Int J Clin Exp Med 2015;8(5):7867-7871
www.ijcem.com /ISSN:1940-5901/IJCEM0006072

Original Article

Effects of angiotensin II type 1 receptor antagonist on rats with septic shock

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Received January 18, 2015; Accepted May 5, 2015; Epub May 15, 2015; Published May 30, 2015

Abstract: Aims: To investigate the effects of angiotensin II type 1 receptor (AT1R) antagonist, losartan, on rats with septic shock induced by endotoxin. Methods: Thirty male SD rats were randomly divided into 3 groups (10 for each group): rats were injected with normal saline in C group, lipopolysaccharide (LPS) of 12 mg kg^{-1} intravenously in LPS group, and losartan of 50 mg kg^{-1} intraperitoneally followed by LPS of 12 mg kg^{-1} intravenously in LOS group. The plasma concentrations of nitric oxide (NO), malondialdehyde (MDA), IL-1β and TNF-α were measured 6 h after LPS administration. Then all the rats were sacrificed immediately before the aortas pectoralis were isolated. Inhibitor of NF-κB (IκB) mRNA and its mRNA expressions in aorta were detected. Results: The plasma concentrations of NO, MDA, IL-1β and TNF-α were all significantly elevated in LPS group compared with the control group (P<0.01), which were markedly reduced in LOS group (P<0.01). Both of the mRNA and protein expressions of IκB in aorta were downregulated after injection of LPS when compared with the control group (P<0.01). However, IκB mRNA and protein expressions in aorta in the LOS group were significantly higher than the LPS group (P<0.01). Conclusion: AT1R antagonist, losartan, has a reverse effect at least partly on circulation dysfunction in rats with septic shock induced by endotoxin.

Keywords: Angiotensin II type 1 receptor, septic shock, losartan

Introduction

Septic shock and its relevant multiple organ dysfunction syndrome (MODS) are the leading causes of death and difficult issues in critical care medicine filed nowadays. It was reported that there were 750,000 severe infectious cases occurring in USA every year, with a mortality of 20%-63% [1]. The foundations of hemodynamic changes in severe infection and septic shock are abnormalities of systolic and diastolic functions of peripheral arteries, which lead to abnormal distribution of blood. A series of excessive release of proinflammatory mediators and compensatory insufficient release of anti-inflammatory mediators after infections cause uncontrolled inflammatory response and vasopermeability changes, which cannot been stopped and gradually lead to MODS even when the primary cause is treated. Septic shock cause a series of complicated nerve humoral responses and pathophysiological changes, among which renin-angiotensin system plays important roles in blood pressure regulation and water electrolyte balance [2, 3].

Circulation dysfunction is the one of the most prominent characteristics of septic shock, which mainly appears as progressive and stubborn decrease of blood pressure. The mortality and morbidity of septic shock remain high, upon which new drugs and therapeutic methods emerged one after another. But no satisfying effect has been achieved. Thus, it is imminent to unveil the mechanism of circulation dysfunction of septic shock. Renin-angiotensin system (RAS) is activated abnormally during septic shock, while the function of RAS in the pathophysiological process of septic shock has not been elucidated. This study aimed to investigate the mechanism of possible protective function of angiotensin II type 1 receptor (AT1R) antagonist in septic shock by applying AT1R

antagonist, losartan, in rats with septic shock induced by endotoxin.

Materials and methods

Animals and grouping

Thirty SPF male SD rats, weighting 200-220 g were provided by Center of Laboratory Animal in our university. The rats were placed with femoral arterial and venous catheters to measure arterial pressure and intravenous injections after intraperitoneal injection of pentobarbital (30 mg kg^{-1}). The rats were kept breathing spontaneously and given additional pentobarbital of 5 mg kg^{-1} during the operation when necessary. The rats were randomly divided into three groups (10 rats for each group): rats were injected with normal saline in C group, lipopolysaccharide (LPS) of 12 mg kg^{-1} intravenously in LPS group, and losartan of 50 mg kg^{-1} intraperitoneally followed by LPS of 12 mg kg^{-1} intravenously in LOS group: the control group (C group): rats were injected with normal saline intravenously; the septic shock group (LPS group): rats were injected with LPS (0127B8, Sigma) of 12 mg kg^{-1} intravenously; and losartan group (LOS group): losartan (MSD) of 50 mg kg^{-1} intraperitoneally followed by LPS of 12 mg kg^{-1} intravenously within 30 minutes. Mean arterial pressure (MAP), heart rate and respiration were monitored during the whole experiment. Successful septic shock models of rats were marked by the standard that MAP decreased by 25~30% and remained on this level after the injection of LPS. Blood samples were collected 6 hours after LPS injections. After rats were sacrificed, aortas pectoralis were taken out and immersed into liquid nitrogen for quick freezing, which were moved to -80°C for following detections.

Detection indicators and methods

Cardiac puncture was applied to draw 5-6 ml blood from rats 6 hours after LPS injection. Blood samples were placed in heparin anticoagulant tubes and then immediately centrifuged at 4°C with a speed of 3000 r min^{-1} for 10 minutes. Supernatants were obtained to measure parameters as follows: (1) concentration of nitric oxide (NO) was tested with nitrate reductase method (Kit: Jiancheng Biological Engineering Institute, Nanjing); (2) concentration of malondialdehyde (MDA) was measured with TBA method, (kit: Jiancheng Biological Engineering Institute, Nanjing); (3) concentration of IL-1β (Rat IL-1β ELISA Kit-Biosource, USA) and TNF-α (Rat TNF-α ELISA Kit-DIACLONE kit, Fance) were measured by enzyme linked immunosorbent assay (ELISA), all the steps were operated according to the instructions of the kits. (4) mRNA expressions of inhibitor of NF-κB (IκB) in thoracic aortic blood samples were measured by RT-PCR. IκB primer sequence: upstream: 5'-CTG GAG CAG CAG AAG TAC AC-3', downstream: 5'-TTC ACT GTT CCG TTC AAG TC-3', amplification fragment: 872 bp. β-actin was set as an internal control. Primer sequence: upstream 5'-ACC CGC GAG TAC AAC CTT-3', downstream 5'-GCT CAG TAA CAG TCC GCC T-3', amplification fragment: 1226 bp. PCR was performed together with β-actin according to the instructions of the kit (TaKaRs Ltd.) under the reactive condition of target mRNA. All the samples were set in the reactant system of 50 ml. Initial denaturation was set at 94°C for 3 min. Amplification was down as follows: 35 cycles of 94°C for 1 min, 56°C for 30 sec and 72°C for 1 min, and then followed by 72°C for 7 min. The negative controls were done in the same conditions, except for using negative reverse transcription reactant mixtures. After amplification, 5 ml PCR reactant mixtures was drawn for 1% agarose gel electrophoresis. The results were scanned by Master VDS scanning analyzer, then optical density was analysed by Totalab image software. mRNA expression levels of IκB were presented by IκB/β-actin gray ratio. (5) IκB protein expressions were measured by Weston blot. Kaumas bright blue staining was used to quantify protein after aortas pectoralis were cleavaged. Samples of same concentration were separated by 10% polyacrylamide gel electrophoresis, then electrotransfered to nitrocellulose filter, blocked overnight in 5% BSA at 4°C, incubated with 1:500 anti-IκB monoclonal antibody (SC-371, Santa Cruz Ltd.), washed by TBST, incubated with secondary antibody of 1:1000, exposed with ECL film, then developed and fixed. Films were performed with density scanning, area integral and relative analysis with β-actin as internal control. IκB protein expressions were represented by IκB/β-actin integral ratio.

Statistical analysis

All data were shown in format of mean ± SD ($\bar{x}$ ± s), analyzed with SPSS 13.0 software, tested

Table 1. Serum levels of NO, MDA, IL-1β and TNF-α in rats of all groups ($\bar{x}$ ± s, n=10)

Group	NO (μmol/L)	MDA (μmol/L)	IL-1β (pg/ml)	TNF-α (pg/ml)
C group	53.71 ± 8.34	5.38 ± 0.48	92.64 ± 10.59	78.40 ± 25.73
LPS group	544.97 ± 78.73*	19.23 ± 3.62*	564.90 ± 51.76*	724.80 ± 74.95*
LOS group	269.73 ± 29.86*,#	10.83 ± 1.06*,#	371.50 ± 47.42*,#	489.80 ± 56.32*,#

Footnotes: *P<0.01, compared with C group; #P<0.01, compared with LPS group.

Table 2. IκB mRNA and protein expressions in aortas pectoralis in all groups ($\bar{x}$ ± s, n=10)

Groups	IκB mRNA	IκB protein
C group	276 ± 35	135 ± 14
LPS group	189 ± 27*	77 ± 12*
LOS group	233 ± 25#	103 ± 13#

Footnotes: *P<0.01, compared with C group; #P<0.01, compared with LPS group.

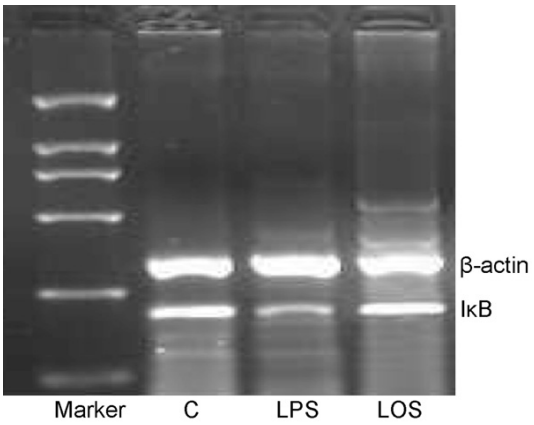

Figure 1. mRNA expression of IκB in aortas pectoralis of all groups.

with single factor analysis of variance (ANOVA), and *P*<0.05 indicated significant difference.

Results

Concentration changes of serum NO, MDA, IL-1β and TNF-α in rats

Concentration of serum NO, MDA, IL-1β and TNF-α increased significantly in rats with septic shock induced by LPS compared with the control group (P<0.01). However, all the indicators in rats of LOS group declined sharply, and the differences were significant compared with LPS group (**Table 1**).

mRNA and protein expressions of IκB in aortas pectoralis

Protein and mRNA expressions of IκB decreased significantly in LPS group compared with C group (P<0.01), while then increased evidently after being treated with LOS (*P*<0.01), which were still less than C group (*P*<0.01) (**Table 2**; **Figures 1** and **2**).

Discussion

Infectious shock, also known as septic shock, is a complicated clinical syndrome characterized by multiple organ dysfunction syndrome caused by systemic infection. Septic shock is a severe stage of a series of pathophysiological changes occurs and the conditions deteriorate continuously, in which uncontrolled inflammation and circulation dysfunction are prominent [4]. The exact mechanisms of infectious shock are still unknown. The most common pathogen causing infectious shock is gram-negative bacteria (mainly including *E.coli, Klebsiella and Pseudomonas aeruginosa*) [5]. LPS, the main component of gram-negative bacteria, is the main trigging factor of sepsis and one of the major mediators causing shock and inflammatory reactions. This study induced the septic shock models successfully by injecting high-dosage of LPS into the femoral vein, which was consistent with previous reports [6].

In the pathological process of infectious shock, proinflammatory cytokines, reactive oxygen series, eicosanoids and so on are produced abundantly and cause a series of reactions of oxidation and inactivation of catecholamin, lipid peroxidation, cellular electrical transportation, slow ATP generation and so on, which is one of the important reasons leading to vascular hyporeactivity in infectious shock [7]. This study indicated that high concentration of serum pro-inflammatory cytokines IL-1β and TNF-α, and significant increases of free radical NO and lipid peroxide MDA, which was also consistent with previous reports [8, 9]. LPS, as pathogen-associated molecular pattern (PAMP), can mediate activation of nuclear transcription factor (NF-κB) through multiple signal transduction pathways after combination with pattern recognition receptor (PRR). NF-κB can

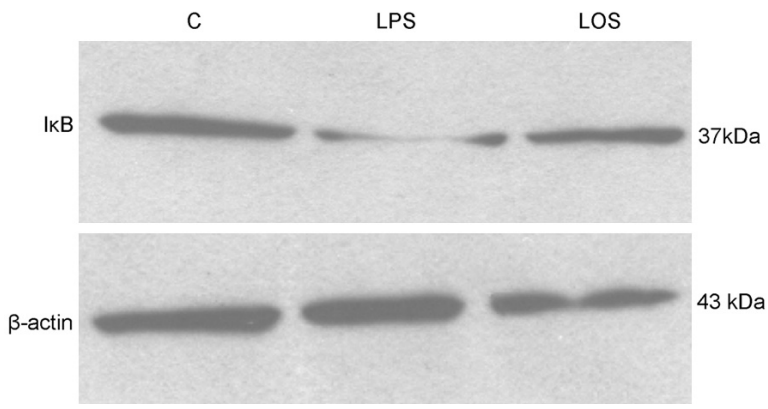

Figure 2. Protein expressions of IκB in aortas pectoralis of all groups.

modulate the production of proinflammatory factors IL-1β, TNF-α and γ-INF, which could lead to uncontrolled inflammatory reactions and immunity dysfunction [10]. Meanwhile induced iNOS can be activated by LPS, NF-κB and proinflammatory factors, which stimulates the epithelium, smooth muscle cells and mononuclear/macrophage cells to synthesize and release large amounts of NO. NO in turn promotes vascular dysfunction by both cGMP-dependent and cGMP-independent pathways [11]. Thus, it suggests that NF-κB, which anticipates in inflammation, immunity, apoptosis and oxidation stress, plays an important part in the genesis and development of sepsis [12]. It has always been a highpoint to study how to interfere activation of NF-κB. Typical NF-κB compound is comprised of two subunits, P50 and P65/c-Rel, and exists in cytoplasm in an inactivated form of combining firmly with its inhibition protein IκB. IκB is the main inhibitory protein of NF-κB and plays its role in the following ways: preventing NF-κB from entering nuclear to activate transcription by combining with NF-κB dimer in cytoplasm; inhibiting combination of NF-κB with DNA in nucleus directly; even removing NF-κB dimer from DNA and combining with NF-κB dimer to exit nucleus [13]. IκB will ablate from the NF-κB/IκB compound if degraded by phosphorylation [13]. In this study, results of RT-PCR and Weston blot showed that expressions of IκB mRNA and protein were downregulated significantly in aortas pectoralis of rats with sepsitic shock, and the downregulated amplitude of protein expressions was more significant than mRNA. It was inferred that the low protein expressions of IκB were also relevant to IκB being phosphorylated into p-IκB besides the downregulation of gene transcription, which caused the decrease of absolute amount of activated IκB and further activation of large amount of NF-κB to achieve series of biological effects.

RAS, as an important endocrine system of human body, could exist independently in local tissues and organs, such as vessels, heart, kidneys and so on. Angiotensin II (Ang II) as a hormone is also considered as a proinflammatory factor [14]. Ang II in some tissues and organs, like plasma, liver and kidney, increases quickly during septic shock [15], which promotes genesis of large amount of proinflammatory factors, activated oxygen series and lipid peroxide, causing severe damage of tissue cells [16]. Abnormal activation of RAS can occur in human bodies at early stage of septic shock, which is in positive correlation with production of NF-κB, proinflammatory factors, NO and lipid peroxide [8], indicating that these factors play an important role in genesis and development of circulation dysfunction in septic shock. The biological function of Ang II is mainly mediated by AT1R [17]. In this study concentration of serum NO, MDA, IL-1β and TNF-α all decreased significantly, and gene and protein expressions of IκB were reversed significantly meanwhile, after AT1R antagonist losartan was used to interfere rats with septic shock, indicating that AT1R antagonist losartan has a reverse effect at least partly on circulation dysfunction in rats with septic shock induced by endotoxin.

In conclusion, losartan can inhibit over-activated RAS by antagonistic actions on AT1R and reducing tissue damages mediated by proinflammatory factors, free radical NO and lipid peroxide to improve circulation dysfunction in sepsis. This therapeutic function of losartan may be relevant to its function of increasing IκB expression levels to reduce the activities of NF-κB.

Acknowledgements

The study was supported by Pudong New Area of Shanghai's leading health system personnel

training plan (PWRI2013-03) and Pudong New Area Health System key disciplines group project (PWZxq2014-06).

Disclosure of conflict of interest

None.

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