Role of Nuclear Transcription Factor-κB in Endotoxin-induced Shock in Rats*

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Summary: To investigate the role of NF-κB in endotoxic shock in rats, the model of endotoxin-shock rats was induced by intravenous infusion of lipopolysaccharide (LPS). 1 h, 2 h, 4 h and 6 h after LPS injection, the activation of NF-κB in blood mononuclear cells and the content of TNF-α and IL-6 in plasma was detected by enzyme-linked immunoadsordent assay (ELISA). The level of mean arterial pressure (MAP) and the histopathological changes of lung and liver were also observed. The activation of NF-κB in mononuclear cells increased 1 h after LPS injection and reached its peak 2 h after the injection, and its level was higher than that of normal group. The level of TNF-α was increased 1 h after the infusion and peaked 2 h after the injection, and its level was higher than that of normal group after LPS infusion. The content of IL-6 increased gradually with time, the IL-6 level was higher than that of normal group after LPS injection. MAP was decreased gradually with time and its level was lower than that of normal group after LPS injection. Pathological examination showed that endotoxic shock could cause pulmonary alveolar hemorrhage, edema and infiltration of inflammatory cell in lung tissue and congestion, edema, capillary dilation and inflammatory cell infiltration in liver tissue. It is concluded that NF-κB can up-regulate the expression of TNF-α and IL-6 in plasma and play an important role in endotoxin-induced shock in rats.

Key words: endotoxic shock; mononuclear cells; lipopolysaccharide; nuclear factor Kappa B; tumor necrosis factor-α; Interleukin-6; mean arterial pressure

Endotoxin is released by *E. coli* and is a major cause in infectious shock[1]. During endotoxic shock, the uncontrolled inflammatory mediators can result in the systemic inflammatory response syndrome, sepsis, MODS and even death[2]. As a transcription factor, NF-κB plays an important part in endotoxic shock[3]. And it is also involved in the transcription of inflammatory mediator genes such as TNF-α and IL-6. In this study, we investigated the role of NF-κB in endotoxic shock by observing the activation of NF-κB in blood mononuclear cells, the level of plasma TNF-α and IL-6, the changes of mean arterial pressure (MAP) and the histopathological changes of lung and liver.

1 MATERIALS AND METHODS

1.1 Animals and Reagents

Twenty-five male Wistar rats, weighing from 180 to 220 g, were supplied by the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China. The reagents used included LPS (*E. coli* O127: B8, Sigma, USA), nuclear extracts kit (Active Motif, USA), TNF-α and IL-6 ELISA kit (Jingmei Co., China), NF-κB TransAM ELISA kit (Active Motif, USA) and lymphocytes separation medium (Shanghai, China).

1.2 Animal Model and Groups

Animals were randomly assigned to two groups: LPS group and normal group (NS group). After an intraperitoneal injection of 2 % pentobarbital sodium (45 mg/kg), the right external jugular vein was cannulated to inject LPS and the right femoral artery was also cannulated for monitoring blood pressure and drawing blood samples. In model establishment, the rats of LPS group were intravenously injected LPS at 12 mg/kg (6 mg/mL) over a period of 2 min. The animals of NS group were given the same volume of normal saline. According to time after LPS injection, the LPS group was divided into 1 h, 2 h, 4 h and 6 h subgroup, with each subgroup having 5 rats. The LPS group has a total of 20 rats and the NS group had 5 rats.

1.3 Sample Collection

Blood (4 mL) was drawn from each group and put into the glass tube containing EDTA anticoagulant, of which 2 mL was used for plasma separation and the other 2 mL blood for separation of mononuclear cells.

1.4 Separation of Mononuclear Cells

The blood mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation.

1.5 Preparation of Nuclear Extracts

By using a commercial kit (Nuclear Extract Kit, Active Motif, USA), mononuclear cells were washed with ice-cold PBS/phosphatase inhibitor three times, then lysed with complete lysis buffer for 30 min on ice. Supernatants were transferred to a pre-chilled microcentrifuge tube, divided and stored at −80 ℃ for NF-κB analysis. Protein concentration in each sample was determined by using a standard Bradford protein assay. 5 μg of protein

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from each extract sample was used for the NF-κB binding assay.

1.6 Activation and Specificity of NF-κB

The activation and specificity of NF-κB was measured with ELISA-based Trans-AM NF-κB kit (Active Motif, USA) by using the nuclear extracts prepared from the isolated mononuclear cells. The Trans-Am NF-κB ELISA kit consists of 96-well microtiter plates precoated with an oligonucleotide containing the appropriate NF-κB binding consensus sequence (5'-GGGACTTTCC- 3'). The active form of the p65 subunit was detected by using a monoclonal antibody only when p65 subunit has bound to its target DNA. A secondary antibody conjugated with horseradish peroxidase was added. The colorimetric readout was quantified by spectrophotometry. Optical density was read at 450 nm, and the results were optical density values after subtracting the blank values. The assay was performed exactly according to the manufacturer's instructions. A wild-type consensus oligonucleotide, serving as a competitor for NF-κB binding, and a mutated consensus oligonucleotide with no effect on NF-κB binding were used to monitor the specificity of the assay.

1.7 Level of Plasma TNF-α and IL-6

Plasma TNF-α and IL-6 were determined by using ELISA kit by following the manufacture's instructions.

1.8 Histopathological Examination of Lung and Liver

The lung and liver were removed for histopathological examination for each subgroup and NS group, and comparison was made in terms of the changes between the 6 h subgroup of LPS group and the NS group.

1.9 Statistical Analysis

Data were expressed as $\bar{x} \pm s$. Differences between LPS group and NS group were analyzed with the unpaired Student's t-test by using SPSS12. 0 and correlation statistics. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Change of MAP

The MAP decreased gradually after LPS injury (table 1, fig. 1).

2.2 Activity of NF-κB in Mononuclear Cells

The activity of NF-κB increased after LPS injury, and the peak appeared 2 h after the injury (table 1, fig. 2). The figure was based on the ratio of LPS group to normal group.

2.3 Level of Plasma TNF-α and IL-6

The level of TNF-α and IL-6 in plasma increased significantly after LPS injury, and TNF-α peaked 2 h after the injury (table 1, fig. 3). But the level of IL-6 was increased gradually with time (table 1, fig. 4).

2.4 Correlation Analysis of NF-κB and TNF-α

The activity of NF-κB was positively correlated with the level of TNF-α ($r= 0.973$, $P<0.01$).

2.5 Histopathological Changes of Lung and Liver

Six hour after LPS injury, pathological examination with light microscope showed pulmonary alveolar hemorrhage, edema, the alveolar septum enlargement, edema and inflammatory cell infiltration in lung tissue, and liver sinus enlargement, congestion and focal necrosis of liver cells. In the NS group, no such changes were found.

Table 1 Changes of activation of NF-κB in PBMC and level of TNF-α, IL-6 in plasma and MAP after LPS injection in rats ($\bar{x} \pm s$, $n=5$)

Groups	NF-κB	TNF-α (pg/mL)	IL-6 (pg/mL)	MAP (mmHg)
NS	0.112±0.017	255.857±19.159	328.000±21.237	125.714±4.231
LPS				
1 h	0.243±0.022$^{\triangle\triangle}$	465.688±26.600$^{\triangle\triangle}$	1947.200±21.845$^{\triangle\triangle}$	113.429±3.409$^{\triangle\triangle}$
2 h	0.604±0.020$^{\triangle\triangle}$	1644.77±25.920$^{\triangle\triangle}$	3553.400±21.893$^{\triangle\triangle}$	98.000±5.394$^{\triangle\triangle}$
4 h	0.442±0.019$^{\triangle\triangle}$	848.474±24.263$^{\triangle\triangle}$	4187.800±24.448$^{\triangle\triangle}$	64.214±4 605$^{\triangle\triangle}$
6 h	0.296±0.018$^{\triangle\triangle}$	489.217±23.923$^{\triangle\triangle}$	6358.800±23.858$^{\triangle\triangle}$	42.857±5.662$^{\triangle\triangle}$

$P<0.01$, as compared with NS group.

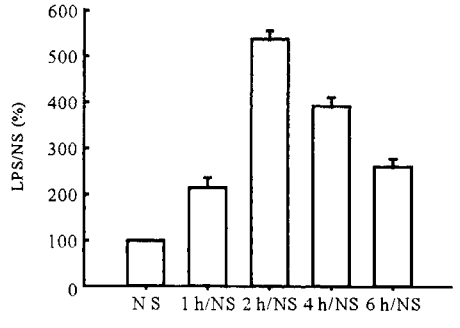

Fig. 1 The activity of NF-κB in blood mononuclear cells after LPS injury

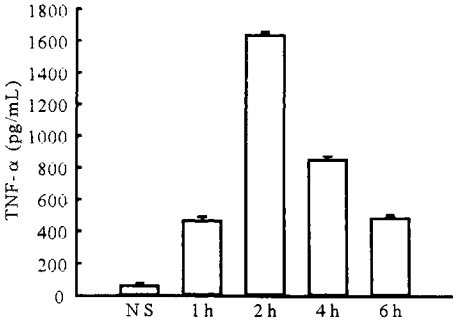

Fig. 2 The level of TNF-α after LPS injury

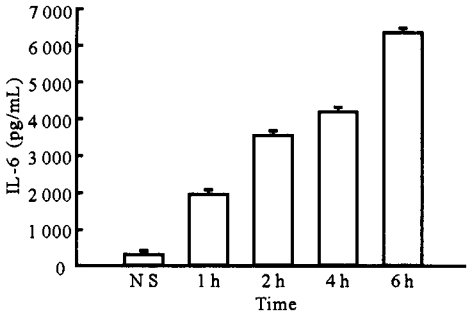

Fig. 3 IL-6 in plasma after LPS injury

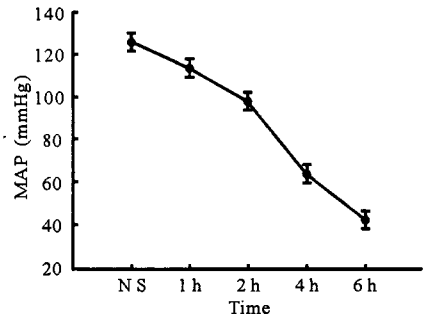

Fig. 4 MAP after LPS injury

3 DISCUSSION

The establishment of infectious shock animal model by *E. coli* LPS is the most commonly used technique. Infection can induce the early protective inflammatory response, but an excessive and uncontrolled inflammatory response can result in systemic waterfall-like inflammatory cascade response, even SIRS and MODS. The endotoxin of bacteria can activate the signal transduction system, induce the translocation of NF-κB and lead to the production and release of harmful cytokines, such as TNF-α, IL-6, etc. These cytokines can cause the leukomonocyte inflammation in lung tissue. The inflammatory mediators, which came from these leukomonocyte can cause hypotension, acidosis and the damage of tissue at first, then lead to organ dysfunctjion and death[4].

NF-κB, which plays a central role in inflammation through its ability to induce transcription of proinflammatory genes, is one of the major transcription factors. On the promoter of many inflammatory mediators, such as iNOS, TNF-α and IL-6, there exists the binding site of NF-κB. NF-κB, which is involved in the transcription regulation of genes of many cytokines, plays a key role in the regulation of the gene expression of inflammatory factors. Many studies showed that the increased NF-κB activity was associated with bad prognosis in sepsis or SIRS[5]. Ross found that the activity of NF-κB of monocytes and neutrophil cells in SIRS patients, especially those who died from SIRS, was obviously higher than that of living SIRS patients[6]. This study demonstrated that the activity of NF-κB increased 1 h and peaked 2 h after LPS injury. Compared with NS group, the activity of NF-κB in the LPS group was obviously higher.

Activation of NF-κB can initiate and regulate the gene expression of inflammatory mediators that are involved in inflammatory reactions. Blackwell *et al* suggested that the activity of NF-κB in lung tissue was increased shortly after hemorrhagic shock [7] and afterwards, the content of all kinds of cytokines increased significantly. This study showed that the activity of NF-κB had a positive correlation with the level of TNF-α, which was increased 1 h and peaked 2 h after LPS injury. The level of TNF-α in LPS group was significantly higher than that of the NS group. TNF-α is an early-expressed cytokine. It is an important pro-inflammatory mediator which is regulated by NF-κB and a central mediator which can activate the cytokine cascade responses in inflammatory reaction. TNF-α appears early in circulation and reaches its peak quickly and then initiates the synthesis and release of other cytokines such as IL-6. It was reported that these pro-inflammatory mediators triggered an irreversible inflammatory reaction in sepsis[8]. TNF-α also induces the activation of neutrophils, enhances the adhesion of leukocytes with endothelial cells, stimulates the secretion of chemokines. Blockade of IL-1β or TNF-α can attenuate inflammation. Moreover, TNF-α can also activate NF-κB, which results in the over-release of inflammatory mediators and cascade reaction, sepsis even MODS. TNF-α can also increase the activity of inducible nitric oxide synthase (iNOS), which induces the production of the NO. The overproduction of NO can cause the persistent hypotension, congestion of microcirculation, ischemia and anoxia of tissues and cells[9].

IL-6 is a delayed release cytokine. It was reported that the elevation of serum IL-6 appeared later than that of TNF-α in infectious shock, and our results were consistent with the findings. Evidence showed that appearance of serum IL-6 was a sign of the activation of cytokine cascade reactions, reflecting the relationship of host inflammatory response with the severity of the disease and serving as a marker of prognosis in sepsis[8]. In this study, the level of MAP was decreased gradually with time. And the pathological examination of lung tissue 6 h after LPS injury showed pulmonary alveolar hemorrhage, edema and enlargement of alveolar septum and infiltration of inflammatory cells. The liver tissue showed sinus enlargement, congestion and local necrosis.

In summary, we found in this study that LPS could activate the NF-κB in blood mononuclear cells, leading to over-release of TNF-α and IL-6, MAP decrease, and organs damage, suggesting that NF-κB plays an important role in endotoxic shock.

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to G_2/M-phase in a dose-dependent manner.

Table 3 Effect of Art on cell cycle and apoptosis of VSMC

Groups	Con (mg/L)	G_0+G_1 (%)	S (%)	G_2+M (%)	Apop (%)
Art	10	58.6	24.6	16.8	4.52
	20	60.9	23.5	15.6	9.14
	40	62	21.1	16.9	12.93
	80	63.6	23.6	12.8	27.53
	160	66.2	22.8	11.0	38.72
Control	—	57.3	26.4	16.3	0.10

3 DISCUSSION

Restenosis results from further accumulation of VSMCs and deposition of extracellular matrix in the neointimal tissue[3]. Natural drugs have significant influences on restenosis after percutaneous coronary intervention (PCI). Art is a semi-synthetic derivative of artemisinin, the active principle of the Chinese herb *Artemisia annua*[4]. It has been proved to be effective in inhibiting the proliferation of fibroblasts in time-dependent and dose-dependent manners[2]. Also, there were several promising studies indicating that Art could inhibit the proliferation of carcinoma cells *in vivo* and *in vitro* [5]. We investigated for the first time the effect of Art on the proliferation of VSMCs *in vitro*. DNA replication is necessary in cell proliferation. In our study, a significant decrease in [^{3}H]-TdR incorporation was observed in Art groups as compared with control group. Art can interfere with cells DNA synthesis and thereby inhibiting the proliferation of VSMCs.

When endothelia of arterial wall were damaged due to the balloon dilation in PCI, VSMCs apoptosis seem to be a major contributor to neointima formation. VSMCs proliferation and apoptosis represent pathogenetic features of atherosclerosis[6]. This study showed that Art increased VSMCs apoptosis in a dose-dependent manner.

Interfering VSMCs cell cycle is an effective method in the prevention of restenosis following PCI[7]. In this study we demonstrated that Art markedly caused a blockage of G_0/G_1-phase of cell cycle, preventing the progress of cell cycle from S-phase to G_2/M-phase in a dose-dependent manner.

In conclusion, in this study, we demonstrated the dose dependent, potent inhibitory effects of Art on VSMCs proliferation *in vitro*. And we believe that it is possible to use Art in drug-eluting stent to prevent the restenosis following PCI.

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