NF-κB inhibition alleviates carbon tetrachloride-induced liver fibrosis via suppression of activated hepatic stellate cells

FEI WANG¹, SHUYUAN LIU², TAIPING DU¹, HAO CHEN¹, ZHIYONG LI¹ and JINGWANG YAN¹

¹Department of General Surgery Ward 1, Xinxiang Central Hospital; ²Department of Infectious Diseases, Third Affiliated Hospital of Xinxiang Medical College, Xinxiang, Henan 453000, P.R. China

Received November 24, 2013; Accepted March 28, 2014

DOI: 10.3892/etm.2014.1682

Abstract. An effective treatment for hepatic fibrosis is not available clinically. Nuclear factor (NF)-κB plays a central role in inflammation and fibrosis. The aim of the present study was to investigate the effect of an NF-κB inhibitor, BAY-11-7082 (BAY), on mouse liver fibrosis. The effects of BAY on hepatic stellate cell (HSC) activation were measured in the lipopolysaccharide-activated rat HSC-T6 cell line. In addition, the therapeutic effect of BAY was studied in vivo using a model of hepatic fibrosis induced by carbon tetrachloride (CCL₄) in mice. BAY effectively decreased the cell viability of activated HSC-T6 cells and suppressed HSC-T6 activation by downregulating the expression of collagen I and α-smooth muscle actin. BAY significantly inhibited the phosphorylation of phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinase-protein kinase B (Akt) in activated HSC-T6 cells. In addition, administration of BAY attenuated mouse liver fibrosis induced by CCL₄, as shown by histology and the expression of profibrogenic markers. BAY also significantly decreased the levels of serum alanine aminotransferase in this model of hepatic fibrosis. Therefore, the results of the present study demonstrate that BAY attenuates liver fibrosis by blocking PI3K and Akt phosphorylation in activated HSCs. Thus, BAY demonstrates therapeutic potential as a treatment for hepatic fibrosis.

Introduction

Liver fibrosis is a common consequence of various chronic liver diseases and the underlying pathology represents the common response of the liver to toxicity, infection or metabolism (1-3). Hepatic fibrosis, characterized by excess deposition of extracellular matrix proteins, is traditionally viewed as an irreversible pathological process involving multiple signaling pathways (4,5). With protracted damage, fibrosis progresses into excessive scarring and organ damage, including liver cirrhosis. However, recent evidence has indicated that liver fibrosis may be dynamic and bidirectional, involving progression and regression (6), offering an opportunity of therapeutic intervention to halt or reverse fibrosis. To date, antifibrotic treatment represents an un conquered area for drug development, with enormous potential but also high risks (7).

During liver fibrosis, hepatic stellate cells (HSCs) are primarily activated by transforming growth factor-β, in addition to other profibrogenic cytokines. Upon activation, HSCs proliferate and differentiate into myofibroblasts which secrete several extracellular matrix constituents, including collagens (8,9). Activated HSCs are the key cells involved in the progression of liver fibrosis (10). Nuclear factor (NF)-κB is a heterodimeric transcription factor that plays a central role in the pathogenesis of a wide variety of conditions affecting the liver, including hepatitis and fibrosis (11). Although the role of NF-κB signaling in the liver has been extensively explored, further studies of NF-κB signaling in liver fibrosis are required to promote translational application in liver disease. Thus, in the present study, the effect of an NF-κB inhibitor, BAY-11-7082 (BAY), was investigated in carbon tetrachloride (CCL₄)-induced mouse model of liver fibrosis.

Materials and methods

Cell culture. HSC-T6 cells, an immortalized rat HSC cell line transfected with the SV40 large T-antigen containing a Rous sarcoma virus promoter, were purchased from the Cancer Institute and Hospital (Chinese Academy of Medical Sciences, Beijing, China). The Chang liver cell line (American Type Culture Collection, Manassas, VA, USA) was used as a normal human cell line derived from normal liver tissue (12). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G and streptomycin at 37°C. Cell passage of the cultures was performed every 3 days and the cells were plated in culture dishes at a density of 1x10⁶ cells. Next, the cells were treated with various concentrations (6.25, 12.5, 25 and 50 µM) of BAY 1 h prior to stimulation with 1 µg/ml lipopolysaccharide (LPS) for 24 h. The study was approved by the ethics committee of Xinxiang Central Hospital (Xinxiang, China).
Measurement of serum alanine aminotransferase (ALT). Mouse sera were collected and enzyme ALT levels were measured using the serum biochemical analyzers Ektachem Hitachi autoanalyzer (Tokyo, Japan), according to the manufacturer's instructions.

Histopathological analysis. Mice were sacrificed at the end of 6 weeks (12). Liver samples from the left lateral and median lobes were separated and fixed in 10% neutral buffered formalin. The samples were then embedded in paraffin, sectioned (5 μm) and stained with Sirius red (Vector Laboratories, Inc., Burlingame, CA, USA) for general observations. A certified histopathologist was blinded to the group distribution throughout the analysis.

Western blotting. Equal amounts of protein were resolved by 12.5% SDS-PAGE and immobilized on polyvinylidene fluoride membranes by wet transfer. Following blocking for 30 min with 5% non-fat dry milk in Tris-buffered saline-Tween 20, the membranes were exposed overnight at 4°C to primary antibodies. This was followed by incubation for 2 h at room temperature with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, Inc.). Equal protein loading was corrected by the immunoblotting of β-actin. Immunoreactive proteins were visualized using a chemiluminescent HRP antibody detection reagent (Denville Scientific, Inc., South Plainfield, NJ, USA) and exposure to X-ray film (Eastman Kodak). Band density was analyzed using ImageJ software. The primary antibodies anti-p-phosphatidylinositol 3-kinase (PI3K)/PI3K, anti-p-Akt/Akt, anti-collagen I, anti-α smooth muscle actin (SMA; 1:1,000) and anti-β-actin antibody (1:2,500), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Hydroxyproline Measurement. Liver tissue was homogenized in ice-cold distilled water (900 µl) using a Power Gen homogenizer (Fisher). Subsequently, 125 µl of 50% (wt/vol) trichloroacetic acid was added, and the homogenates were incubated further on ice for 20 min. Precipitated pellets were hydrolyzed for 18 h at 110°C in 6 N HCl. After hydrolysis, the samples were filtered and neutralized with 10 N NaOH, and the hydrolysates were oxidized with Chloramine-T (Sigma) for 25 min at room temperature. The reaction mixture then was incubated in Ehrlich's perchloric acid solution at 65°C for 20 min and cooled to room temperature. Sample absorbance was measured at 560 nm in duplicate. Purified hydroxyproline (Sigma) was used to set a standard. Hydroxyproline content was expressed as microgram of hydroxyproline per g liver.

Statistical analysis. Data are expressed as mean ± SD. Animal experiments were performed with 12 animals in each treatment and control group. All in vitro data is reported as the result of three independent experiments, including three replicates per experiment. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and statistical differences between the groups were analyzed using the Student's T test or one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of BAY on cell viability. Various concentrations of BAY (6.25-50 µM) significantly reduced the cell viability of HSC-T6 cells in a dose-dependent manner within 24 h following LPS stimulation (Fig. 1A). To determine whether BAY was cytotoxic to normal hepatocytes, normal human Chang liver cells were selected as a normal control to test the cell viability in the presence of various concentrations of BAY. At concentrations between 6.25 and 50 µM, BAY exhibited insignificant toxicity in normal Chang liver cells (Fig. 1B).
Effect of BAY on the protein expression of collagen I and α-SMA. Activation of HSCs plays a central role in liver fibrosis and α-SMA is an established indicator of HSC activation (3). Collagen I is the principal collagen responsible for fibrosis and is generated by activated HSCs. The levels of α-SMA and collagen I were upregulated in LPS-activated HSC-T6 cells, indicating that HSCs were activated upon LPS administration. By contrast, BAY decreased the protein levels of α-SMA and collagen I in the LPS-treated cells (Fig. 2). These results demonstrated that BAY reduced HSC activation.

Effect of BAY on LPS-induced phosphorylation of PI3K/Akt. To investigate the antifibrotic mechanism of BAY and the possible association with the PI3K/Akt signaling pathway, PI3K/Akt expression was observed in activated HSC-T6 cells. PI3K and Akt phosphorylation was upregulated following LPS stimulation; however, the phosphorylation levels of PI3K/Akt were significantly reduced by BAY treatment in a dose-dependent manner (Fig. 3).

Effect of BAY on CCl₄-induced hepatic fibrosis. Mouse hepatic fibrosis was determined using Sirius red staining. As expected, marked bridging fibrosis was observed in the mice treated with vehicle (Fig. 4B). BAY significantly attenuated the CCl₄-induced liver fibrosis (Fig. 4D). Further analysis demonstrated that the area of hepatic fibrosis was significantly reduced in BAY and CCl₄-treated mice compared with that in the mice treated with CCl₄ alone (Fig. 4E). The effect of BAY on hepatic hydroxyproline, which is indicative of hepatic fibrosis, was then studied. CCl₄ administration significantly increased the hepatic hydroxyproline content in the mice, while BAY administration significantly reduced this CCl₄-induced increase in hepatic hydroxyproline content (Fig. 4F).

Effect of BAY on serum ALT levels and expression of collagen I and α-SMA in CCl₄-induced mouse liver injury. Serum ALT levels were determined as an indicator of liver function and the ability of BAY to reduce serum ALT levels in CCl₄-induced liver injury was investigated. ALT levels were significantly elevated in the CCl₄ group (Group 1 vs. Group 2, 38.96±5.88 vs. 448.45±78.40 U/l; P<0.001). However, BAY treatment significantly attenuated the CCl₄-induced increase in ALT levels.
WA NG et al: NF-κB INHIBITION ALLEVIATES LIVER FIBROSIS

98

In addition, CCl\textsubscript{4}-induced liver injury revealed high expression levels of collagen I and α-SMA by western blotting (Fig. 5) and BAY was shown to decrease the protein expression levels of collagen I and α-SMA in the liver injury model. These in vivo results were consistent with the in vitro results.

**Figure 4.** Effect of BAY on CCl\textsubscript{4}-induced liver fibrosis. Mice were intraperitoneally injected with 1 ml/kg CCl\textsubscript{4} twice a week in combination with 5 mg/kg BAY three times a week for 6 weeks. (A-D) Liver fibrosis was detected by Sirius red staining. Representative micrographs of histology from (A) normal, (B) vehicle (10% DMSO/PBS), (C) CCl\textsubscript{4}, and (D) CCl\textsubscript{4} + BAY treated mice (magnification, x100). (E) Morphometrical analysis for evaluating the percentages of α-SMA-positive areas in 12 random fields. (F) Hepatic hydroxyproline was detected. All data are expressed as the mean ± SD of 12 mice. *P<0.01, vs. control; ^P<0.01, vs. CCl\textsubscript{4}. BAY, BAY-11-7082; SMA, smooth muscle actin; CCl\textsubscript{4}, carbon tetrachloride; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

**Figure 5.** Effect of BAY on the protein expression levels of collagen I and α-SMA in CCl\textsubscript{4}-induced mouse liver injury. CCl\textsubscript{4}-induced liver injury revealed high expression levels of collagen I and α-SMA by western blotting. BAY decreased the protein expression levels of collagen I and α-SMA in the liver injury model. The in vivo data were consistent with the in vitro results. *P<0.01, vs. control; ^P<0.01, vs. CCl\textsubscript{4}. BAY, BAY-11-7082; SMA, smooth muscle actin; CCl\textsubscript{4}, carbon tetrachloride.

**Discussion**

Chronic inflammation and the associated regenerative wound-healing response are strongly associated with the development of fibrosis and cirrhosis (16). In the past decade, numerous inflammatory mediators have been shown to contribute to the progression of chronic liver disease, a number of which are targets or activators of NF-κB (17-20). Studies targeting this molecule as an appropriate therapeutic agent in various diseases are ongoing. However, it is necessary to demonstrate whether NF-κB antagonism effectively treats pre-existing hepatic fibrosis and the potential mechanism of action. In the present study, the NF-κB inhibitor, BAY, effectively suppressed HSC-T6 activation by downregulating the...
expression of collagen I and α-SMA. BAY also inhibited PI3K and Akt phosphorylation in activated HSC-T6 cells.

Kupffer cells contribute to HSC activation and liver fibrosis (21). Inhibition of NF-κB in Kupffer cells results in decreased liver fibrosis; however, the underlying mechanisms remain largely elusive (22). While the role of NF-κB activation in hepatocytes and Kupffer cells leading to liver fibrosis is not completely understood, there is growing evidence that NF-κB functions as a key mediator of fibrosis. A wide range of proinflammatory mediators activate NF-κB in HSCs, including LPS, tumor necrosis factor and interleukin-1β (23-26). In addition, HSCs activate NF-κB during culture activation (27) and in human and mouse models of liver fibrosis, as demonstrated by the presence of Ser 536-phosphorylated p65 (25). Notably, NF-κB activation is almost exclusively observed in HSCs, indicating that these cells are an important site of inflammation in a chronically injured and fibrotic liver (28). Notably, the results of the present study demonstrate that the administration of BAY attenuates liver fibrosis induced in mice by the administration of CCl₄. BAY also significantly decreased the levels of serum ALT in the model mice.

PI3K is a key signaling molecule that controls numerous cellular functions (29). In the liver, PI3K activation promotes cytokine production and subsequent hepatocyte proliferation following partial hepatectomy (30). Hepatocyte-associated PI3K regulates hepatocyte growth by a process involving Akt activation (30). In the present study, fibrogenesis, which may be promoted by PI3K, was inhibited in association with reduced α-SMA expression and collagen production. PI3K/Akt signaling activation was also inhibited by BAY in LPS-treated HSCs and the fibrotic mouse liver. Inhibition of PI3K/Akt signaling may be strongly associated with the antifibrogenic effect.

In summary, the present study demonstrates that NF-κB signaling is activated in the pathogenesis of CCl₄-induced hepatic fibrosis. BAY, an NF-κB inhibitor, inhibits CCl₄-induced hepatic PI3K/Akt signaling activation. In addition, BAY attenuates CCl₄-induced HSC activation and effectively alleviates CCl₄-induced hepatic fibrosis in mice. Thus, NF-κB inhibition may have potential therapeutic value against hepatic fibrosis.

References

1. Friedman SL: Mechanisms of hepatic fibrogenesis. Gastroenterology 134: 1655‑1669, 2008.
2. Jiao J, Friedman SL, and Aloman C: Hepatic fibrosis. Curr Opin Gastroenterol 25: 223‑229, 2009.
3. Bataller R and Brenner DA: Liver fibrosis. J Clin Invest 115: 209‑218, 2005.
4. Friedman SL: Reversibility of hepatic fibrosis and cirrhosis - is it all hype? Nat Clin Pract Gastroenterol Hepatol 4: 236‑237, 2007.
5. Tangkijvanich P and Yee HF Jr: Cirrhosis - can we reverse hepatic fibrosis? Eur J Surg Suppl 587: 100‑112, 2002.
6. Povero D, Busletta C, Novo E, et al: Liver fibrosis: a dynamic and potentially reversible process. Histol Histopathol 25: 1075‑1091, 2010.
7. Schuppan D and Kim YO: Evolving therapies for liver fibrosis. J Clin Invest 123: 1887‑1901, 2013.
8. Gressner AM and Weiskirchen R: Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-β as major players and therapeutic targets. J Cell Mol Med 10: 76‑99, 2006.
9. Gressner AM, Weiskirchen R, Breitkopf K and Dooley S: Roles of TGF-beta in hepatic fibrosis. Front Biosci 7: d793‑d807, 2002.
10. Gressner OA and Gressner AM: Connective tissue growth factor: an autocrine master switch in fibrotic liver diseases. Liver Int 28: 1065‑1079, 2008.
11. Robinson SM and Mann DA: Role of nuclear factor kappab in liver health and disease. Clin Sci (Lond) 118: 691‑705, 2010.
12. Gao Q, Wang XY, Zhou J and Fan J: Cell line misidentification: the case of the Chang liver cell line. Hepatology 54: 1894‑1895, 2011.
13. Hao ZM, Cai M, Lv YF, Huang YH and Li HH: Oral administration of recombinant adeno-associated virus-mediated bone morphogenetic protein-7 suppresses CCl₄-induced hepatic fibrosis in mice. Mol Ther 20: 2043‑2051, 2012.
14. Kar S, Ukil A and Das PK: Cystatin cures visceral leishmaniasis by NF-κB-mediated proinflammatory response through co-ordination of TLR/MyD88 signaling with p105-TpI2-ERK pathway. Eur J Immunol 41: 116‑127, 2011.
15. Zhao J, Zhang H, Huang Y, et al: Bay11-7082 attenuates murine lupus nephritis via inhibiting NLRP3 inflammasome and NF-κB activation. Int Immunopharmacol 17: 116‑122, 2013.
16. Luedde T and Schwabe RF: NF-κB in the - linking injury, fibrosis and hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 8: 118‑118, 2011.
17. Bonacchi A, Petrai J, Defranco RM, et al: The chemokine CCL21 modulates lymphocyte recruitment and fibrosis in chronic hepatitis C. Gastroenterology 125: 1060‑1076, 2003.
18. Seki E, De Minicis S, Osterreicher CH, et al: TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 13: 1324‑1332, 2007.
19. Seki E, De Minicis S, Gwak GY, et al: CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest 119: 1858‑1870, 2009.
20. Miura K, Kodama Y, Inokuchi S, et al: Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology 139: 323‑334, 2010.
21. Dufield JS, Forbes SJ, Constantindou CM, et al: Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 115: 56‑65, 2005.
22. Son G, Iimuro Y, Seki E, Hirano T, Kaneda Y and Fujimoto J: Selective inactivation of NF-kappaB in the liver using NF-kappaB decoy suppresses CCl₄-induced liver injury and fibrosis. Am J Physiol Gastrointest Liver Physiol 293: G631‑G639, 2007.
23. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C and Brenner DA: Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology 57: 1043‑1055, 2003.
24. Hellerbrand C, Jobin C, Iimuro Y, Licato L, Sartor RB and Brenner DA: Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. Hepatology 27: 1285‑1295, 1998.
25. Oakley F, Teoh Y, Ching-A-Sue G, et al: Angiotensin II activates 1 kappaB kinase phosphorylation of RelA at Ser 536 to promote myofibroblast survival and liver fibrosis. Gastroenterology 136: 2334‑2344, 2009.
26. Schwabe RF, Schnabl B, Kweon YO and Brenner DA: CD40 activates NF-kappa B and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. J Immunol 166: 6812‑6819, 2001.
27. Elsharkawy AM, Wright MC, Hay RT, et al: Persistent activation of nuclear factor-kappaB in cultured rat hepatocellular cells involves the induction of potentially novel Rel-like factors and prolonged changes in the expression of IkappaB family proteins. Hepatology 30: 761‑769, 1999.
28. Kluwe J, Pradere JP, Gwak GY, et al: Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology 138: 347‑359, 2010.
29. Cushing TD, Metz DP, Whittington DA and McGee LR: PI3Kδ and PI3Kγ as targets for autoimmune and inflammatory diseases. J Med Chem 55: 8559‑8581, 2012.
30. Jackson LN, Larson SD, Silva SR, et al: PI3K/Akt activation is critical for early hepatic regeneration after partial hepatectomy. Am J Physiol Gastrointest Liver Physiol 294: G1401‑G1410, 2008.