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Original Research Article

Aromadendrin protects mouse liver from sepsis-induced injury by inhibiting NF-κB signaling pathway

Zhihui Zhou[1], Qun Yin[2]*
[1]Department of Critical Care Medicine, Chongqing General Hospital, Chongqing 401147, [2]Department of Hospital Infection Management, Wuxi Second People's Hospital, Wuxi, Jiangsu Province 214001, China

For correspondence: **Email: qu_yin1635@163.com; **Tel:** +86-0510-68182476*

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Abstract

Purpose: *To investigate the protective role of aromadendrin in septic liver injury in mice, and to determine its mechanism of action.*
Methods: *Eight-week-old male C57BL/6 mice (n=6 for each group) were administrated with aromadendrin (SMB00175, Sigma-Aldrich) at 0 mg/kg, 30 mg/kg and 60 mg/kg via a hypodermic intraperitoneal injection. HE staining was used to examine liver histopathological structural changes in the liver while DAPI/Tunel staining was employed to evaluate liver cell apoptosis. The mRNA expression levels of TNF-α, IL-1β and IL-6 were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Moreover, enzyme-linked immunosorbent assay (ELISA)was applied to assess the levels of TNF-α, IL-1β and IL-6, as well as the activities of catalase (CAT), antioxidant glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA). Moreover, the protein levels of p65, p-p65, p-IκBα and IκBα were analyzed by Western blotting.*
Results: *The liver tissues exhibited severe structural damages, with edema, necrosis, and neutrophil infiltration, but recovered as a result of aromadendrin treatment ($p < 0.05$). The increased serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in CLP mice were reduced by aromadendrin, which also attenuated liver injury and cell apoptosis. Aromadendrin inhibited the levels of TNF-α, IL-1β and IL-6 in the mice, while the activities of GSH and antioxidant enzymes (SOD and CAT) were also significantly lowered in the mice, but attenuated by aromadendrin ($p < 0.05$). Aromadendrin also prevented the increased level of MDA, and suppressed the phosphorylation of p65 and IκBα ($p < 0.05$).*
Conclusion: *Aromadendrin protects mouse liver from sepsis-induced injury by inhibiting NF-κB signaling in vivo, thus suggesting a potential strategy for the therapy of sepsis-induced liver injury.*

Keywords: *Aromadendrin, Sepsis, NF-κB, Liver injury, Cell apoptosis*

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INTRODUCTION

Sepsis is a systemic life-threatening symptom in response to infection [1]. Patients with severe sepsis suffer from microvascular thrombosis, which leads to multiple organ dysfunction [1]. Sepsis-related deaths represent 19.7 % of all deaths worldwide [1], and it is caused by infections of bacteria, fungi or viruses Unfortunately though, there are currently no

specific therapeutic strategies in existence [2]. Multiple organ failure and hypotension are the main causes of the high mortality of sepsis [2,3]. Septic liver dysfunction is a severe risk factor for multiple organ dysfunction and sepsis-related deaths [4]. Therefore, an elucidation of the molecular mechanisms of liver injury in sepsis is important and urgent.

Aromadendrin, a flavonoid from Chionanthus retusus, has demonstrated tremendous pharmacological activities which include anti-proliferation, anti-cardiac hypertrophy, anti-inflammation, anti-oxidant and anti-hyperglycemia [5-7]. Aromadendrin exhibits a protective role in neuronal cells through methamphetamine-induced autophagy and apoptosis via the promotion of endoplasmic reticulum stress and PI3K/Akt/mTOR signaling pathway [8]. Aromadendrin can attenuate lipopolysaccharide-induced inflammation by the inhibition of nuclear translocation of NF-κB and the phosphorylation of JNK [9]. However, the role of aromadendrin in septic liver injury has not been studied.

Emerging studies have demonstrated that NF-κB signaling pathway play a critical role in the progress of sepsis-induced multi-organ dysfunction [10]. Upregulation of NF-κB related signaling is linked with liver injury induced by sepsis [11]. NF-κB is composed by five members namely, p65 (RelA), RelB, NF-κB1, NF-κB2 and c-Rel [12]. NF-κB signaling can be activated in response to pathogens and proinflammatory cytokines [12]. NF-κB binds to its inhibitor IκB and forms a complex in cytosol in a resting state [10]. When the IκB kinase is activated in response to inflammatory stimuli, this stimulates the phosphorylation of IκB, and then it moves to the nucleus, thus allowing its regulatory role in various gene expressions [10]. The inflammatory cytokines including TNFα, IL-1β and IL-6 stimulates the activity of NF-κB, which also regulates the expression levels of inflammatory cytokines, and forming an amplified feedback loop [13].

The objective of this work was to investigate the ameliorative role of aromadendrin in septic liver injury.

EXPERIMENTAL

Mice and reagents

C57BL/6 were obtained from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). The protocol conformed to International Guidelines for the Humane Care and Use of Laboratory Animals. All animal experiments were approved by the Ethics Committee of Chongqing General Hospital (Approval no. S2020-072-01) for the use of animals, and were conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [14]. Aromadendrin was supplied by Sigma-Aldrich (St. Louis, USA); Antibodies against p65 (# 8242S), p-p65 (# 3033) and p-IκBα (# 9246) were supplied by Cell Signaling Technology (Cambridge, UK); Antibodies against IκBα (sc-1643) and β-actin (sc-47778) were supplied by Santa Cruz Biotechnology. Antibodies were diluted 1:1000 with 5 % BSA for working solution.

Cecum ligation and puncture (CLP)-induced sepsis model

C57BL/6 were administered with aromadendrin for five days. On day 6, a midline abdominal incision was made on the mice after the administration of intraperitoneal anesthesia. The cecum was then ligated and punctured with a needle, and the peritoneum and skin were sutured. Sterile saline solution was then injected into the mice via a hypodermic intraperitoneal injection. In the sham group, a laparotomy was conducted on the mice, and after 12 h, blood and liver tissue were collected.

Hematoxylin-eosin (HE) staining

Fixed liver tissue was dehydrated with xylene, then embedded in paraffin, sliced and dewaxed. The sample was stained with hematoxylin and eosin, dehydrated, cleared, dried, and mounted. Images of the histological changes were observed with a microscope, and the histopathological changes score was calculated based on these criteria: 1, congestion; 2, edema; 3, infiltration of polymorphonuclear leukocytes and monocytes; 4 necrosis. The sum of the score ranged from 0 to 10.

Liver injury assessment

The serum levels of ALT and AST in CLP mice were assessed using the respective assay kits according to the manufacturer's instructions.

TUNEL assay

Tunel assay was performed using Promega Tunel assay kit (cat no. G3250). Liver tissue was embedded with paraffin, and Ethanol was used to deparaffinize and rehydrate. The sample was fixed with 4 % paraformaldehyde, and treated with proteinase K. The tissue was treated with rTDT incubation buffer and counterstained with

DAPI. The images were captured with fluorescence microscope (Nikon).

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β, IL-6, CAT, GSH, SOD, MDA in liver tissues were assessed with ELISA kit (eBioscience, America) according to the manufacturer's instructions.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen), RT Reagent kit was used for reverse transcription, while the mixture for qPCR was performed using SYBR Green mix (Takara, Japan). The reaction was initiated and measured with ABI PRISM 7500 Real-Time PCR system, and the relative mRNA expression level was quantified with $2^{-\triangle\triangle Ct}$ method [15]. The primer sequences used are shown in Table 1.

Western blotting

Liver tissue was collected and washed once with cold PBS, and protein was extracted with lysis buffer. The supernatant was collected after centrifugation at 12000 × *g* for 30 min. Protein concentration was evaluated with a bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Protein was separated by SDS-PAGE, and the blots were evaluated by immunoblotting analysis. The bands were then determined using enhanced chemiluminescence.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was analyzed by one-way ANOVA. Statistical significance between different groups were analyzed by two-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Aromadendrin alleviates CLP-induced liver injury

CLP method was used to generate sepsis in a mice model. Compared to that of the sham group, the liver tissue of the CLP group exhibited severe structural damage with edema, necrosis, and neutrophil infiltration (Figure 1 B). The administration of aromadendrin alleviated the injury induced by CLP in a dose-dependent manner (Figure 1 B). The histopathological score was significantly increased after CLP treatment, which was significantly attenuated by the administration of aromadendrin (Figure 1 C). The serum levels of AST and ALT were significantly increased in the CLP group, compared to the sham group (Figure 1 D). The administration of aromadendrin significantly reduced the serum levels of AST and ALT (Figure 1 D). TUNEL staining revealed that aromadendrin treatment alleviated liver injury and cell apoptosis (Figure 1 E - F). These observations demonstrated that the administration of aromadendrin attenuated CLP-induced liver injury.

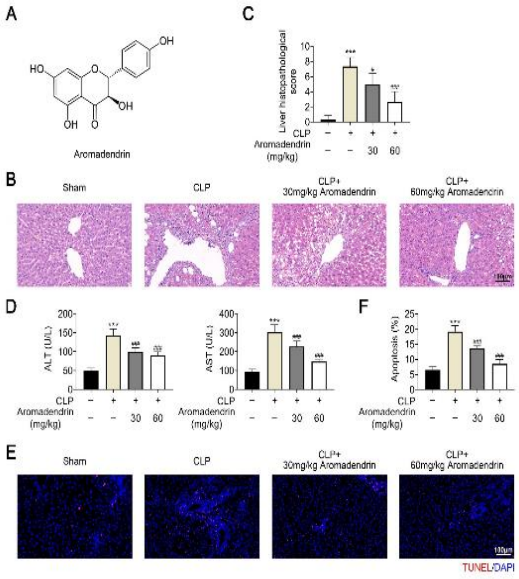

Figure 1: Aromadendrin alleviates CLP-induced liver injury. (A) Aromadendrin structure. (B) Representative image of HE staining of liver tissue. (C) Liver histopathological scores. $^{***}p<0.001$ *vs* sham, $^{\#}p<0.05$ *vs* CLP, $^{\#\#}p<0.01$ *vs* CLP. (D) The serum levels of ALT and AST in CLP mice. $^{***}p<0.001$ *vs* sham, $^{\#\#\#}p<0.001$ *vs* CLP. (E) Representative image of TUNEL and DAPI staining. (F) Percentage of apoptotic cells. $^{***}p<0.001$ *vs* sham, $^{\#\#\#}p<0.001$ *vs* CLP. All the above data are mean ± S.E.M. of at least three independent experiments

Table 1: Primers used in PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TNF-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG

Aromadendrin reduces the production of inflammatory cytokine induced by CLP

To further investigate the effects of aromadendrin on the production of inflammatory cytokine induced by CLP, the levels of TNF-α, IL-1β and IL-6 were measured by qRT-PCR and ELISA. qRT-PCR analysis revealed that the levels of TNF-α, IL-1β and IL-6 were significantly enhanced in the CLP group, which was significantly lowered by aromadendrin treatment (Figure 2 A). The ELISA assay data showed that the levels of TNF-α, IL-1β and IL-6 were significantly increased in the CLP group (Figure 2 B). Aromadendrin treatment attenuated the inducing effect of CLP on the production of inflammatory cytokine (Figure 2 B). These data demonstrated that aromadendrin could reduce the production of inflammatory cytokine, such as TNF-α, IL-6 and IL-18, induced by CLP.

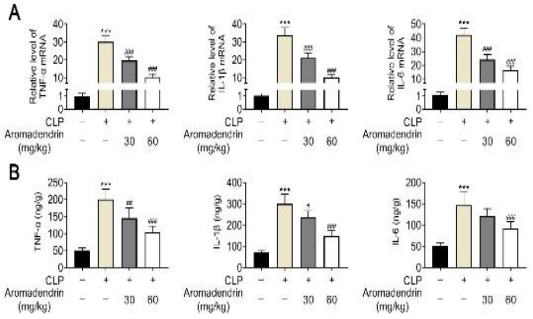

Figure 2: Aromadendrin reduces the production of inflammatory cytokine induced by CLP. (A) Relative mRNA levels of TNF-α, IL-1β and IL-6 measured by qRT-PCR. $^{***}p$<0.001 *vs* sham, $^{###}p$<0.001 *vs* CLP. (B) ELISA was used to assess the levels of TNF-α, IL-6 and IL-18 in liver tissue; $^{***}p$<0.001 *vs* sham, $^{\#}p$<0.05 *vs* CLP, $^{\#\#}p$<0.01 *vs* CLP, $^{###}p$<0.001 *vs* CLP. All the above data are mean ± S.E.M. of at least three independent experiments

Aromadendrin ameliorates oxidative stress in CLP mice

To future investigate the mechanism through which aromadendrin alleviated CLP-induced liver injury, the biomarker of oxidative stress was determined by ELISA assay. The activities of antioxidant glutathione (GSH) and antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), were significantly reduced in CLP mice (Figure 3). Aromadendrin treatment attenuated the inhibitory effects of CLP on the activities of CAT, GSH and SOD (Figure 3). In CLP mice, a lipid peroxidation marker, malondialdehyde (MDA) which was inhibited by aromadendrin treatment, was significantly increased (Figure 3). The data revealed that aromadendrin was able to ameliorate oxidative stress induced by CLP.

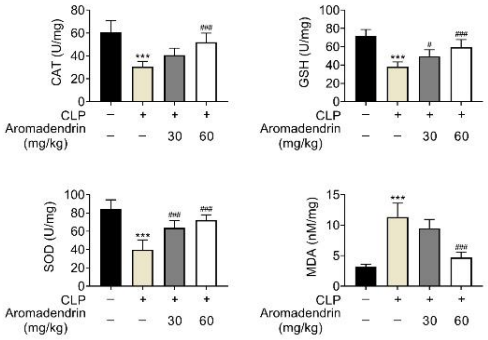

Figure 3: Aromadendrin ameliorates oxidative stress in CLP mice. The activities of CAT, GSH, SOD and MDA in liver tissue measured by ELISA assay. $^{***}P$ < 0.001 *vs* sham, $^{\#}p$<0.05 *vs* CLP, $^{###}p$<0.001 *vs* CLP. Data are mean ± SEM (n = 3)

Aromadendrin suppresses NF-κB signaling pathway

NF-κB signaling pathway is activated by oxidative stress. Therefore, its activity was assessed by western blotting. CLP treatment also significantly increased the phosphorylation p65 and IκBα, which was inhibited by the administration of aromadendrin (Figure 4). These data demonstrated that aromadendrin could suppress the activation of NF-κB signaling pathway in CLP mice.

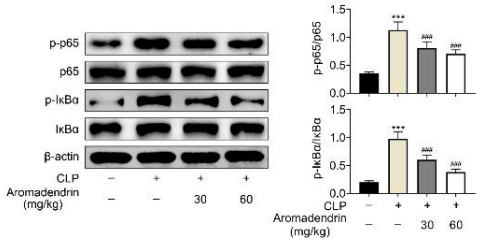

Figure. 4: Aromadendrin suppresses NF-κB signaling pathway. Western blotting analysis was used to detect the protein levels of p65, p-p65, p-IκBα and IκBα in liver tissues. *Left,* representative blots of p65, p-p65, p-IκBα, IκBα and β-actin. *Right,* histograms represent the protein expression ratio of p-p65/p65 and p-IκBα/IκBα. $^{***}P$ < 0.001 *vs* sham, $^{###}p$ < 0.001 *vs* CLP. Data are mean ± SEM (n = 3)

DISCUSSION

Sepsis is a systemic infection resulting in life-threatening situations [1,2]. Sepsis leads to multiple organ failure, which is a main reason for its high mortality rate [1,2]. Liver injury induced by sepsis is a severe risk factor in sepsis-related

deaths [4]. Further research into the molecular mechanisms of liver injury caused by sepsis is important and urgent. NF-κB signaling pathway is vital in the development of sepsis-induced multi-organ dysfunction [10], and in a case of liver injury induced by sepsis, NF-κB related signaling is upregulated [11]. The proinflammatory cytokines stimulates the activity of NF-κB, and NF-κB also regulates the gene expressions of the proinflammatory cytokines [13].

Cellular oxidative stress also contributes to the process of sepsis [16]. Oxidative stress causes vascular permeability impairment and mitochondrial dysfunction [16]. Excess reactive oxygen species (ROS) can cause oxidative stress, resulting in the damage of DNA, proteins, and lipids [17,18]. Mitochondrial is the main source of ROS, and excess ROS accumulated in the mitochondrial matrix may eventually lead to mitochondrial malfunction and impaired respiration [16]. Lipid peroxidation occurs in the membranes and subcellular organelles [16], and the peroxidative metabolic pathways are regulated by a GSH shuttle system [16]. SOD and CAT protect cells or tissues against the cellular toxicity of ROS [16]. MDA, a lipid peroxidation marker, is a substrate produced in the oxidative reaction [17]. The antioxidant enzymes including GPX and SOD act as the defense system against ROS [17].

In this study, the role of aromadendrin in septic liver injury, and the underlying potential mechanism have been investigated. In the CLP mice, the liver tissue exhibited severe structural damage with edema, necrosis, and neutrophil infiltration, which were attenuated by the administration of aromadendrin. The liver injury and cell apoptosis were also attenuated by aromadendrin. Therefore, aromadendrin can alleviate CLP-induced liver injury. The levels of TNF-α, IL-1β and IL-6 were increased in CLP mice, and aromadendrin treatment significantly reduced these productions of inflammatory cytokines. The activities of GSH, SOD and CAT were significantly reduced in CLP mice, which was then rescued by aromadendrin. Aromadendrin inhibited the production of MDA, therefore, aromadendrin can ameliorate oxidative stress in CLP mice. The phosphorylation levels of p65 and IκBα was enhanced in CLP mice, which was suppressed by aromadendrin treatment, indicating that aromadendrin could suppress NF-κB signaling pathway.

CONCLUSION

Aromadendrin exhibits a protective role in mouse liver injury induced by sepsis, via the inhibition of NF-κB signaling pathway *in vivo*. Thus, this compound can potentially be developed as a therapeutic strategy for the management of septic liver injury.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhihui Zhou and Qun Yin designed the experiments, carried them out, analyzed and interpreted the data and prepared the manuscript with contributions from all co-authors.

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