of the pathophysiology of PHT and liver cirrhosis has resulted in improved management for patients with cirrhosis (2). As a novel NSBB, carvedilol reduces portal pressure more effectively than traditional NSBBs, such as propranolol and nadolol (21). For patients with compensated cirrhosis, the goal of treatment is to delay the development of liver cirrhosis and PHT. Therefore, the effects of carvedilol on liver cirrhosis were explored in vivo and in vitro to discover the potential role of carvedilol in treating early-stage liver cirrhosis.

Previous studies have reported that carvedilol has antioxidant, anti-proliferative, anti-inflammatory, anti-angiogenic and antifibrogenic effects (14,15,22-24). Hamdy et al demonstrated that carvedilol had potent antifibrotic effects in chronic CCl4-induced liver damage, but the underlying mechanisms...
were not described (25). In the present study, carvedilol not only improved the hepatotoxicity indicators, but also improved hepatic architectural distortion and liver fibrosis in cirrhotic rats. Structural changes are important factors in the development of IHVR, which is the determinant factor of PHT (6). Previous studies have demonstrated that improvement of liver fibrosis can reduce portal pressure (17,26). Therefore, the antifibrogenic effect of carvedilol may be involved in reducing portal pressure. HSCs are the target of the antifibrogenic therapy for hepatic fibrosis (27,28). A number of agents targeting activated HSCs have demonstrated their antifibrogenic effect in animal models (29‑32). Preventing HSC activation, proliferation, migration and collagen synthesis are major objectives in the treatment of liver fibrosis (27,28,33). The present study demonstrated that carvedilol may exert antifibrogenic effects in cirrhotic rats by inhibiting the proliferation, migration, activation and collagen synthesis in HSCs.

HSCs are the resident perisinusoidal cells in the space of Disse, and are the central effector in hepatic fibrosis (7). In response to liver injury, HSCs are activated and undergo phenotypic transformation to a myofibroblastic phenotype characterized by proliferation, migration to sites of injury, increased production of profibrogenic cytokines, and elevated accumulation of ECM components including Col I and FN (10,34). Among the profibrogenic cytokines, TGFβ1 is the most potent profibrogenic cytokine; TGFβ1 promotes the accumulation of ECM proteins in the progression of liver fibrosis (12). TGFβ1 was used in the present study to stimulate LX-2 cells to explore the mechanisms underlying the antifibrogenic effect of carvedilol. The results demonstrated that TGFβ1 upregulated the collagen synthesis in LX-2 cells, which was consistent with previous studies (12,35).

The present study results demonstrated that pretreatment with carvedilol decreased TGFβ1-induced collagen synthesis in LX-2 cells. TGFβ1 activates SMAD-dependent and SMAD-independent pathways, including PI3K/AKT and MAPK pathways such as ERK (36‑38). The TGFβ1/SMAD pathway is a major signaling pathway in the liver in both normal and pathological conditions (36,37). In the SMAD-dependent pathway, members of the SMAD family transmit signals from the cell surface into the nucleus. Following stimulation by TGFβ1, SMAD 2 and SMAD3 are phosphorylated and form a heterotrimeric complex with SMAD4 (36,37). This complex translocates into the nucleus and regulates the expression of target genes (36,37). The results of the present study revealed that TGFβ1 activated the SMAD-dependent pathway in LX-2 cells, and pretreatment with carvedilol decreased the TGFβ1-induced phosphorylation of SMAD2 and SMAD3. As a potent and selective inhibitor of SMAD3, SIS3 blocked the upregulation of collagen synthesis in TGFβ1-stimulated LX-2 cells. These results demonstrated that carvedilol may reduce the TGFβ1-induced increase of collagen synthesis in LX-2 cells by inhibiting the TGFβ1/SMAD pathway.

In conclusion, the present study demonstrated that carvedilol may improve liver cirrhosis in rats by inhibiting HSCs proliferation, invasion, activation and collagen synthesis. Furthermore, carvedilol may inhibit collagen synthesis in HSCs by suppressing the TGFβ1/SMAD pathway. Therefore, the application of carvedilol in chronic liver diseases may be extended beyond the pharmacological treatment of PHT in patients with decompensated cirrhosis, and carvedilol may be applied in the treatment of early-stage liver cirrhosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

LL performed experiments and wrote the manuscript. GL and GW performed the experiments. DM and ZL maintained the animals and established the liver cirrhosis animal model. CZ...
designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures and protocols were approved by the Animal Medical Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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