Angiotensin-(1-7) prevents lipopolysaccharide-induced hepatocellular inflammatory response by inhibiting the p38MAPK/AP-1 signaling pathway

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Abstract. The pathological mechanism of lipopolysaccharide (LPS)-induced liver injury involves a number of inflammatory mediators and cytokines. Angiotensin (Ang)-(1-7), a ligand for the proto-oncogene Mas (Mas) receptor, antagonizes the actions of Ang II in the renin angiotensin system and exerts an anti-inflammatory effect on LPS-induced macrophages. The present study investigated the potential role of Ang-(1-7) in the regulation of inflammatory responses in LPS-induced hepatocytes using the rat liver BRL cell line. The results of the present study demonstrated that the inflammatory mediator, tumor necrosis factor (TNF)-α, its upstream transcriptional regulatory factor activator protein (AP)-1 and p38 mitogen activated protein kinase (MAPK) which were detected by reverse transcription-quantitative polymerase chain reaction and western blotting were upregulated in LPS-induced hepatic cells in a time-dependent manner, peaking 12 h following LPS stimulation. By contrast, treatment with Ang-(1-7) significantly attenuated the expression of TNF-α, AP-1 and p38MAPK in a concentration-dependent manner. The anti-inflammatory effect of Ang-(1-7) was reversed by the Mas receptor selective antagonist, A779, in BRL cells. Furthermore, the p38MAPK inhibitor, SB 203580, abolished the protective effects of Ang-(1-7), suggesting the involvement of the p38MAPK pathway in the anti-inflammatory activity of Ang-(1-7). The results of the present study indicated that Ang-(1-7) may serve an anti-inflammatory role in LPS-induced hepatocyte injury via the regulation of the p38MAPK/AP-1 signaling pathway.

Introduction

Lipopolysaccharide (LPS), present in the external part of the cell wall of Gram (-) bacteria, can induce sepsis characterized by an uncontrolled hyper-inflammatory response that frequently results in multiple organ failure (1). The liver serves a role in the reduction of inflammation and is responsible for the initiation of multiple organ failure (2,3). Upon exposure of the liver to LPS, several inflammatory responses are initiated, including the release of inflammatory cytokines, and the activation of the renin-angiotensin (Ang) system (RAS) and the associated Ang converting enzyme (ACE)-Ang II-Ang I type 1 receptor (AT1R) (4-6).

Ang-(1-7) is currently recognized as a biologically active component of non-classic RAS, which, counteracts Ang II-AT1R by upregulating the production of nitric oxide and prostaglandins, and mediating anti-fibrosis, vasodilation and anti-diuretic responses (7-11). It has also been demonstrated that Ang-(1-7) decreases LPS-induced inflammatory responses in macrophages (12). However, the anti-inflammatory effect of Ang-(1-7) on LPS-induced liver injury and the underlying molecular mechanism remain to be elucidated.

Tumor necrosis factor-α (TNF-α) is a cytokine involved in the progression of numerous inflammatory diseases, including LPS-induced liver injury (4,13). Activator protein (AP)-1 is a transcription factor that regulates the expression of TNF-α in cells (14). In addition, p38 mitogen activated protein kinase (p38MAPK) serves a role in mediating inflammatory responses from the extracellular space to the cytoplasm and nucleus (15). p38MAPK is activated in the liver by LPS via the phosphorylation and activation of components of the MAPK signaling pathway, that in turn promote the activation of AP-1 (16-18). Therefore, targeted inhibition of the MAPK/AP-1 signaling pathway has been hypothesized to serve a role in potential anti-inflammatory therapeutic approaches.

In the present study, the anti-inflammatory effect of Ang-(1-7) was investigated in LPS-induced hepatocytes, in order to elucidate whether the anti-inflammatory...
effect of Ang-(1-7) is mediated via the modulation of the p38MAPK/AP-1 signaling pathway.

Materials and methods

Cell culture. Immortalized rat liver BRL cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 µg/ml streptomycin (Beijing Suolaobao Biotechnology Co., Ltd., Beijing, China). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All experiments were carried out following 24 h once cells were seeded. The number of cells in each 25 ml culture flask was 4.5x10⁵.

Treatment groups. All cell treatments were performed at 37°C. LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to induce inflammation in BRL cells as a model of sepsis. Initially, 10 µg/ml LPS was applied for 0, 6, 12 and 24 h to determine the optimal time point to induce inflammation in BRL cells. Subsequently, as the maximum effect was recorded following 12 h of stimulation, 12 h of LPS stimulation was applied in the following experiments. The cells were divided into the following 5 groups: i) Control-untreated cells; ii) LPS-treated cells (10 µg/ml LPS for 12 h); iii) the LPS+Ang-(1-7) group, in which, cells were treated with 10⁻⁷, 10⁻⁶ or 10⁻⁵ mol/l Ang-(1-7) for 30 min followed by incubation with 10 µg/ml LPS for 12 h; iv) the LPS+A779 group, in which, cells were treated with 10⁻⁷, 10⁻⁶ and 10⁻⁵ mol/l of the Ang-(1-7) antagonist, A779, for 30 min followed by incubation with 10 µg/ml LPS for 12 h; and v) the LPS+Ang-(1-7)+SB 203580 group, in which, cells were pretreated with 10⁻⁵ mol/l Ang-(1-7) and 10⁻⁵ mol/l of the p38MAPK inhibitor, SB 203580, for 30 min followed by incubation with 10 µg/ml LPS for 12 h. Cells in each group were harvested 12 h following LPS stimulation. Ang-(1-7) and A779 were supplied by Sigma-Aldrich (Merck KGaA) and SB 203580 was obtained from Beyotime Institute of Biotechnology (Haimen, China).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured BRL cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using random primers, M-MLV reverse transcriptase and RNase inhibitor (Fermentas; Thermo Fisher Scientific, Inc.). qPCR was performed using the Power SYBR Green PCR Master Mix and an ABI 7500 instrument (both of Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) with the Green PCR Master Mix and an ABI 7500 instrument (both of Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using random primers, M-MLV reverse transcriptase and RNase inhibitor (Fermentas; Thermo Fisher Scientific, Inc.). qPCR was performed using the Power SYBR Green PCR Master Mix and an ABI 7500 instrument (both Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using random primers, M-MLV reverse transcriptase and RNase inhibitor (Fermentas; Thermo Fisher Scientific, Inc.). Expression levels of all transcripts were normalized to the expression level of GAPDH. 

Results

LPS treatment induces time-dependent alterations in AP-1 and p38MAPK expression in hepatocytes. To characterize the effect of LPS on signaling pathways mediating inflammation, BRL cells were stimulated with LPS for 6, 12 and 24 h. When compared with the control-untreated cells, LPS significantly increased the expression levels of AP-1 (Fig. 1A), and p38MAPK and p-p38MAPK (Fig. 1B), following 6 and 12 h of stimulation. By contrast, LPS demonstrated no effect on the expression levels of these proteins following 24 h of stimulation (Fig. 1). As the maximum LPS effect was recorded following 12 h of stimulation, this time point was used for subsequent experiments.

Inhibitory effect of Ang-(1-7) on LPS-induced AP-1 and p-p38MAPK expression. Activation of p-p38MAPK and AP-1
serves an important role in the production of TNF-α (14,17). Therefore, the aim of the present study was to determine the inhibitory effect of Ang-(1-7) treatment on the activation of p-p38MAPK and AP-1. BRL cells were pretreated with...
different concentrations (10^(-7), 10^(-6) and 10^(-5) mol/l) of Ang-(1-7) for 30 min and were subsequently stimulated with 10 µg/ml LPS for 12 h. Pretreatment with Ang-(1-7) neutralized the effect of LPS (Fig. 2). Protein and mRNA levels of AP-1 (Fig. 2A and B), and p38MAPK and p-p38MAPK (Fig. 2C and D) were significantly reduced following pretreatment with Ang-(1-7) in a concentration-dependent manner, compared with the LPS-treated cells.

**A779 further upregulates LPS-induced AP-1 and p-p38MAPK expression.** In order to further elucidate the protective anti-inflammatory role of Ang-(1-7), the effect of the Ang-(1-7) proto-oncogene Mas (Mas) receptor (MasR) selective antagonist, A779, on AP-1 and p-p38MAPK expression was evaluated. BRL cells were pretreated with 10^(-7), 10^(-6) and 10^(-5) mol/l A779 for 30 min and then stimulated with 10 µg/ml LPS for 12 h. AP-1 mRNA expression was significantly increased by treatment with A779 in a concentration-dependent manner, with the peak mRNA level observed at 10^(-5) mol/l, when compared with the control-untreated cells and cells stimulated with LPS only (Fig. 2B). A779 at 10^(-6) mol/l induced the most elevated protein level of AP-1 (Fig. 3A). A779 at concentrations between

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**Figure 3.** Dose-dependent effects of A779 on LPS-induced AP-1, p38MAPK and p-p38MAPK expression. (A) AP-1 protein expression in BRL cells treated with or without A779 at a dose of 10^(-7) and 10^(-6) mol/l. (B) p38MAPK and p-p38MAPK protein expression in BRL cells treated with or without A779 at a dose of 10^(-7) and 10^(-6) mol/l. Data are presented as the mean ± standard deviation. **p<0.01 and ***p<0.001 vs. the control group; &p<0.05 and &&&p<0.001 vs. the LPS group; ^p<0.05 and ^^^p<0.001 vs. the LPS+A779 10^(-7) mol/l group; *p<0.05 vs. the LPS+A779 10^(-6) mol/l group. LPS, lipopolysaccharide; AP-1, activator protein 1; p-p38MAPK, phosphorylated-p38 mitogen activated protein kinase; A779, Mas receptor selective antagonist.

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**Figure 4.** Effect of Ang-(1-7) and the phosphorylated-p38 mitogen activated protein kinase inhibitor, SB 203580, on AP-1 and TNF-α expression. (A) AP-1 protein expression. (B) AP-1 mRNA expression levels. (C) TNF-α mRNA expression levels. Data are presented as the mean ± standard deviation. ***p<0.001 vs. the control-untreated cells; ###p<0.001 vs. the LPS-treated cells; &&&p<0.001, vs. the LPS+Ang-(1-7) 10^(-5) mol/l group. LPS, lipopolysaccharide; AP-1, activator protein 1; Ang-(1-7), Angiotensin-(1-7); TNF-α, tumor necrosis factor-α.
10⁻²-10⁻⁵ mol/l enhanced the LPS-induced increase in the mRNA levels of p-p38MAPK, with peak levels observed with A779 at 10⁻⁴ mol/l (Fig. 2D). Pretreatment with A799 resulted in a significant increase in the protein levels of p38MAPK and p-p38MAPK, when administered at concentrations of 10⁻³ and 10⁻² mol/l, when compared with the control or LPS groups (Fig. 3B). However, A779 at 10⁻³ mol/l did not affect p38MAPK and p-p38MAPK protein levels.

**SB-203580 eliminates the inhibitory effects of Ang-(1-7) on the LPS-induced expression of AP-1 and TNF-α.** SB-203580, a specific p38MAPK signaling pathway inhibitor, was used to further verify the anti-inflammatory effect of Ang-(1-7) on MAPK signaling. Ang-(1-7) was added to BRL cells alone or with SB-203580 prior to stimulation with LPS. When compared with the LPS group, incubation with LPS+Ang-(1-7) significantly decreased the protein expression of AP-1 by 69.89% at 12 h; whereas, incubation with LPS in combination with Ang-(1-7) and SB-203580 significantly increased the protein expression of AP-1 by 3.25-fold compared with the LPS+Ang-(1-7) group (both P<0.05; Fig. 4A). In addition, LPS+Ang-(1-7)+SB-203580 treatment increased the mRNA expression of AP-1, when compared with the control and Ang-(1-7)+LPS groups (Fig. 4B). Ang-(1-7) inhibited the LPS-induced expression of TNF-α compared with the LPS group, which was marginally ameliorated by treatment with SB 203580 (Fig. 4C). The above results suggested that Ang-(1-7) may be protective against LPS-induced liver injury through the inhibition of the p38MAPK signaling pathway.

**Discussion**

In the present study, LPS induced a significant upregulation of the inflammatory response in hepatic cells. A significant increase was observed in the expression levels of transcriptional regulatory factors, AP-1 and p38MAPK. The BRL cell model was used to investigate the anti-inflammatory role of Ang-(1-7) in hepatocytes. Ang-(1-7) significantly reduced the LPS-induced inflammatory response. However, the Ang-(1-7) MasR antagonist, A779, inhibited the protective effect of Ang-(1-7) on LPS-induced inflammatory responses. Furthermore, the protective effects of Ang-(1-7) were abolished by treatment with the p38MAPK inhibitor. Therefore, the results of the present study suggest that the p38MAPK/AP-1 signaling pathway may be an important molecular mechanism underlying the liver-protective effects of Ang-(1-7) against LPS-mediated injury.

A previous study on the pathological mechanism of LPS-induced liver injury demonstrated that a number of inflammatory mediators and cytokines, including TNF-α, nitric oxide, interleukin (IL)-1, IL-6, transforming growth factor-β and reactive oxygen species, are released as a result of upregulated MAPK signaling in activated hepatic Kupffer cells (16). In addition, LPS activated RAS and associated ACE-Ang II-AT1 to affect hepatic blood flow redistribution, microcirculation disturbances and the production of pro-inflammatory cytokines (5,6).

Ang-(1-7) functions as a ligand for the G protein-coupled receptor Mas and has been demonstrated to antagonize the activity of Ang II (20,21). Specifically, Ang-(1-7) has demonstrated anti-inflammatory properties through its antagonistic effects on the pro-inflammatory factor Ang II (22,23). A previous study also revealed the anti-inflammatory effect of Ang-(1-7) on LPS-induced macrophages (12). Furthermore, it has been indicated previously that Ang-(1-7) prevents acute respiratory distress syndrome in rats following intratracheal administration of LPS, and that Ang-(1-7) receptor Mas deficiency exacerbates LPS-induced cerebral and systemic inflammation in mice (24,25). A recent study indicated that Ang-(1-7) inhibits LPS-induced acute lung inflammation in alveolar epithelial cells (26).

In the present study, the anti-inflammatory activity of Ang-(1-7) was demonstrated in hepatic cells stimulated with LPS. Since TNF-α is a pro-inflammatory mediator of liver damage in response to LPS, the inhibitory effects of Ang-(1-7) on TNF-α production were determined by investigating the activation of AP-1, an upstream transcriptional regulator of TNF-α (27). The results revealed that Ang-(1-7) reduced the expression of TNF-α when administered at a concentration of 10⁻³ mmol/l and reduced AP-1 expression in a concentration-dependent manner. A779 increased AP-1 mRNA expression when administered at concentrations of 10⁻²-10⁻³ mmol/l to LPS-stimulated hepatocytes. The above results suggested that the inhibitory effect of Ang-(1-7) on TNF-α production may be associated with AP-1.

p38MAPK serves an important role in the regulation of LPS-induced inflammation by controlling AP-1 activation and has been associated with LPS-induced liver injury (4,28). In the present study, pretreatment of hepatocytes with Ang-(1-7) significantly inhibited the effect of LPS by reducing the expression of p38MAPK and p-p38MAPK. However, this inhibitory effect was counteracted by the Ang-(1-7) antagonist, A779, and the p38 MAPK inhibitor, SB 203580. The results of the present study are supported by those previously obtained by Zhou et al (29) and Akhter et al (30). The above results suggest that Ang-(1-7) may inhibit AP-1 and TNF-α activation by inhibiting the p38MAPK signaling pathway in LPS-induced hepatocytes.

In conclusion, the present study demonstrated that Ang-(1-7) significantly reduced the levels of LPS-induced pro-inflammatory cytokines, and the expression of MAPK and AP-1 in hepatocytes. Inhibition of the p38MAPK/AP-1 signaling pathway serves a role in the anti-inflammatory effect of Ang-(1-7) treatment. Therefore, Ang-(1-7) could potentially be used for the hepatoprotective treatment of LPS-induced liver injury.

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**References**