Resveratrol attenuates the progress of liver fibrosis via the Akt/nuclear factor-κB pathways

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Abstract. Liver fibrosis is a wound-healing response to chronic liver injury that results in the accumulation of extracellular matrix proteins. It eventually leads to cirrhosis of the liver and liver failure, and it is a critical threat to the health and lives of patients with chronic liver diseases. No effective treatment is currently available. Resveratrol is a polyphenol with anti-oxidant, anti-cancer and anti-inflammatory properties. It has been reported that resveratrol prevents liver fibrosis, possibly by inhibiting NF-κB activation. The present study investigated the mechanisms by which resveratrol prevented liver fibrosis, focusing on the possible involvement of the NF-κB pathway. Mice with carbon tetrachloride (CCl4)-induced liver fibrosis were treated with various concentrations of resveratrol. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and tumor necrosis factor (TNF)-α were detected by ELISAs. Expression of α-smooth muscle actin (α-SMA), collagen I, inhibitor of NF-κB (IκB) and NF-κB were detected by western blot analysis. In addition, the present study examined the effects of resveratrol on the expression of fibrosis markers in LX-2 cells. Western blot analysis was further used to detect the levels of Akt and phosphorylated Akt, as well as the nuclear levels of IκB, phosphorylated IκB and NF-κB p65. The expression of α-SMA in resveratrol-treated LX-2 cells was detected by immunofluorescence and flow cytometry, which demonstrated that resveratrol decreased the expression of α-SMA in LX-2 cells. Resveratrol also decreased CCl4-induced upregulation of serum AST, ALT, TNF-α, α-SMA and collagen I. Finally, resveratrol prevented the activation of NF-κB and Akt. The results of the present study therefore indicated that resveratrol attenuates liver fibrosis via the Akt/NF-κB pathways.

Introduction

Liver fibrosis is the pathophysiological consequence of the excessive accumulation of extracellular matrix (ECM) proteins in response to chronic liver injury or disease (1,2). Advanced liver fibrosis results in liver cirrhosis and can eventually progress to liver failure and hepatocellular carcinoma, diseases which have a poor outcome and high mortality (3,4). As early liver fibrosis is asymptomatic, a large percentage of patients present with advanced and irreversible liver fibrosis or even cirrhosis at the time-point of diagnosis (5). Therefore, anti-fibrotic therapies that are capable of halting or reversing the progression of liver fibrosis in patients with advanced disease are urgently required (5,6).

Liver fibrosis is a continuous remodeling process involving numerous cell types, inflammatory cytokines and signaling pathways (5,6). The key step in the genesis of liver fibrosis is the activation of hepatic stellate cells (HSCs), which are the primary source of ECM and are characterized by the expression of α-smooth muscle actin (α-SMA) (1,4). Following the activation of HSCs, a number of cytokines are secreted to activate associated intracellular signaling pathways and regulate liver fibrosis (6); these secreted cytokines include transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, adiponectin and leptin. Target signaling pathways include the TGF-β/SMAD, TNF-α/NF-κB, leptin, IFN-γ/signal transducer and activator of transcription 3, adipor/mitogen-activated protein kinase and peroxisome proliferator-activated receptor-α signaling pathways. These signaling pathways are all potential targets for anti-fibrotic treatments.

Resveratrol is a plant-derived polyphenol that has anti-oxidant and anti-inflammatory properties (7-9). Evidence has suggested that resveratrol protects against heart diseases (10), autoimmune diseases (11), skin disorders (12), diabetes (13) and numerous cancer types (14). Furthermore, it has been evidenced that resveratrol protects against numerous liver diseases, including alcoholic fatty liver disease (15,16), non-alcoholic fatty liver disease (17), high-fat diet-induced fatty liver (18), liver fibrosis (19,20) and hepatocellular
canceroma (9). It is thought that resveratrol primarily prevents liver damage by increasing the hepatic glutathione content, scavenging free radicals and inhibiting the expression or activity of inflammatory factors, including TNF-α and NF-κB (19-22).

It has been reported that resveratrol can prevent liver fibrosis by inhibiting the activity of NF-κB (19); however, the mechanisms by which resveratrol modulates NF-κB have remained elusive. It has also remained elusive whether other signaling pathways are involved in the preventive effects of resveratrol against liver fibrosis and the implication of these signaling pathways in the pathology of liver fibrosis. The present study used a mouse model of carbon tetrachloride (CCL4)-induced liver fibrosis to study the inhibitory effects of resveratrol on liver fibrosis and to reveal the underlying mechanisms.

Materials and methods

Cell lines and treatments. The human stellate cell line LX-2 was obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) and maintained in RPMI 1640 culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen Life Technologies) and 1% penicillin/streptomycin (Invitrogen Life Technologies) in 5% CO₂ at 37°C. Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of resveratrol in dimethylsulfoxide (DMSO; Aladdin Reagents Co., Ltd., Shanghai, China) at a concentration of 100 mg/ml was prepared.

MTT assay. LX-2 cells were seeded at a density of 5x10⁴ cells per well in 96-well plates. After 24 h, various concentrations of resveratrol were added to the wells (0, 3.125, 6.25, 12.5, 25.0, 50.0, 75.0, 100 and 125 µg/ml) and the plates were incubated for 72 h. After treatment, MTT (Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/ml. Plates were incubated at 37°C for an additional 4 h. After incubation, the supernatant was removed and the cells were lysed in 150 µl DMSO. Absorbance of the blue formazan derivative was measured at 570 nm using a microplate reader (VICTOR X Multilabel; PerkinElmer, Waltham, MA, USA). All measurements were repeated three times.

Immunofluorescence assay. Cells were seeded in 96-well plates at a density of 5x10⁴ cells per well. After 24 h, the cells were incubated for 48 h with resveratrol (0, 10, 20 and 50 µg/ml). Cells were then washed with PBS and the nuclei were counterstained with DAPI (Invitrogen Life Technologies) in PBS for 10 min at RT. Immunofluorescently labelled cells were observed and images were captured under a fluorescence microscope (BX71; Olympus, Tokyo, Japan) equipped with a DP70 digital camera (Olympus). All measurements were performed in triplicate and all experiments were repeated three times.

Flow cytometric analysis. Cells were seeded in 6-cm dishes at a density of 5x10⁴ cells per well. After 24 h, the cells were incubated with resveratrol (0, 10, 20 and 50 µg/ml) for 48 h. For fluorescence detection, a single-cell suspension was prepared by treatment with 0.25% trypsin (Invitrogen Life Technologies). Single cells were fixed in ice-cold methanol (Aladdin Reagents Co., Ltd.) for 30 min and then washed three times in PBS. For antibody staining, ~0.2x10⁶ cells were incubated with α-SMA primary antibody at 4°C for 1 h in independent reactions. Afterwards, cells were washed three times with PBS buffer, followed by incubation at 4°C for 30 min in the dark with AlexaFlour 488-labeled rabbit-specific secondary antibody (Invitrogen Life Technologies, Inc.). Subsequently, cells were washed and re-suspended in 0.2 ml sheath fluid. Flow-cytometric analysis was performed using a BD FACSCalibur fluorescence-assisted cell sorting machine (BD Biosciences, Franklin Lakes, NJ, USA) using FlowJo software 7.6 (FlowJo LLC, Ashland, OR, Canada). All measurements were performed in triplicate and all experiments were repeated three times.

Animals and liver fibrosis model. Male C57BL/6 mice (weight, 20-25 g; age, 8-12 weeks; n=25) were obtained from the Animal Division of Fudan University, Shanghai Medical College (Shanghai, China). The mice were maintained at 24°C on a 12-h light/dark cycle and had access to rodent chow and water ad libitum. All experimental procedures were approved by the Ethics Committee for Animal Care of Fudan University (Shanghai, China). Mice were randomly divided into five groups, including a normal group, a resveratrol (50 mg/kg) treatment group, a CCl4 (Aladdin Reagents Co., Ltd.) treatment group, a combined resveratrol (20 mg/kg) plus CCl4 treatment group and a combined resveratrol (50 mg/kg) plus CCl4 treatment group. Resveratrol was dissolved in 2% DMSO and saline prior to administration. To induce liver fibrosis, mice were intraperitoneally injected with 0.3 ml/kg CCl4 (mixed 1:1 with vegetable oil) twice a week for four weeks and then once a week for the following four weeks. In the combined resveratrol plus CCl4 treatment groups, mice were given an intragastric administration of resveratrol (20 or 50 mg/kg) everyday, and were also intraperitoneally injected with CCl4 three times per week, for a total co-administration time of eight weeks. Resveratrol dosages were selected according to guidelines established by previous studies (18,23). Mice were sacrificed at eight weeks and blood samples were collected for serum biochemistry. The liver was dissected, weighed, frozen in liquid nitrogen and stored at -80°C until analysis.

ELISA. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and TNF-α were determined using ELISAs according to the manufacturer's instructions. The ELISA kit for ALT, AST and TNF-α was
obtained from Shanghai Kemin Bioscience Ltd. (Shanghai, China). All measurements were performed in triplicate and all experiments were repeated three times.

Western blot analysis. Cells and tissues were homogenized in a commercial lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). For isolation of cytoplasmic and nuclear fractions, all tissue samples were processed using a Cytoplasmic/Nuclear Extraction kit (Fermentas, Thermo Scientific, Pittsburg, PA, USA) according to the manufacturer’s instructions. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Sangon Biotech Co., Ltd.) with bovine serum albumin (Wuhan Boster Biological Technology, Ltd.) as the standard. Protein was denatured by heating at 100°C for 5 min and the cellular debris was removed by centrifuging at 12,000 x g for 10 min. Equal amounts of protein (30 µg) were loaded and subjected to 10% SDS-PAGE followed by electrophoretic transfer onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST; Sigma-Aldrich) and 5% (w/v) non-fat dry milk (Wuhan Boster Biological Technology, Ltd.) for 1 h. After blocking, the membranes were incubated overnight at 4°C with primary antibodies against α-SMA (1:400; rabbit polyclonal; cat. no. ab5694; Abcam), Collagen I (1:800; mouse monoclonal; cat. no. ab6308; Abcam), NF-κB (1:200; rabbit polyclonal; cat. no. ab7972; Abcam); IκB-α (1:500; mouse monoclonal; cat. no. sc-1643; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), IκB-α (1:500; mouse monoclonal; cat. no. sc-101713; Santa Cruz Biotechnology, Inc.), GAPDH (1:10,000; rabbit monoclonal; cat. no. ab181603; Abcam), p65 (1:500; rabbit polyclonal; cat. no. ab7970; Abcam), α-tubulin (1:500; rabbit polyclonal; cat. no. ab126165; Abcam), β-tubulin (1:1,000; rabbit monoclonal; cat. no. ab179513; Abcam). After washing with TBST, the membranes were incubated with constant agitation with horseradish peroxidase-conjugated secondary antibodies (Sangon Biotech Co., Ltd.) at a dilution of 1:2,000 at RT for 1 h. The membranes were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer’s instructions.

Figure 1. Resveratrol decreases the expression of α-SMA in LX-2 cells. (A) Effects of resveratrol on the viability of LX-2 cells determined via MTT assay following 72 h of incubation. (B) Immunohistochemical analysis of α-SMA expression (green) in LX-2 cells incubated with resveratrol (10 or 20 µg/ml) for 48 h. Nuclei were counterstained with DAPI (blue). Scale bars, 10 µm. (C) Flow cytometric analysis of α-SMA expression in LX-2 cells incubated with resveratrol for 48 h. The frames indicate α-SMA-positive cells and the numbers next to the frames indicate the percentages of α-SMA-positive cells. SMA, smooth muscle actin; FITC, fluorescein isothiocyanate; SSC, side scatter; OD570, optical density at 570 nm.
Chemiluminescent signals were captured digitally using a chemiluminescence imaging system (Shanghai Clinx Science Instruments, Shanghai, China). The intensity of each band was quantified by densitometric analysis and normalized to α-tubulin. Values are expressed as the mean ± standard deviation. Data were analyzed using SPSS 20 (IBM, Armonk, NY, USA). Comparisons between groups were performed using analysis of variance with Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol decreases the expression of α-SMA in LX-2 cells. The cytotoxicity of resveratrol in LX-2 cells was determined using an MTT assay. Resveratrol was not cytotoxic below a concentration of 20 µg/ml. The IC_{50} value of resveratrol on LX-2 cells was 51.8 µg/ml (Fig. 1A). Therefore, the non-cytotoxic concentrations of 10 and 20 µg/ml were selected as the experimental conditions of all subsequent experiments.

The effects of resveratrol on α-SMA expression in LX-2 cells was determined using immunofluorescence and flow cytometric analyses. Immunofluorescent microscopic observation revealed that resveratrol (20 µg/ml) markedly decreased α-SMA expression (Fig. 1B). Similarly, flow cytometric analysis demonstrated that resveratrol treatment decreased the expression of α-SMA in LX-2 cells (Fig. 1C). The percentage of α-SMA-positive cells was 65.1, 31.0 and 15.1% when cells were treated with 0, 10 and 20 µg/ml resveratrol, respectively. These results demonstrated that resveratrol decreased α-SMA expression in LX-2 cells.

Resveratrol reduces the expression of liver fibrosis markers in a CCl_4-induced mouse model of liver fibrosis. To investigate the potential anti-liver fibrosis activity of resveratrol in vivo, the present study used a CCl_4-induced mouse model of liver fibrosis. Serum levels of ALT and AST were determined in animals treated with or without CCl_4 and resveratrol. As shown in Fig. 2A, CCl_4-induced mice had significantly higher levels of ALT and AST when compared with those in the untreated control mice. Resveratrol treatment decreased the levels of ALT and AST in a dose-dependent manner in the CCl_4-induced mice; however, in mice that were not treated with CCl_4, resveratrol treatment had no effect on ALT or AST expression. Serum levels of the inflammatory factor TNF-α as well as other markers of liver fibrosis were also detected. The levels of TNF-α were significantly decreased by resveratrol treatment in CCl_4-induced mice when compared with those in the untreated mice (Fig. 2B). The effects of resveratrol treatment on the expression of the liver fibrosis markers α-SMA and collagen-I were also examined. As shown in Fig. 2C and D, the expression of α-SMA and collagen-I was markedly increased in the CCl_4-induced mice, while resveratrol treatment significantly inhibited the CCl_4-induced
increase of α-SMA and collagen-I in a dose-dependent manner. Treatment with 50 µg/ml resveratrol decreased the expression levels of α-SMA and collagen-I to almost basal levels of the normal control.

**Resveratrol inhibits the activation of NF-κB in a mouse model of CCl₄-induced liver fibrosis and LX-2 cells.** As it has been reported that resveratrol prevents liver fibrosis by inhibiting the activity of NF-κB (19), the effects of resveratrol on NF-κB activity were investigated in the mouse model of liver fibrosis as well as in LX-2 cells. As NF-κB is activated through phosphorylation of IκB and the translocation of p65 from the cytoplasm to the nucleus (24), the levels of IκB, pIκB and p65 were assessed. As shown in Fig. 3A and B, the expression of IκB-α was markedly decreased in CCl₄-induced mice; however, resveratrol treatment partially rescued the expression of IκB-α. Furthermore, NF-κB was markedly increased by CCl₄ stimulation, while resveratrol treatment reduced NF-κB to levels below those of the control group. In a further experiment, LX-2 cells were induced with CCl₄. While resveratrol or CCl₄ treatment had no significant effects on the expression of IκB-α, the levels of pIκB-α were markedly increased in CCl₄-induced cells, which was attenuated by resveratrol treatment (Fig. 3C and D). Furthermore, the expression of NF-κB in CCl₄-induced LX-2 cells was assessed (Fig. 3E and F). The levels of the NF-κB p65 sub-unit were markedly increased in the nuclei of CCl₄-induced cells, which was significantly inhibited by resveratrol treatment. All of these results suggested that resveratrol inhibits the activation of NF-κB during liver fibrosis.

**Resveratrol inhibits the activation of Akt in LX-2 cells.** The effects of resveratrol on Akt were also examined in vitro. As shown in Fig. 4A and B, neither CCl₄-induced nor resveratrol-treated cells showed a change in total Akt expression.
compared with that in the control group. However, Akt phosphorylation was markedly increased in CCl₄-induced cells, which was attenuated by resveratrol treatment. These results indicated that resveratrol inhibited the activation of Akt during liver fibrosis.

**Discussion**

The present study reported that resveratrol downregulated the expression of α-SMA in HSCs. Furthermore, resveratrol decreased the serum levels of ALT, AST and TNF-α, and the protein expression of α-SMA and collagen-I in a mouse model of liver fibrosis. Furthermore, resveratrol inhibited the activation of NF-κB and Akt during liver fibrosis.

Liver fibrosis is a dynamic wound-healing response to chronic liver injury that can result in serious and life-threatening consequences for affected patients; however, it has been indicated that even advanced fibrosis is a potentially reversible process (2). Activation of HSCs is the initial step in the process of liver fibrosis and is characterized by the expression of α-SMA (1). Downregulation of α-SMA expression is widely thought to be a promising potential method of liver fibrosis inhibition (25). Using immunofluorescence and flow cytometry, the present study revealed that resveratrol decreased the expression of α-SMA in HSCs, indicating that resveratrol inhibited liver fibrosis. Furthermore, resveratrol decreased serum levels of ALT, AST and TNF-α as well as the expression of α-SMA and collagen-I in a CCl₄-induced mouse model of liver fibrosis. The results of the present study were in accordance with those of previous studies and indicate that resveratrol inhibits liver fibrosis (19,20).

Inflammation is an integral part of the wound-healing response in the liver and chronic inflammation is tightly associated with liver fibrosis (26). The NF-κB signaling pathway is a highly evolutionarily conserved pathway that has a pivotal role in the regulation of immune and inflammatory responses (24). In accordance with this known function, the NF-κB signaling pathway appears to have a central role in liver homeostasis (24). The NF-κB family of proteins are Rel family proteins. They are transcription factors that can exist as either heterodimers or homodimers, and they regulate the transcription of genes with the common κB binding motif. There are five DNA-binding Rel family sub-units: p50, p52, cRel, p65 and RelB. The most common form of NF-κB is the p50:p65 heterodimer. NF-κB is activated through two different pathways - the classical pathway, which depends on the phosphorylation of IkB and the translocation of p65 from cytoplasm to nucleus, and the non-canonical pathway, which is based on the inducible processing of NF-κBp2/p100 to p52:RelB (24,26). It was recently reported that inhibition of NF-κB alleviated CCl₄-induced liver fibrosis via suppression of activated HSCs (27).

A previous study postulated that the prevention of liver fibrosis by resveratrol is likely to be associated with its ability to reduce NF-κB activation (19). While this previous study observed DNA-binding activity of NF-κB in liver tissue, it did not elucidate the regulatory mechanism of NF-κB activation. The present study demonstrated that in the CCl₄-induced mouse model of liver fibrosis, resveratrol attenuated the fibrosis-induced decrease in IkB-α expression and the increase in NF-κB expression. Furthermore, in activated LX-2 cells, resveratrol attenuated the CCl₄-induced increase in pIkB-α levels and inhibited the nuclear translocation of NF-κB p65. These results indicated that resveratrol reduces liver fibrosis via the inhibition of NF-κB through the classical pathway.

A recent study showed that NF-κB is inhibited by the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway during liver fibrosis (27). The PI3K/Akt signaling pathway has a critical role in cell growth and survival (28). PI3K and Akt are also involved in the activation of innate immune cells via the regulation of key inflammatory cytokines (29). Accumulating evidence indicated that the de-regulation of the PI3K/AKT pathway in hepatocytes is a common molecular event in liver diseases (28). Liver-specific activation of the PI3K/Akt pathway promotes cytokine production and regulates the liver’s early regenerative response (30). Therefore, the present study investigated a possible link between the PI3K/Akt signaling pathway and the effects of resveratrol in CCl₄-induced cells. The results showed that the phosphorylation of Akt was increased in activated LX-2 cells, and that treatment with resveratrol reversed this activation. This result suggested that the PI3K/Akt signaling pathway is involved in the protective effects of liver fibrosis by resveratrol.

In conclusion, the present study indicated that resveratrol may help prevent CCl₄-induced liver fibrosis and that this effect is associated with the inhibition of Akt as well as NF-κB activation. This mechanism may provide promising potential targets in the treatment of human liver fibrosis. Further study is required to verify the ability of resveratrol to prevent or possibly reverse liver fibrosis in vivo.

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**References**