

• BASIC RESEARCH •

ACEI attenuates the progression of CCl₄-induced rat hepatic fibrogenesis by inhibiting TGF- β 1, PDGF-BB, NF- κ B and MMP-2,9

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activity markedly increased in model group, perindopril treatment considerably reduced NF- κ B DNA binding activity.

CONCLUSION: Perindopril attenuates CCl₄-induced hepatic fibrogenesis of rat by inhibiting TGF- β 1, PDGF-BB, NF- κ B and MMP-2,9

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Abstract

AIM: Angiotensin II has pro-fibrotic function in the liver. Blockade of the renin-angiotensin-aldosterone-system (RAAS) attenuates hepatic fibrosis. The aim of the present study was to determine the mechanism of angiotensin-converting enzyme inhibitor (ACEI) on the progression of rat hepatic fibrosis.

METHODS: Forty male Wistar rats were divided into three groups. Model group (Mo): The rats were injected subcutaneously with 40% of CCl₄ 0.25 mL/100 g. Perindopril group (Pe): The rats were injected subcutaneously with 40% of CCl₄. Perindopril, equivalent to 2 mg/(kg•d), was administrated. Control group (Nc): the rats were treated with olive oil only. After 4 and 6 wk, the rats were killed. The liver sections were stained with Masson. The protein expressions of AT1R, TGF- β 1 and PDGF-BB were examined by Western blot. Nuclear factor κ B (NF- κ B) DNA binding activity was examined by EMSA (Electrophoretic gel mobility shift assay). Matrix metalloproteinase-2,9 (MMP-2,9) activity was assessed by zymography. Serum laminin (LN) and hyaluronic acid (HA) were measured using radioimmunoassays.

RESULTS: Using Western blot, we clearly provided direct evidence for the expression of AT1R in liver. The expression was up-regulated when fibrogenesis occurred. Perindopril treatment significantly reduced mean fibrosis score, protein levels of AT1R, TGF- β 1 and PDGF-BB, serum levels of HA and LN, and the activity of MMP-2,9. NF- κ B DNA binding

INTRODUCTION

It has been known that renin-angiotensin-aldosterone system (RAAS) plays a key role in the fibrogenesis of local tissue. Angiotensin II (Ang II) exerts local effects on tissue growth and fibrosis by interaction with angiotensin II type 1 receptor (AT1R), leading to a series of pathological changes such as myocardial hypertrophy, renal interstitial fibrosis, and pulmonary fibrosis. In recent years, much attention has been focused on the novel relationship between activation of RAAS and fibrosis of liver. Recent evidence indicates that Ang II may be an important mediator in liver fibrosis. Intra-hepatic RAAS is upregulated in experimental hepatic fibrosis, and serum Ang II levels are frequently elevated in patients with cirrhosis^[1,2]. Patients with chronic hepatitis C and a genetic polymorphism associated with increased Ang II synthesis have been reported to develop more severe fibrosis^[3]. More over, inhibition of Ang II synthesis or the blockade of Ang II type 1 (AT1) receptors markedly reduce fibrosis in experimental models of hepatic fibrosis^[4-7]. However, the mechanisms underlying the anti-fibrotic effect of angiotensin-converting enzyme inhibitor (ACEI) on liver fibrosis remain unclear. The present study was undertaken to investigate the effect of ACEI on hepatic fibrosis of rat and the potential mechanism.

MATERIALS AND METHODS

Animal model

Male Wistar rats (250-280 g, $n = 40$, purchased from Animal Center of the First Military Medical University) were randomly

divided into three groups. Model group (Mo): The rats were injected with 40% of CCL₄ (the mixture of CCL₄ and olive oil) at the dosage of 0.25 mL/100 g subcutaneously three a week. Perindopril group (Pe): The rats were treated with CCL₄ same to model group, and at the same time, perindopril, equivalent to 2 mg/(kg·d), was given ig. Control group (Nc): the rats were injected with olive oil only.

Histology

At the end of the 4th and 6th wk, the rats were killed. The tissue of liver was regularly fixed, embed, sliced and stained with Masson. Fibrosis was staged 0-4 based on Scheuer's scoring system^[8] as follows: stage 0: no fibrosis; stage 1: expansion of the portal tracts without linkage; stage 2: portal expansion with portal to portal linkage; stage 3: expansive portal to portal and focal portal to central linkage; and stage 4: cirrhosis.

Serum HA and LN assays

Serum levels of LN and HA were determined by radioimmunoassays (kit purchased from Northern Biot Co., China) according to the instruction.

Western blot analysis of AT1R, TGF- β 1 and PDGF-BB

Six separate liver tissues from each group were homogenized in 1× cell lysis buffer (Cell Signaling, USA). Fifty micrograms of protein were electrophoresed on 10% or 15% sodium dodecyl sulfate-polyacrylamide under denaturing conditions, and then electrotransferred to PVDF membranes. Nonspecific protein binding was blocked by incubating the membranes with blocking solution (1×TBS, 0.1% Tween-20 with 5% nonfat dry milk) over night at 4 °C. Polyantibody specific for AT1R, TGF- β 1 and PDGF-BB (Santa Cruz, USA; 1:700 in 1×TBS containing 0.1% Tween-20 with 5% nonfat dry milk) was applied to the membrane for 2 h at room temperature. After rising with washing buffer (1× TBS, 0.1% Tween-20), HRP-conjugated anti-rabbit IgG antibody (Santa Cruz, USA) diluted at 1:2000 was applied to the membrane for 1 h at room temperature. The detection of specific signal was performed using the Luminol Reagent Solution (Santa Cruz, USA) according to the instruction of the vendor. The protein signal intensity was quantified by a computerized medical image-processing system (GDS-7500, UVP, UK).

EMSA (electrophoretic gel mobility shift assay) for NF- κ B DNA binding activity

The nuclear extracts were prepared either by treating the rats with perindopril for 4 or 6 wk. The nuclear extracts (6 μ g) were incubated with 100 pg of ³²P-labeled double-stranded nuclear factor κ B (NF- κ B) oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC3'; 5'-AGTTGCCTGGGAAAGTCCCCCTC 3') in binding buffer (25 mmol/L Hepes (pH 7.9), 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 1% Nonidet P-40, 5% glycerol, and 50 mmol/L NaCl) containing 2 μ g of polydeoxyinosinic deoxycytidylic acid (poly(dI-dC)). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography. In separate experiments, the nuclear extracts were preincubated with 100-fold excess of unlabeled NF- κ B oligonucleotide for 15 min prior to the addition of labeled probe and the

samples were further analyzed.

Zymography

Liver samples were centrifuged at 6 000 r/min for 30 min. Samples were then mixed with an equal volume of 2× non reducing sample buffer, and 50 μ g was loaded per well. MMP-2 and MMP-9 were analyzed on gelatin containing gels (7.5% polyacrylamide gel containing 2 mg/mL gelatin). Gels were electrophoresed at 90 V at 4 °C in 1× running buffer. After electrophoresis, SDS was removed from the gel by washing 1 h in 2.5% Triton X-100 solution. This allows the MMPs to renature and digest the surrounding substrate after being incubated overnight at 37 °C in zymogram incubation buffer (50 mmol/L Tris buffer at pH 7.6, 2.5% Triton X-100, 500 mmol/L NaCl, 0.02% NaN₃, and 5 mmol/L CaCl₂). After incubation, the gel was stained with a solution of 0.25% Coomassie blue R250, 40% methanol, and 10% acetic acid for 2 h at room temperature and destained with 40% methanol, 10% acetic acid until the bands of lysis became clear.

Statistical analysis

Analysis of data was performed with oneway ANOVA (SPSS 11.0®) and rank sum test. Results were expressed as mean±SD. A value of $P<0.05$ was regarded as statistical significance.

RESULTS

Histology

Histologic examination showed that marked and extensive fatty degeneration was present around the central veins and portal tracts except in the control group. At the end of the 4th wk, in model group, fibroblasts were obviously proliferated in the portal tracts, and collagen was invaded into the hepatic lobules along with the injured limiting laminae. In one case of this group, the formation of pseudolobules could be seen. At the end of the 6th wk, all of the untreated animals had developed severe damage (stage 3 or 4), characterized by extensive portal-portal and portal-central fibrous linkage, distortion of liver architecture, and entrapment of groups of hepatocytes by fibrosis. In contrast, perindopril treatment decreased the score of fibrosis at the end of 4th and 6th wk ($P<0.05$, Table 1, Figure 1).

Serum levels of LN and HA

Serum LN and HA levels in rats of model group were significantly higher than those of the control group ($P<0.05$). Treatment with perindopril significantly reduced serum levels of LN and HA ($P<0.05$, Table 2).

Western blot analysis of AT1R, TGF- β 1 and PDGF-BB

Western blot revealed that AT1R protein expression was upregulated in model group rats compared with control group rats. Perindopril treatment significantly reduced protein levels of AT1R, TGF- β 1 and PDGF-BB (Figure 2A-D).

EMSA

NF- κ B DNA binding activity markedly increased in model group, perindopril treatment considerably reduced NF- κ B DNA binding activity (Figure 3).

Table 1 Grades of fibrosis in rat liver

Groups	4 wk						6 wk					
	0	1	2	3	4	n	0	1	2	3	4	n
Mo	0	1	5	0	1	7	0	0	0	3	3	6
Pe ^{a,e}	1	3	2	0	0	6	0	1	1	4	0	6
Nc ^{b,c}	6	0	0	0	0	6	6	0	0	0	0	6

^a $P < 0.05$ vs Mo; ^b $P < 0.05$ vs Pe; ^c $P < 0.05$ Pe (4 wk) vs Pe (6 wk). Mo: model group; Pe: perindopril group; Nc: control group.

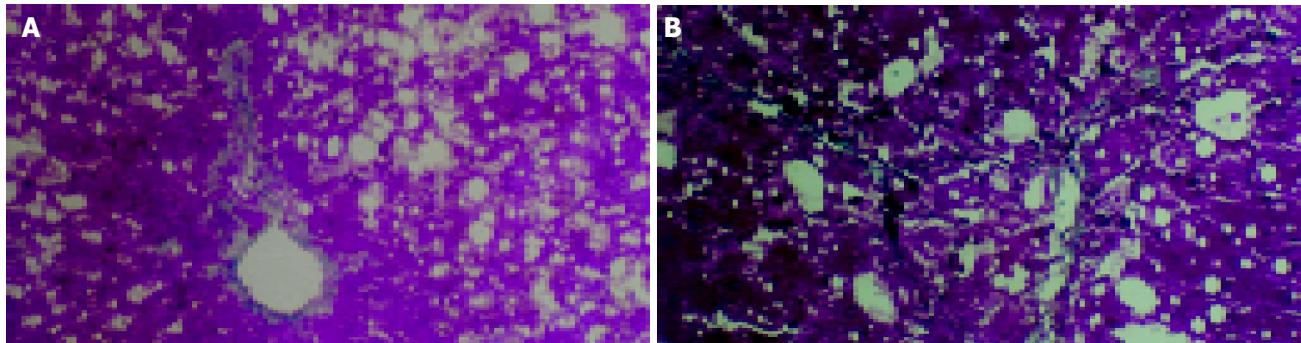


Figure 1 Masson staining of liver sections in perindopril treated-group (6 wk, panel A) and model group (6 wk, panel B). Perindopril [2 mg/(kg·d)] was administered for 6 wk. Less proliferation of fibrotic septa and marked fatty degeneration in the portal tract are evident in panel A (final magnification 100×).

While in panel B, the lobule is disorderly, and wide portal to portal fibrotic septa is evident. Extensive fatty degeneration is present around portal tracts and the central veins (final magnification 100×).

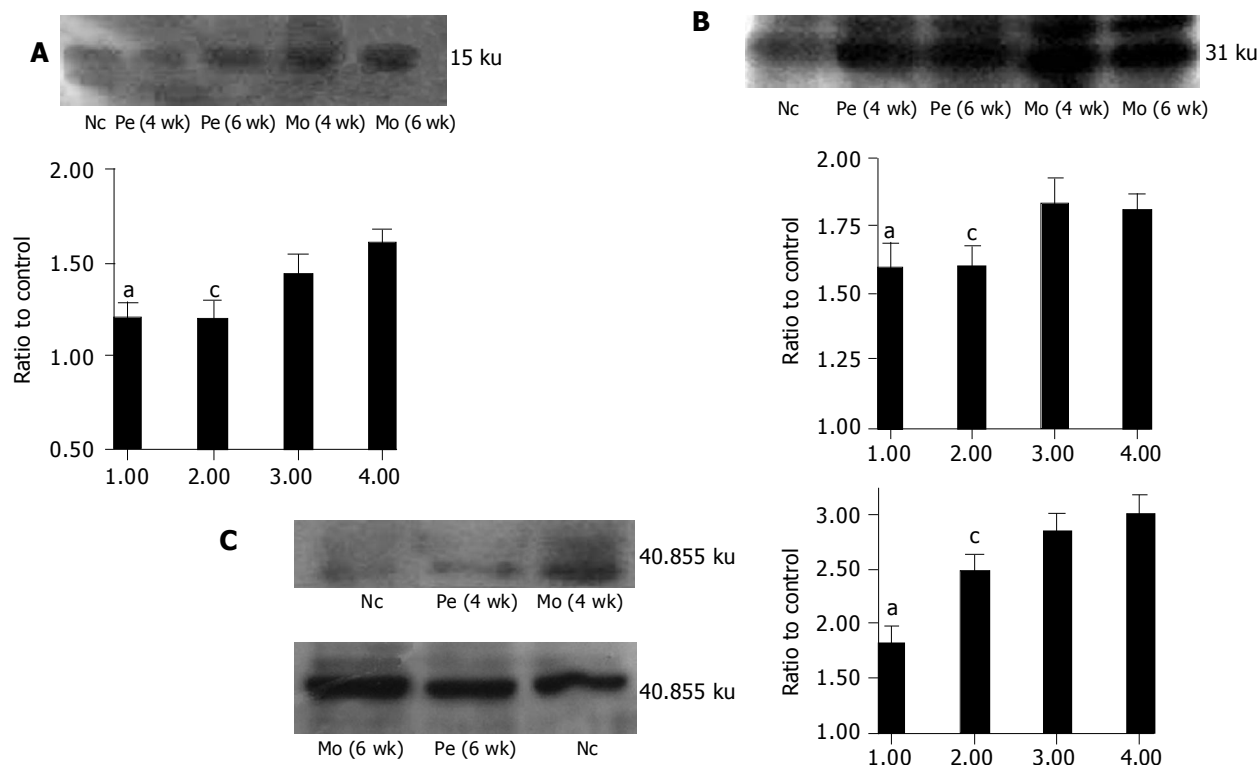


Figure 2 Western blot analysis **A**: Western blot analysis of TGF-β1 protein expression. Effect of perindopril on TGF-β1 expression in CCl₄-induced hepatic fibrosis of rat. Cell lysates were obtained as described in Methods. Protein (50 μg/lane) were separated by 15% SDS-PAGE under denaturing conditions, transferred to PVDF membranes and then incubated with specific antibody (1:700). Ratio to control presents TGF-β1 protein level. 1: Pe (4 wk); 2: Pe (6 wk); 3: Mo (4 wk); 4: Mo (6 wk) Results are the mean±SD from three independent experiments. ^a $P < 0.05$ vs Mo (4 wk); ^c $P < 0.05$ vs Mo (6 wk); **B**: Western blot analysis of PDGF-BB protein expression. Effect of perindopril on PDGF-BB expression in CCl₄-induced hepatic fibrosis of rat. Cell lysates were obtained as described in Methods. Protein (50 μg/lane) were separated by 10% SDS-PAGE under denaturing conditions,

transferred to PVDF membranes and then incubated with specific antibody (1:700). Ratio to control presents PDGF-BB protein level. 1: Pe (4 wk); 2: Pe (6 wk); 3: Mo (4 wk); 4: Mo (6 wk) Results are the mean±SD from three independent experiments. ^a $P < 0.05$ vs Mo (4 wk); ^c $P < 0.05$ vs Mo (6 wk); **C**: Western blot analysis of AT1R protein expression. Effect of perindopril on AT1R expression in CCl₄-induced hepatic fibrosis of rat. Cell lysates were obtained as described in Methods. Protein (50 μg/lane) were separated by 10% SDS-PAGE under denaturing conditions, transferred to PVDF membranes and then incubated with specific antibody (1:700). Ratio to control presents AT1R protein level. 1: Pe (4 wk); 2: Pe (6 wk); 3: Mo (4 wk); 4: Mo (6 wk) Results are the mean±SD from three independent experiments. ^a $P < 0.05$ vs Mo (4 wk); ^c $P < 0.05$ vs Mo (6 wk).

Table 2 Effect of perindopril on serum levels of HA and LN (mean±SD)

Groups	n	HA (μg/L)	LN (μg/L)
Nc	7	74.58±10.51	100.29±4.99
Pe (4 wk)	7	81.59±12.60 ^c	101.95±2.27 ^c
Pe (6 wk)	8	82.07±13.66 ^e	94.84±3.54 ^b
Mo (4 wk)	7	142.41±31.34 ^a	114.99±2.28 ^a
Mo (6 wk)	7	147.86±18.92 ^a	113.72±2.14 ^a

^aP<0.05 vs Nc; ^bP<0.01 vs Mo (6 wk); ^cP<0.05 vs Mo (4 wk); ^eP<0.05 vs Mo (6 wk).

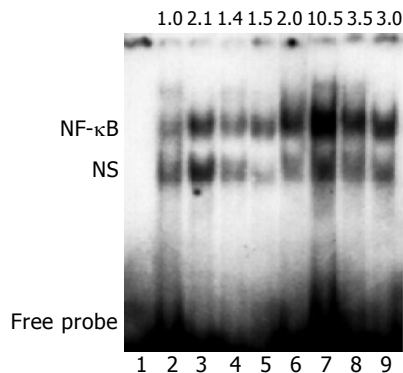


Figure 3 EMSA for NF-κB DNA binding activity. EMSA for NF-κB DNA binding activity. Lane 1: 100 folded excess unlabeled oligonucleotides; lane 2: control group; lanes 3 and 4: Pe (4 wk); lanes 5 and 6: Pe (6 wk); lanes 7 and 8: Mo (4 wk); lane 9: Mo (6 wk). NF-κB DNA binding activity markedly increased in model group, perindopril treatment considerably reduced NF-κB activity. Binding specificity was demonstrated by competition of excess unlabeled oligonucleotides containing the κB site. Results are representative of three independent experiments.

Activities of MMP-2 and MMP-9 assay

Perindopril treatment considerably reduced the increased MMP-2 and MMP-9 activities that were seen in model group rats (Figure 4).

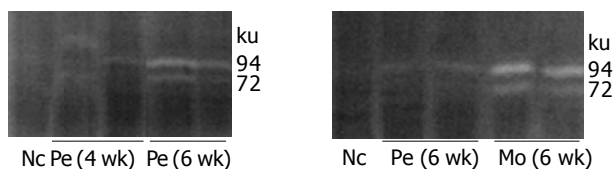


Figure 4 Zymography analysis of activities of MMP-2 and MMP-9. Effect of perindopril on the activities of MMP-2 (72 ku) and MMP-9 (94 ku) in CCL₄-induced hepatic fibrosis of rat. Liver samples were obtained as described. Protein (50 μg/lane) were separated by 7.5% gelatin containing SDS-PAGE, then remove SDS and incubate in zymogram incubation buffer as described. The clear band presents the activity of MMP-2,9. The activity of MMP-2,9 was elevated in Mo (4, 6 wk) compared with Nc and Pe (4, 6 wk).

DISCUSSION

The RAAS is an important regulator of cardiovascular homeostasis and systemic blood pressure. In recent years, AngII has been shown to have pro-sclerotic actions at many organs including heart, lung and kidney. The RAAS has also been demonstrated in liver with the potential for a functioning intra-hepatic paracrine RAAS. Stimulation of AT1R, belonging to the seven transmembrane, G-protein coupled receptor

families, leads to vasoconstriction, fibroblast growth, and aldosterone secretion with synthesis of collagen type I, III, all deem to have a detrimental component in cardiac hypertrophy and organ fibrosis^[9,10]. In contrast, ACEI exert effects on inhibiting the growth of cardiac fibroblast and pulmonary fibroblast, resulting in reduction of collagen deposition^[11,12]. *In vitro* studies suggest that AngII may directly stimulate proliferation and enhance bioactivation of TGF-β1, being reduced by RAAS blockade, in cardiac^[9] and pulmonary fibroblasts^[10].

It is well known that RAAS is always activated in chronic hepatopathy. Powell^[3] recently elucidates the inheritance of polymorphisms in the TGF-β1 and angiotensinogen genes and the influence of genotypes on the stage of hepatic fibrosis in patients with chronic hepatitis C. Patients who inherit neither of the pro-fibrogenic genotypes have no or only minimal fibrosis. The documentation raises the novel suggestion that Ang-II may be a mediator of fibrosis in the liver. Hepatic stellate cell (HSC), the main resource of ECM, is the key fibrogenic effector of the liver. The *in vitro* finding suggest that AT1 receptors are expressed on activated human HSC, and the binding of Ang-II to the receptor induces contraction and proliferation of these cells^[13]. Recently, our previous study^[14-16] demonstrated that aldosterone synthase gene-CYP11B2 expression was upregulated in HSC when liver fibrosis occurred, and antisterone could partly exert a fibrogenesis-inhibiting effect in the early stage of liver fibrosis. Therefore, RAAS indeed plays a pivotal role in liver fibrosis development through activation of HSC.

In the present study, we examined the *in vivo* effect of perindopril on hepatic fibrosis of rat. Administration of perindopril can partly alleviate hepatic fibrosis. The fibrosis score and serum fibrosis markers are suppressed by perindopril treatment. Moreover, using Western blot, we clearly provided direct evidence for the expression of AT1R in rat liver. The expression was up-regulated when fibrogenesis occurred. The protein levels of AT1R were suppressed by perindopril. Furthermore, perindopril treatment can inhibit the secretion of Angiotensin II-mediated TGF-β1 and PDGF-BB proteins, which are key factors conducive to the synthesis and the deposition of collagen products by autocrine and paracrine action^[17,18]. Further studies are required to elucidate the potential mechanism.

NF-κB is a family of transcription factor that have been shown to be involved in gene regulation of cellular processes like inflammation, fibrogenesis, cell proliferation, and apoptosis^[19-21]. This family of proteins is particularly interesting, due to its implication for activation of hepatic stellate cell, the key cellular element involved in the development of hepatic fibrosis. This DNA-binding protein binds to the κB sequence. It promotes transcription of cell adhesion molecules, angiotensinogen and varieties of cytokines such as IL-6, IL-8, TNF-α, which promote hepatic fibrogenesis^[22-24]. The present study firstly showed that perindopril treatment significantly reduced NF-κB DNA binding activity in fibrotic liver induced by CCL₄. Consequently, perindopril attenuate hepatic fibrosis by inhibiting NF-κB DNA binding activity.

Matrix metalloproteinases (MMPs) degrade the extracellular matrix (ECM) and play critical roles in tissue repair and fibrogenesis. In human chronic liver diseases, there is a

upregulation of MMP-2 and MMP-9, which results in increased degradation of basement membrane collagen. Previous study^[25] showed that captopril can inhibit MMP-2 and MMP-9 by interacting with the zinc at their active sites, which contribute to the relief of fibrosis. In the current study, MMP-2 and MMP-9 were significantly suppressed by perindopril. In consideration of the action of NF- κ B mediated-induction of MMP-2^[26], we may demonstrate that perindopril attenuated MMP-2/9 level by inhibiting NF- κ B DNA binding activity.

Consequently, the dual effects of ACEI to inhibit NF- κ B activity and MMP-2/9, in addition to reducing protein expression of TGF- β 1 and PDGF-BB, are likely to contribute to the anti-fibrotic actions of these agents. These findings are in accordance with recent experimental studies that have demonstrated anti-fibrotic effects of RAAS blockade in liver^[27].

The effect of existing anti-fibrotic medicines such as interferon and colchicine is limited. However, ACEI have been proven to be an effective anti-fibrotic drug with low incidence of serious side effects. Thus, these medicines may be suitable agents for trials in human chronic liver diseases associated with progressive fibrosis. The present study provides a novel pathway for attenuation of liver fibrosis.

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