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Activation and Clinical Significance of p38 MAPK Signaling Pathway in Patients With Severe Trauma

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Background. **Organ dysfunction or multiple organ dysfunction syndrome caused by developing immunological dysfunction and subsequent sepsis or the systemic inflammatory response syndrome after trauma is the leading cause of death in trauma patient. It is believed that mitogen-activated protein kinase) (p38MAPK) is one of the most important kinases in inflammatory signaling. In this study, the change of p38 MAPK signaling pathway in trauma patient with different severity and its clinical significance in trauma inflammation were investigated.**

Methods. **One hundred fifty major trauma patients were included in the study and divided into three groups according to injury severity score (ISS). All data required to calculate ISS and determine organ function were registered on admission and during the ICU-stay. Peripheral blood samples were collected from trauma patients 6 h, 1 d, 3 d, 5 d, and 7 d after injury. RQ-PCR and Western blot was used to examine the changes in gene expression, protein expression, and activation level of leukocyte p38 MAPK. Plasma IL-6 and TNFα were assayed by ELISA.**

Results. **Organ dysfunction in 33 trauma patients developed and eight deaths occurred after 24 h in ICU. The causes of death included severe ARDS, MODS, and irreversible brain injury. Incidence of organ dysfunction was related to the increase of injury severity ($P < 0.01$). Compared with healthy control, the gene expression of p38 MAPK in trauma patients increased significantly 6 hours after injury ($P < 0.05$), and reached a maximum in 1 d ($P < 0.01$). The expression maintained a high level for 7 d ($P < 0.05$). One day after injury, significant elevation was observed in protein expression and activation level of p38 MAPK ($P < 0.05$), as well as the plasma TNFα and IL-6 level ($P < 0.01$). Further investigation found that the gene expression, protein expression, and activation levels of p38 MAPK increased with higher ISS ($P < 0.05$), and the elevation of plasma TNFα and IL-6 level was associated with the increase of activated p38 MAPK and ISS ($P < 0.05$).**

Conclusion. **p38 MAPK signal pathway was activated in trauma patients. The severity of trauma had highly positive correlation with the expression and activation of p38 MAPK, as well as the elevation of plasma TNFα and IL-6 expression. These findings indicate that p38 MAPK signaling pathway plays an important role in the pathological mechanism of trauma.**

Key Words: **trauma; signal pathway; p38 MAPK; IL-6;TNFα.**

INTRODUCTION

Trauma is still one of the main reasons for death among the population worldwide. Extensive trauma causes the release of stress hormones, the generation of inflammatory cytokines, and the absorption of necrotic tissue. Harmful factors including ischemia, hypoxia, detrimental nerve transmitter and free redicals following severe trauma can lead to cell necrosis and apoptosis, and result in multi-organ dysfunction syndrome (MODS) with a high possibility of death [1].

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Recently, a number of in vitro studies have shown that the production of inflammatory mediators is strongly affected by mitogen-activated protein kinases (MAPKs), such as p38MAPK, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Among these MAPK groups, p38MAPK is believed to be one of the most important kinases in stress signaling which can be activated by a variety of cellular stresses such as ischemia-reperfusion and causes inflammatory response as well as apoptosis [2, 3]. Researches revealed remarkable p38 MAPK activation in ischemia-reperfusion models including kidney [4], liver [5], vascular and myocardial cells [6], and lung [7]. In addition, studies showed that inhibition of p38 MAPK decreased ischemia-reperfusion induced apoptosis. Furuichi *et al.* [8] proved that FR167653, a specific inhibitor of p38 MAPK, could reduce cell infiltration into the outer medulla, the extent of acute tubular necrosis, and the expression of TNF-α and IL-l in renal ischemia-reperfusion injury in mice. These data showed that the p38 MAPK signaling pathway plays a vital role in multiple organ injury caused by ischemia-reperfusion in animal models. However, few studies have been reported on the activation of MAPKs in clinical aspect, especially about the relationship between p38 MAPK signaling pathway and the severity of traumas. This study discussed the changes of p38 MAPK signaling pathway in trauma patients with various degrees of severity and investigated the pathogenic mechanism of trauma at a molecular biology level.

MATERIALS AND METHODS

Patients

Trauma patients admitted to the surgical intensive care unit (ICU) in our hospital were evaluated in this study between January 2005 and December 2006. Patients who died before arrival at the emergency department or within 24 h in ICU, and who had a less-than-9 injury severity score (ISS) were excluded.

Study Design

On admission, we registered age, gender, blood pressure, mechanism and pattern of injury, and calculated ISS. During the ICU-stay, all data required to determine organ function (ventilator settings, hemodynamic parameters, laboratory data) were registered and documented in the patient's chart. All trauma patients were divided into three groups by ISS: ISS 9 ~ 15 (L-ISS), ISS l6 ~ 25 (M-ISS), and ISS $\geq$ 25 (H-ISS), each group consisted of 50 patients. A control group included 30 healthy blood donors. Peripheral blood samples (5mL each) were collected in heparinized tubes from patients 6 h, 1 d, 3 d, 5 d, and 7 d after trauma. Normal donors were sampled once. All the blood samples were stored at 4°C.

Real-time Quantitative Polymerase Chain Reaction (RQ-PCR)

Total RNA was extracted from leukocytes separated from heparinized blood (TRIZOL reagent; Gibcol BRL, Shanghai, China). ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Beijing, China) was used in reverse transcription to synthesize cDNA according to the manufacturer's instructions. RQ-PCR employed SYBR Green I, a highly specific double-stranded DNA dye. When SYBR green I binds to double-stranded DNA, the resulting complex greatly increases the fluorescence. The fluorescence intensity represents the amount of double-stranded DNA. All primers used in RQ-PCR were synthesized by Shanghai Sangon Biological Engineering Technology and Services Company, Shanghai, China. RQ-PCR reaction solutions included: (1) p38 MAPK forward primer and reverse primer (Table 1) 0.4 μL, respectively; SYBR green PCR master mix 10 μL; cDNA 1 μL; DEPC water 8.2 μL. (2) β-actin forward primer and reverse primer (Table 1) 0.4 μl, respectively; SYBR Green PCR master mix 10 μL; cDNA 1 μL; DEPC water 8.2 μL. PCR protocol was followed under the condition: preheating at 50°C for 2 min, degeneration at 95°C for 10 min prior to amplification, then at 95°C for 15 s, at 55°C for 30 s, and 72°C for 30 seconds. The amplification was carried out for 40 cycles. The whole process was performed on 7300 real-time PCR System (Applied Biosystems, Shanghai, China). Fluorescent intensities were analyzed by SDS software in 7300 System.

Western-Blot Analysis

Leukocytes separated from heparinized blood were lysed with cell lysis buffer (20 mM Tris PH7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na3VO4, 0.5 μg/mL leupeptin, Beyotime, Haimen, Jiangsu, China). Lysates were then centrifuged and the supernatants collected. Protein concentrations of the extracts were measured by BCA protein assay (Beyotime) according to the manufacturer's instructions. Equal amounts of leukocyte extracts (50 μg protein) were then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel. The separated proteins were blotted on to a polyvinylidene fluoride (PVDF) membrane. Blocked at room temperature (RT) for 1 h in 5% (wt/vol) dry skim milk, the target proteins were incubated at 4°C overnight with a 1:1000 dilution of specific primary antibody. Primary antibodies used were rabbit antibodies specific for p38 MAPK, phospho-p38 MAPK, and β-actin (Cell Signaling, Beverly, MA), followed at RT for 1 h with a 1:1000 dilution of secondary antibody conjugated with horseradish peroxidase (Beyotime). The peroxidase reaction was visualized by an enhanced chemiluminescence method using EZ-ECL chemiluminescence detection kit (Biological Industries, Kibbutz Beit Haemek, Israel). The relative intensities of the protein bands were quantified by scanning densitometry in a Bio-Rad (Shanghai, China) Fluor-S multi-imager using the program Quantity One. Relative expression levels of target protein were expressed as target protein (p38 MAPK or phosphorylated p38 MAPK)/β-actin ratios.

TABLE 1

Primers of p38 MAPK and β-Actin

Genes	Forward	Reverse
p38 MAPK	5'-GAAGAGCCTGACCTACGAT-3'	5'- ACTGCCAAGGAGCATCTA-3'
β-Actin	5'-AACTCCATCATGAAGTGTGA-3'	5'-ACTCCTGCTTGCTGATCCAC-3'

TABLE 2

Clinical Data of the Trauma Patients

Parameters	L-ISS	M-ISS	H-ISS
n	50	50	50
Age (range)	35.84±12.66 (19-61)	37.76±13.85 (18-71)	38.54±15.57 (19-69)
Gender (female/male)	20/28	16/34	14/36
ISS (range)	12.7±3.6 (9–16)	21.2±4.5 (16–25)	32.3±7.6 (25–54)
Trauma mechanism			
Traffic accidents	14	27	29
Falls	25	14	13
Stabbings	12	9	8
Injury pattern			
Lung contusion	0	5	14
Intraabdominal injury	1	4	21
Traumatic brain injury	2	3	7
Others	47	38	8
Shock*	9	25	47
Surgical procedure	14	33	47
Mortality	0	2	6†

*Shock was defined as systolic pressure less than 90 mmHg.
†$P < 0.05$, compared with L-ISS.

ELISA

The concentrations of plasma TNFα and IL-6 were measured by sandwich ELISA using commercially available antibodies (R and D Co., Minneapolis MN). ELISA was performed according to the manufacturer's instruction. Briefly, after 2 h incubation of standard, sample, or controls in testing wells, the TNFα or IL-6 conjugate was added for 2 h followed by substrate solution for 20 min. The reactions were read by using a microplate auto-reader (Bio-Tek Instruments, Winooski, VT) at A450nm after adding stop solution.

Statistics

All the analysis was performed by SPSS10.0 (SPSS Inc., Chicago, IL) statistics software. Data were expressed as mean ± SEM. Student's t-test or χ^2 test were applied for comparison between groups. Correlation matrices were used in the correlation coefficient tests. $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical Characteristics

One hundred fifty major trauma patients with a mean age of 37.38 (rang 18–71 y) were studied (Table 2); 65% of the patients were male ($n = 98$). There were no significant differences in gender and age distribution between the groups. Fifty-four percent of the trauma patients were admitted with traumatic or hemorrhagic shock ($n = 81$) and 62.7% of hospitalized patients underwent at least one surgical procedure ($n = 94$). Mechanisms of injury consisted of traffic accidents, falls, and stabbings with no statistical difference in constituent ratio. Two deaths in M-ISS and six in H-ISS occurred after 24 h in ICU. The causes of death included severe ARDS ($n = 3$), MODS ($n = 4$), and irreversible brain injury ($n = 1$). Incidence of organ dysfunction increased significantly with the increase of injury severity ($P < 0.01$, Table 3).

Gene Expression Changes of Leukocyte p38 MAPK from Trauma Patients

To test the hypothesis that p38 MAPK is activated in peripheral leukocytes in response to trauma in patients, expressions of p38 gene in leucocytes were detected by RQ-PCR (Table 4). The absolute quantification of p38 MAPK gene expression was obtained by dividing the gene copy numbers of p38 MAPK detected in each sample by corresponding internal control gene β-actin. The results showed that p38MAPK gene was expressed in the leucocytes of 150 patients with different stages of trauma as well as 30 normal donors. The p38 mRNA levels increased

TABLE 3

Organ Dysfunction Occurred in ICU

Organ dysfunction△	L-ISS	M-ISS	H-ISS
ARDS	0	0	6
ARF	0	1	3
Cardiovascular failure	0	0	3
Acute stress ulcer	0	4	4
Hepatic failure	0	1	5
MOD	0	0	6
Total	0	6†	27*,‡

△Organ dysfunction was defined as follows: acute renal failure (ARF): creatinine > 2 mg/dL or requirement for hemodialysis; acute respiratory distress syndrome (ARDS): $PaO_2/FiO_2 < 200$ mmHg; cardiovascular failure: need for inotropes other than dopamine; stress ulceration: acute mucosal ulcerations were observed by endoscopic examination; hepatic failure: bilirubine > 2 mg/dL; multiple organ failure: failure of three or more organs.
†$P < 0.05$
*$P < 0.01$, compared with L-ISS.
‡$P < 0.01$, compared with M-ISS.

TABLE 4

Gene Expression Levels of Leukocyte p38 MAPK in Trauma Patients (p38MAPK/β-Actin)

Groups	*n*	Gene expression levels of leukocyte p38 MAPK at different times				
		6h	1d	3d	5d	7d
Control	30	0.28±0.08	0.29±0.09	0.29±0.08	0.28±0.08	0.28±0.09
L-ISS	50	0.34±0.12[*]	0.39±0.13[△]	0.36±0.12[*]	0.35±0.13[*]	0.35±0.14[*]
M-ISS	50	0.40±0.14[*,†]	0.48±0.13[△,†]	0.43±0.13[*,†]	0.41±0.12[*,†]	0.40±0.12[*,†]
H-ISS	50	0.46±0.13[*,‡]	0.53±0.11[△,‡]	0.50±0.12[*,‡]	0.48±0.12[*,‡]	0.45±0.13[*,‡]

[*] $P < 0.05$,
[△] $P < 0.01$, compared with control.
[†] $P < 0.05$, compared with L-ISS.
[‡] $P < 0.05$, compared with M-ISS.

significantly in patients with each stage of trauma compared with normal donors ($P < 0.05$), and the p38 mRNA levels increased with the severity of the trauma stage, i.e., group H-ISS > group M-ISS > group L-ISS ($P < 0.05$). The expression level of p38 MAPK gene was up-regulated within 6 h after trauma ($P < 0.05$), reached the peak value on day 1 ($P < 0.05$), and kept on high expression at day 3, 5, and 7 after trauma ($P < 0.05$).

Expression and Activation Changes of Leukocyte p38 MAPK from Trauma Patients

The results from RQ-PCR showed that the gene expression levels of leukocyte p38 MAPK in trauma patients increased and reached a maximum at day 1; we further determined whether the expression and activation levels of leukocyte p38 MAPK increased in response to trauma. Each sample at day 1 was examined by Western blot analysis to detect the expression of p38 MAPK using p38 MAPK antibody and activation-associated phosphorylation of p38 MAPK (pp38 MAPK) using a phospho-specific antibody. The expression or activation level of p38MAPK detected in each sample was standardized by dividing with that of the corresponding internal control β-actin for absolute quantification (Fig.1). The leukocyte p38 MAPK protein expressions were found in 150 patients with different severity of trauma as well as in 30 healthy donors, and significantly higher in trauma patients than those in healthy donors ($P < 0.05$). Elevation of p38 MAPK was associated with the increase of ISS, i.e., group H-ISS > group M-ISS > group L-ISS ($P < 0.05$). Then, the activation levels of p38 MAPK were analyzed in trauma patients and healthy blood donors. Phosphorylated p38 MAPK was at very low level in healthy donors. In contrast, phosphorylated p38 MAPK and the activation ratio of phosph-p38MAPK/p38MAPK were elevated significantly in trauma patients compared with healthy donors ($P < 0.05$). There were no differences in the activation ratios of phosph-p38MAPK/p38MAPK between ISS groups, and elevation of phosphorylated p38 MAPK was associated with the increase of injury severity, i.e., group H-ISS > group M-ISS > group L-ISS ($P < 0.05$, Table 5).

Changes of Plasma TNFα and IL-6 from Trauma Patients

Plasma TNFα and IL-6 were evaluated by ELISA (Table 6). The results indicated that the levels of TNFα and IL-6 also increased significantly in patients 1 d after trauma ($P < 0.01$). Furthermore, the level of plasma TNFα and IL-6 in trauma patient was elevated with the increase of ISS ($P < 0.05$).

Correlation Analysis

Correlation matrices were used to reveal the relevance of p38 MAPK gene expression, the protein expression level of p38 MAPK or p38 MAPK phosphorylation with the ISS stages, and plasma IL-6 and TNFα with phospho-p38 MAPK expression and the ISS stages (Table 7). Correlation analysis on the data 1 d after trauma showed positive correlations between the p38 MAPK gene expression and ISS ($P < 0.01$), the protein expression level of p38 MAPK and ISS ($P < 0.01$), the expression of phosphorylated p38 MAPK and ISS ($P < 0.01$), the activation of p38 MAPK and the expression of TNFα, IL-6 ($P < 0.01$), as well as the expression of TNFα, IL-6, and ISS ($P < 0.01$).

DISCUSSION

Mortality after major trauma has been divided into three separate time periods after injury [1]. The first peak of death after injury presenting at the scene or within the first h is usually due to massive head injury or bleeding. The second smaller peak in the first 24 h is

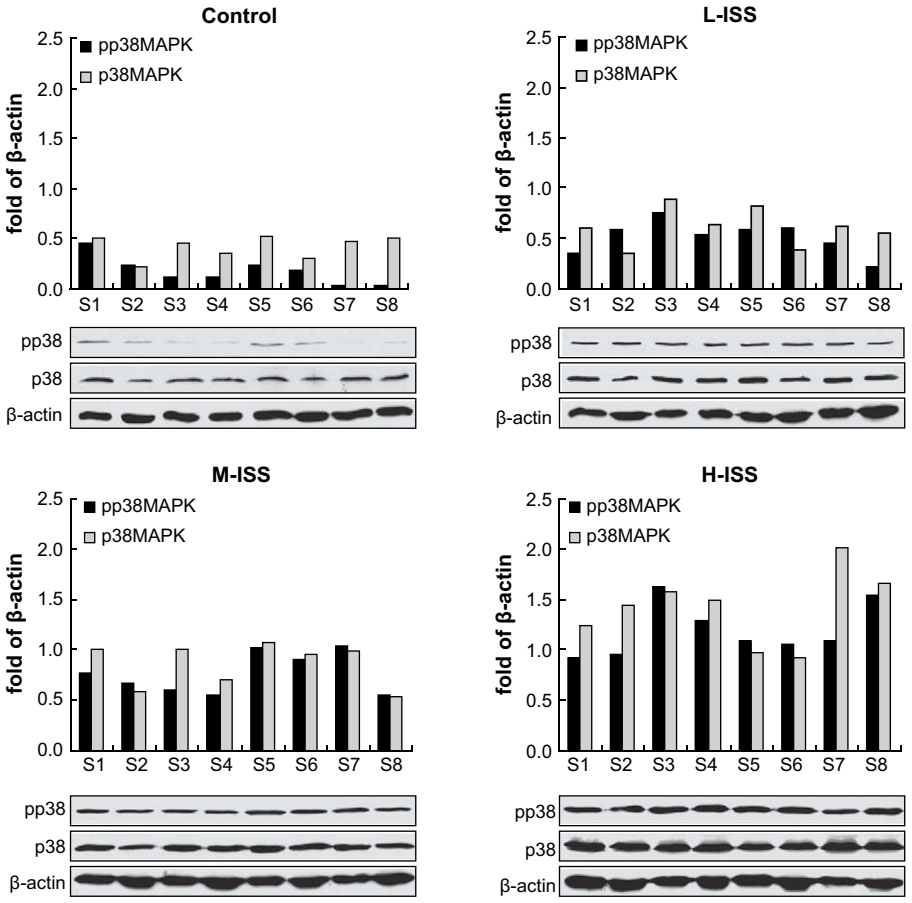

FIG. 1. Detection of protein expression and phosphorylation of p38 MAPK. The expressions of phosphorylated and nonphosphorylated p38 MAPK at 1 d after injury were monitored by Western-blot analysis. An antihuman β-actin antibody was used as a loading control. Eight peripheral blood samples from each group are shown.

normally due to hypoxia, hypovolemia, or severe head trauma. Patients who died before arrival at the emergency department or within 24 h in ICU were excluded in this study. The third peak of death is caused by a high risk of developing immunological dysfunction and subsequent sepsis or the systemic inflammatory response syndrome (SIRS), which can lead to MODS. In the study, incidence of organ dysfunction occurring 24 h after trauma was found to be related with the increase of injury severity.

MAPK, which was discovered in 1994 and initially regarded as a kinase that can be activated by lipopolysaccharide (LPS) [9], is an important signal transduction system that regulates the proliferation, apoptosis, and gene expression of the cells. Animal experiments suggested that p38 MAPK signaling pathway plays an important role in traumatic injuries. This study revealed that the gene expression of p38 MAPK in trauma patients increased significantly 6 h after injury and reached a maximum in 1 d, and protein expression

TABLE 5

The Expression and Activation of p38MAPK in Trauma Patients

Groups	n	p38MAPK/β-Actin	pp38MAPK/β-Actin	pp38MAPK/p38MAPK
Control	30	0.40±0.12	0.18±0.13	0.50 ±0.33
L-ISS	50	0.53±0.19*	0.37±0.17*	0.76±0.42*
M-ISS	50	0.63±0.25*,†	0.46±0.23*,†	0.73±0.23*
H-ISS	50	0.85±0.33*,‡	0.63±0.31*,‡	0.74±0.20*

*$P < 0.05$, compared with control.
†$P < 0.05$, compared with L-ISS.
‡$P < 0.05$, compared with M-ISS.

TABLE 6

Plasma IL-6 and TNFα Levels in Various ISS One Day After Trauma (pg/mL)

Groups	Patients	IL-6	TNFα
Control	30	43.7±20.3	38.2±16.4
L-ISS	50	67.8±31.6*	55.3±23.4*
M-ISS	50	85.3±38.5*,†	68.6±25.1*,#
H-ISS	50	101.2±32.2*,‡	90.6±35.7*,△

*$P < 0.01$, compared with control.
†$P < 0.05$.
#$P < 0.01$, compared with L-ISS.
‡$P < 0.05$.
△$P < 0.01$, compared with M-ISS.

TABLE 7

Correlation Analyses for p38 MAPK Gene Expression, p38 MAPK, and Phospho-p38 MAPK Protein Expression, Plasma TNFα, IL-6 Levels, and ISS 1 Day After Trauma

Groups	p38 MAPK gene	p38 MAPK	pp38 MAPK	TNFα	IL-6
ISS	0.993	0.981	0.987	0.935	0.988
pp38 MAPK	—	—	—	0.912	0.941

and activation level of p38 MAPK increased significantly 1 d after trauma. This study also found that the activation ratio (phospho-p38 MAPK/p38 MAPK) was obviously higher than its basal level in healthy control and kept an elevated level in trauma patients. These findings indicated that p38 MAPK signaling pathway is activated after injury, and the expression and activation of p38 MAPK has highly positive correlation with the severity of trauma.

p38 MAPK can be activated in response to a variety of extracellular stimuli including UV [10], osmolarity [11], inflammatory cytokines, growth factor, and shock. p38 MAPK is transduced through highly conserved 3-level kinase cascade [12, 13]. When activated by extracellular stimuli, MAPK kinase kinase (MAPKKK) phosphorylates a MAPKK on its threonine and tyrosine residues, and then MAPKK activates MAPK. The activation of p38 MAPK requires phosphorylation of both serine and tyrosine. Upon activation, p38 MAPK can enter nucleus or other places, and activates downstream kinases or various transcription factors.

Activated transcription factor-2 (ATF-2) is a major substrate of p38 MAPK. p38 MAPK can regulate the gene expression of inflammatory cytokines such as TNFα, IL-1, and IL-6 by activating ATF-2 [14]. Myocyte enhancer factor 2C (MEF2C) is also a major downstream substrate of p38 MAPK. p38 MAPK increases c-jun gene transcription by activating MEF2C. Under inflammatory states, phosphorylated c-jun augments the expression of inflammation-related genes by binding to activator protein-1 (AP-1) sites located in cytokine promoter.

p38 MAPK signaling pathway is associated with trauma-induced inflammation by up regulating cytokines expression. Plasma TNFα and IL-6 were found to be associated with activated p38 MAPK and the injury severity in trauma patients in this study. Cytokines, including TNFα and IL-6, known collectively as pro-inflammatory cytokines, are the central events triggering the cascades of inflammatory reactions. Expression and release of the pro-inflammatory cytokines increase in the development of inflammation and their overproduction is the leading cause of tissue damage [15, 16]. p38 MAPK inhibitors could significantly reduce the release of LPS-induced cytokines [17, 18]. Sato *et al.* [19] found that blocking p38 by pretreatment with FR167653 suppressed the stress-promoted TNF-α and IL-1β production and improved inflammation-related lung dysfunction following hemorrhagic shock in rats.

Numerous researches revealed that the p38 MAPK signaling pathway resulted in multiple organ injury caused by ischemia/reperfusion [4–8, 20]. When severe trauma or blood loss leads to shock, ischemia happens in all major organs, and reperfusion injury tends to happen during resuscitation, which aggravates illness and results in MODS. p38 inhibitor could effectively prevent myocardial ischemia-reperfusion injury [21].

p38 MAPK also has close relation with cell apoptosis caused by trauma [2, 3, 22]. p38 MAPK can regulate apoptosis through at least the following ways: increases c-myc expression; phosphorylates p53; involves in Fas/FasL-mediated apoptosis; activates c-jun and c-fos; induces Bax translocation.

However, it was reported that after traumatic bleeding, up-regulation of p38 MAPK signaling pathway can activate heat shock protein such as HSP27 and α B-crystallin by phosphorylation, and induces the cell protective effect of the body [23]. In addition, activation of p38 MAPK is associated with wound healing because p38 MAPK influences the motions of many types of cells that take an important part in the healing process, such as immune cells, fibroblasts, epithelial cells. Experiments showed that over-suppression or over-activation of p38 MAPK would cause scar formation or delayed wound healing, such as chronic skin ulcers [24].

CONCLUSION

Our research suggested that p38 MAPK signal pathway has close relation with both the severity and the pathology mechanism of the trauma. Effective

detection of p38 MAPK signaling pathway could help predict the severity and prognosis of trauma. It is also suggested that precise control of p38 MAPK signaling pathway is a new approach to treat severe trauma, which has great significance in reducing trauma-led disability and mortality.

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