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Full Length Research Paper

Role of glucocorticoid receptor and nuclear factor kappa B in rat hepatic injury after traumatic hemorrhagic shock

Donglin Luo[1], Xiankai Huang[2]*, Baohua Liu[1], Xiaobao Ren[3], Renping Xiong[1], Tao Li[1] and Guangyan Li[1]

[1]Department of General Surgery, Institute of Battle Surgery Research, Daping Hospital, Third Military Medical University, Chongqing 400042, China.

[2]Trauma Center, Institute of Battle Surgery Research, Daping Hospital, Third Military Medical University, Chongqing 400042, China.

[3]Department of Emergency, Southwestern Hospital, Third Military Medical University, Chongqing 400038, China.

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The present study investigated expressions and functions of glucocorticoid receptor (GR) and nuclear factor kappa B (NF-κB) in rat livers after traumatic hemorrhagic shock. The rat model of bilateral femur fracture accompanied with traumatic hemorrhagic shock was established. 96 male Wistar rats were randomly divided into normal control group (n = 6), traumatic shock group (n = 30), GR blocking group (n = 30) and NF-κB inhibiting group (n = 30). 10 g/L of Ru486 (Mifepristone) was given via intramuscular injection 1.5 h before injury to block GR expression in GR blocking group, and 200 mg/kg of pyrrolidine dithiocarbamate (PDTC) was given via intraperitoneal injection 1 h before injury to inhibit the activity of NF-κB. The expression of GR, TNF- and IL-6, the activity of NF-κB, the hepatic pathology and the hepatic function were dynamically observed 0.5, 2, 4, 6, 8 h after trauma. Electrophoretic mobility shift assay (EMSA) was used to detect the bind activity of NF-κB. The content of GR protein in liver tissue started to decrease 2 h after traumatic hemorrhagic shock, and was significantly lower than the normal control group after 4 h ($P < 0.01$). The activity of NF-κB was significantly increased after injury, and peaked after 6 h ($P < 0.01$). After blocking GR expression, NF-κB expression was significantly increased at each time point after reinjury. Two hours after injury, inflammatory cell infiltration was observed in the *Sinus hepaticus*. The expressions of TNF - , IL-6, ALT and TB were significantly increased 2 h after injury ($P < 0.01$). After inhibiting NF -κB, GR expression was increased in liver tissue after reinjury. TNF-and IL-6 were rapidly decreased at each time point after injury. The liver cell degeneration was significantly recovered 4 - 8 h after injury under light microscope and the congestion in the *S. hepaticus* was relieved. ALT and TB expressions in serum were significantly decreased 4 h after injury. GR and NF-κB have a close relationship and play an important role in the hepatic injury after traumatic hemorrhagic shock.

Key words: Trauma, hemorrhagic shock, glucocorticoid receptor, nuclear factor kappa B, liver injury.

INTRODUCTION

Increased glucocorticoid (GC) secretion is the most important response under stresses such as trauma, blood loss and infection. The effect of GC depends not only on its concentration in plasma, but also on the number and affinity of the glucocorticoid receptor (GR) on target cells (Li 1999). NF-kB (Nuclear factor-kappa B) can induce the transcription and expression of a variety of immune- and inflammation-induced cytokines and genes, thus causing serious damage to tissues and organs after injury (Lee et

*Corresponding author. E-mail: huang024326@qq.com. Tel: +8623-68765115.

Abbreviation: GC, glucocorticoid; **GR,** glucocorticoid receptor.

al., 2006).

The liver plays an important role in multiple organ failure induced by traumatic hemorrhagic shock. GR and NF-kB play important roles in the procedure of anti-injury of the liver cells after traumatic shock. Nuclear factor kappa might prove to be essential for timely and complete regeneration. However, whether they are involved in the occurrence and development of traumatic shock is still rarely reported. Therefore, in the present study, we established a rat model of bilateral femur fracture accompanied with traumatic hemorrhagic shock (Sun et al., 2002), and observed the expression of GR and the activity of NF-κB in liver tissue and their roles in liver injury.

MATERIALS AND METHODS

Ethics statement

All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of The Third Military University and conformed to the National Institute of Health guidelines on the ethical use of animals.

Experimental animals and grouping

A total of 96 healthy male Wistar rats weighing 280 ± 30 g were used in our study. They were fasted for 12 h, but given free access to water before the experiment. The rats were randomly divided into normal control group (n = 6), bilateral femur fracture accompanied with hemorrhagic shock group (trauma group, n = 30), GR blocking group (GR treatment group, n = 30) and NF-κB inhibiting group (n = 30).

Model of traumatic hemorrhagic shock after trauma

After anesthesia for 12 h with 1.5 g/L sodium pentobarbital intraperitoneally injected at 30 mg/kg, the rats were fixed on the platform in the supine position. The middle of the bilateral femur fracture was broken and right femoral arterial cannula was carried out for blood collection and the pressure transmitter was linked by a three-way joint. The blood pressure was monitored with a polygraph, and maintained at 40 mmHg (1 mmHg = 0.133 Kpa) for 12 h, and the model of hemorrhagic shock after trauma was established.

Blocking GR expression

One hour before injury, 10 g/L Ru486 (mifepristone) (Sigma) was given via intramuscular injection at 5 mg/kg (Fan et al., 1994).

Inhibiting NF-κB expression

One hour before injury, 200 mg/kg pyrrolidine dithiocarbamate (PDTC) (Sigma) was given via intraperitoneal injection (Yin et al., 2004).

Observation indexes

Six rats were sacrificed 0.5, 2, 4, 6, 8h after trauma, respectively.

Western blot

The liver tissues were obtained and GR expression was determined by Western blot. Denatured cytoplasmic proteins (50 g per sample) were separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) along with molecular weight markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), which were blocked with 5% nonfat dried milk in Tris-buffered saline that contained 0.1% Tween-20. The membranes were probed with the specific primary antibody (anti- GR or anti- -actin antibody) and an appropriate secondary horseradish peroxidase-conjugated antibody (Boster Biological Technology, China) and visualized using the ECL detection system.

Emsa

NF-κB binding activity was examined with electrophoretic mobility shift assay (EMSA). EMSA was performed using a light shift chemiluminescent EMSA kit (Pierce Biotechnology, USA) according to the manufacturer's protocol. Briefly, 8 g of the nuclear proteins and 1 l of biotin-labeled NF- B probe were incubated. For competition assays, 1 l of unlabeled NF- B or AP-1 oligouncleotides (200-fold excess of biotin-labeled probe) were added and incubated for 15 min before the addition of the biotin-labeled probe. Samples were separated on a 6% non- denaturing acrylamide gel, pre-run for 45 min at 100 V, in 0.5 × Tris boric acid-EDTA buffer at room temperature for 2 h at 100 V. Gels were transferred to positively charged nylon membranes where the gel shifts were visualized by a chemiluminescent detection system (Pierce Biotechnology, USA).

Expression with optical density

The image was analyzed with an image analyzer and the results were expressed with banged density. The liver tissues were obtained at each corresponding time point and fixed through the perfusion of 40 g/L paraformaldehyde. The tissues were progressively dehydrated with alcohol and xylene. The tissues were embedded with paraffin and the slices were made, and the HE staining was performed. The sections were observed under light microscopy.

Elisa

The content of serum alanine aminotransferase (ALT) and total bilirubin (TB) were examined with an automatic biochemical analyzer. TNF- and IL-6 contents were detected with ELISA. An ELISA kit was purchased from ADL, USA. All harvested serum specimens were preserved at room temperature for 2 h and then were centrifugated at 1000 g for 20 min, and supernatant was collected and preserved at -20°C and the supernatant were frozen and thawed as less as possible. There were blank holes, standard holes and sample holes. 100 l sample diluent was added in blank holes, and 100 l supernatant and standard solution was added into sample holes and standard holes, respectively. After uniform mixing by gently swaying, the enzyme-coated plates were incubated at 37°C for 2 h. Then, the liquid was discarded, and the plates were dried and 100 l/hole test buffer A was added and incubated at 37°C for 1 h. After the liquid was discarded, the plates were washed with PBS for 3 times and 100 l/hole test buffer B was added and incubated at 37°C for 1 h. Then, the liquid was discarded, and the plates were dried, and 90 l/hole substrate solution was added and incubated at 37°C and in dark room for 30 min. Subsequently, 50 l/hole stop buffer was added to terminate reaction and then mixed

Table 1. Content of GR protein (OD) in rat liver tissue ($\bar{x}$ ± s, n = 6).

Groups	Before injury	Time after injury (h)				
		0.5	2	4	6	8
Traumatic group	21.7 ± 2.0	19.9 ± 2.0	15.2 ± 2.5*	11.6 ± 2.0*	9.8 ± 1.7*	11.3 ± 2.0*
NF-κB inhibiting group	21.7 ± 2.0	20.9 ± 2.1	19.2 ± 2.9*	16.0 ± 1.5*	12.4 ± 1.8*	12.0 ± 2.2*

Note: Compared with that before injury * P < 0.01. Compared with the traumatic group P < 0.05

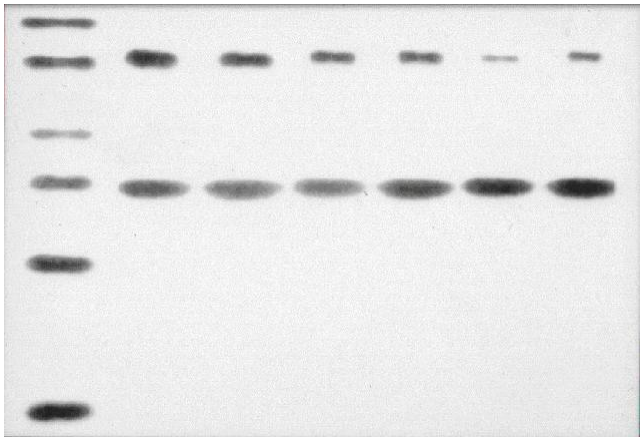

Figure 1. Content of GR protein in liver tissue with traumatic shock. Note: 1-6 were the control, 0.5, 2, 4, 6 and 8h after injury, respectively.

liquid presented yellow from blue. Then, the optical density (OD) of each hole was detected with a microplate reader at 450 nm.

Statistical analysis

All data were analyzed with SPSS12.0 software. Experimental data were expressed by mean ± standard deviation. Paired *t* test was used to compare the results before and after treatment. Multi-group comparison was tested by analysis of variance. *q* test was used for comparison between two means. P < 0.05 was considered statistically significant.

RESULTS

Content of GR protein

The content of GR protein in liver tissues started to decrease 1 h after bilateral femur fracture companied with hemorrhagic shock and was significantly lower than the normal control group after 2 h (P < 0.01), and was decreased to the minimum after 6 h (P < 0.01). After inhibiting the activity of NF-κB, GR expression recovered to some extent after reinjury, and was higher than the traumatic shock group at each time point after injury, but it was still lower than the normal control group 4 ~ 8 h after injury (P < 0.01) (Table 1, Figures 1 and 2).

Activity of NF-kB

NF-kB activity was very low in normal liver tissues. 2 h after bilateral femur fracture accompanied with hemorrhagic shock, NF-kB activity was significantly increased as compared with the control (P < 0.01), and peaked after 6 h (*P* < 0.01). After blocking the expression of GR, NF-κB expression was higher than the traumatic shock group at each time point after reinjury and it was gradually increased with the time. The peak was at 6 h after injury and it was still maintained at a high level 8 h after injury (Table 2, Figures 3 and 4).

Pathological changes of the liver

Four to eight hours after hemorrhagic shock, mild congestion could be seen in the *Sinus hepaticus* accompanied with sporadic inflammatory cell infiltrate under the light microscope. After blocking GR, expanded

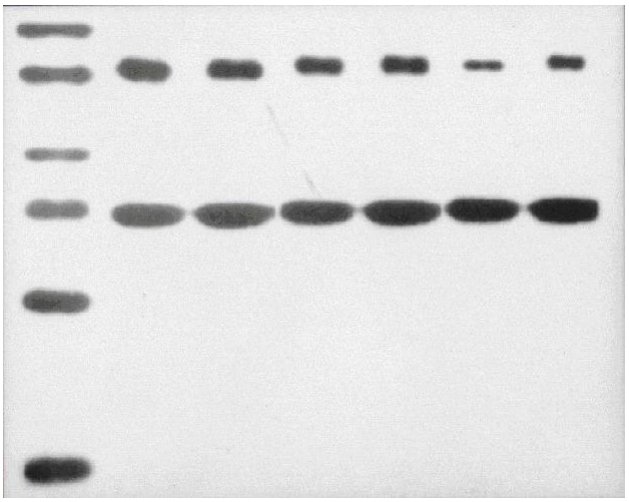

Figure 2. Content of GR protein in liver tissue of rat with traumatic shock after inhibiting NF-kB.

Table 2. Activity of NF-κB (OD) in rat liver tissue ($\bar{x}$ ± s, n=6)

Groups	Before injury	Time after injury (h)				
		0.5	2	4	6	8
Traumatic group	2.3 ± 0.4	3.6 ± 0.4	8.4 ± 0.7*	19.3 ± 2.7*	43.4 ± 4.6*	38.2 ± 3.4*
GR blocking group	2.3 ± 0.4	4.5 ± 0.4	15.2 ± 1.8*	27.6 ± 4.1*	57.9 ± 7.0*	48.5 ± 1.1*

Note: Compared with that before injury * P < 0.01. Compared with the traumatic group P < 0.05.

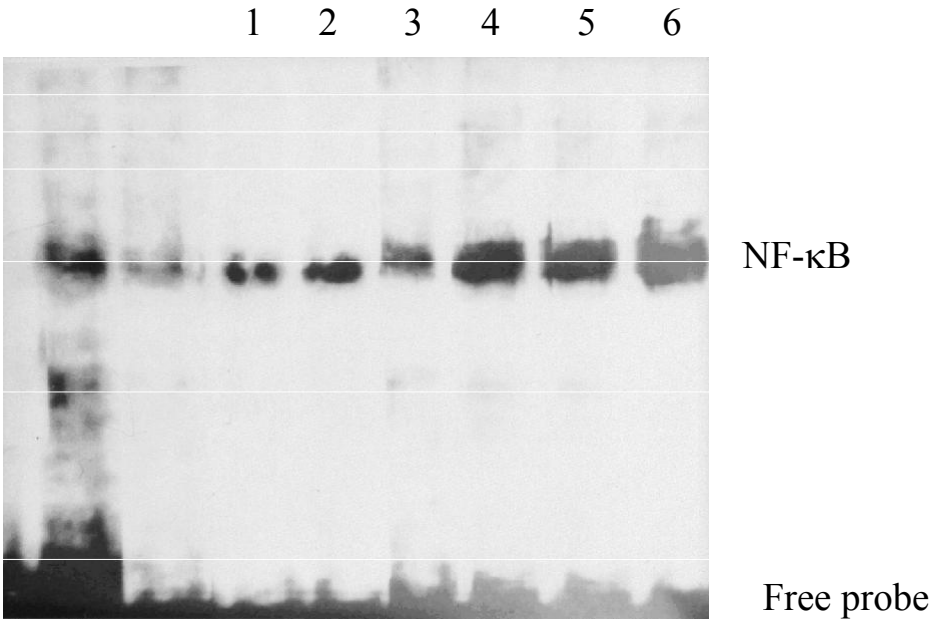

Figure 3. Activity of NF-κB in liver tissue of rat with traumatic shock. Note: 1-6 were the control, 0.5, 2, 4, 6 and 8 h after injury, respectively.

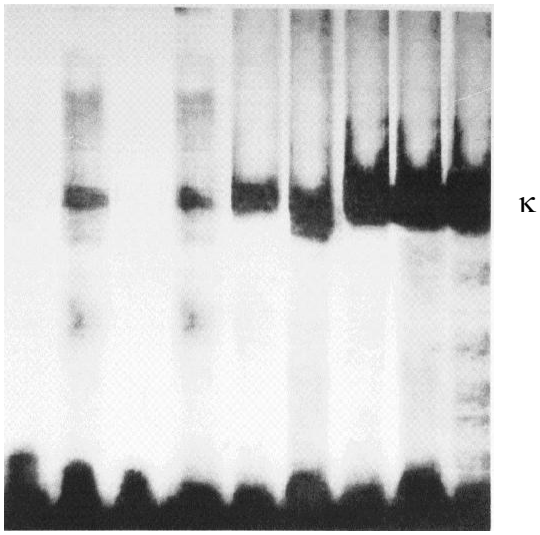

Figure 4 Activity of NF-kB in liver tissue of rat with traumatic shock after blocking GR expression. Note: 1-6 were the control, 0.5, 2, 4, 6 and 8h after injury, respectively.

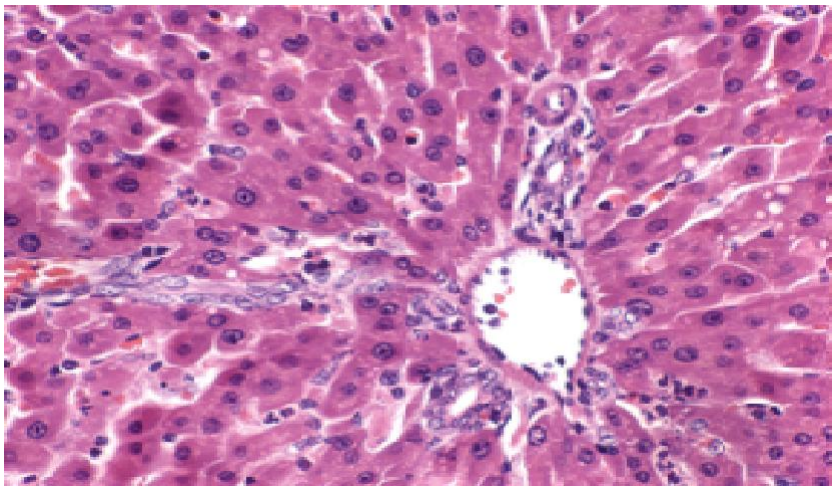

Figure 5. Four hours after blocking GR expression, a large number of Kupffer cells and neutrophils infiltration and liver cells adipose degeneration could be seen in the *Sinus hepaticus*. HE × 400.

S. hepaticus and obvious congestion were found. The structure of the liver lobular was disordered and some liver cells showed cell degeneration. Kupffer cell infiltration was shown between the hepatic sinusoid and the liver cell cord and neutrophils were seen on the wall. 6 – 8 h after injury, the above changes became more significant. After inhibiting NF-κB, the liver cell degeneration was significantly better than the traumatic shock group after reinjury. 4 h after injury, only a small number of neutrophils and lymphocytes could be seen between the hepatic sinusoid and the liver cell cord (Figures 5 and 6).

Changes of liver function

The expression levels of serum ALT and TB started to increase 4 h after hemorrhagic shock. After blocking GR expression, the expressions of serum ALT and TB were

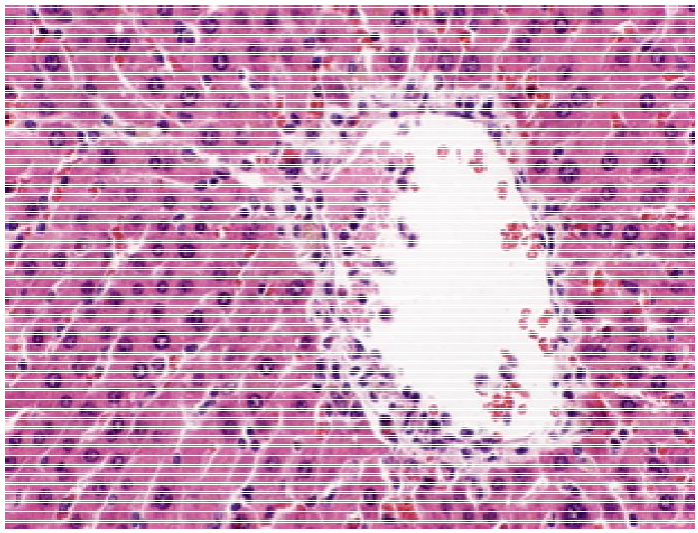

Figure 6. Four hours after inhibiting NF-κB, the sporadic congestion, sporadic neutrophils and lymphocytes infiltration could be seen in the *Sinus hepaticus*. HE × 400.

Table 3. Changes of ALT and TB in rat serum ($\bar{x}$ ± s, n = 6).

Groups	Before injury	Time after injury (h)				
		0.5	2	4	6	8
ALT(nmol$^{-1.L-1}$)						
Traumatic group	536.8 ± 60.0	542.3 ± 62.2	575.6 ± 63.2	640.6 ± 80.2*	720.6 ± 87.2*	830.6 ± 106.2*
GR blocking group	536.8 ± 60.0	582.6 ± 66.2	655.6 ± 71.2*	732.6 ± 88.6*	845.6 ± 108.4*	968.8 ± 116.5*
NF-κB inhibiting group	536.8 ± 60.0	525.5 ± 63.2	533.1 ± 63.7	540.8 ± 66.2≥	587.2 ± 69.5≥	602.4 ± 72.2≥
TB (mol/L)						
Traumatic group	1.6 ± 0.2	2.2 ± 0.5	3.4 ± 0.9*	4.7 ± 1.1*	6.5 ± 1.6*	8.8 ± 1.9*
GR blocking group	1.6 ± 0.2	3.5 ± 0.5*	4.9 ± 1.0*	7.3 ± 1.2 *	8.7 ± 1.0*	11.7. ± 1.9*
NF-κB inhibiting group	1.6 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	2.3 ± 0.3≥	2.8 ± 0.6≥	4.5 ± 1.1*≥

Note: Compared with that before injury * P<0.01. Compared with the traumatic group P < 0.05.

significantly increased 2 h after injury and more significant 6 – 8 h after injury (P < 0.01). After inhibiting the activity of NF-κB, the expressions of serum ALT and TB were significantly decreased after injury and more significant 6 – 8 h after injury (P < 0.01) (Table 3).

TNF- , IL-6 content in serum

The levels of serum TNF- and IL- 6 were low in normal rats. However, they peaked 6 h after traumatic hemorrhagic shock. After blocking GR expression, the expressions of serum TNF - and IL-6 were rapidly increased at each time point and they were significantly higher than the traumatic group 2 h after injury (P < 0.05). They still maintained a high level 6 and 8 h after shock. After inhibiting NF-κB, the expressions of TNF- and IL-6 were rapidly reduced, and was significantly decreased as compared with the traumatic group (P < 0.05), and they were still reduced 6 and 8 h after injury (Table 4).

DISCUSSION

This study showed that apparent liver damage appeared both in morphology and function 8h after hemorrhagic shock. GR sustained a low expression after injury during this pathological process, while NF- kB consistently showed high expression, indicating that GR and NF-kB play an important role in secondary liver injury following

Table 4. Changes of TNF- and IL-6 in rat serum (pg/ml) ($\bar{x} \pm s$, n = 6)

Groups	Before injury	Time after injury (h)				
		0.5	2	4	6	8
TNF–						
Traumatic group	30.8 ± 1.8	34.9 ± 3.2	48.1 ± 3.3*	93.9 ± 5.3*	173.7 ± 12.1*	156.6 ± 12.9*
GR blocking group	30.8 ± 1.8	37.3 ± 2.0	73.1 ± 6.2*	131.4± 10.8*	282.3 ± 17.9*	262.3± 18.9*
NF-κB inhibiting group	30.8 ± 1.8	38.2 ± 2.0	47.4 ± 2.7*	59.8± 2.1*	135.2 ± 10.2*	107.4± 10.8*
IL-6						
Traumatic group	10.4 ± 0.7	12.8 ± 1.0	28.3 ± 1.6*	78.4 ± 5.2*	175.5 ± 12.5*	162.1 ± 8.2*
GR blocking group	10.4 ± 0.7	18.3 ± 1.6	47.1 ± 3.7*	117.9± 10.2*	234.5 ± 20.8*	197.9± 10.9*
NF-κB inhibiting group	10.4 ± 0.7	16.7 ± 1.0	28.4 ± 2.5*	50.7± 3.5*	113.0 ± 10.8*	92.4± 5.0*

Note: Compared with that before injury *P < 0.01.Compared with the traumatic group P < 0.05.

traumatic hemorrhagic shock.

It is also found that in the reinjury after blocking GR, many pathological manifestations appear more apparently, such as apparent liver congestion and swelling, inflammatory cell infiltration in hepatic sinusoids, hepatic lobule structural disorder, degenerations in some liver cells, more conspicuous liver damage and increase in serum TNF - , IL-6 levels. The fact that NF - kB expression increases significantly in various steps after reinjury and the expression increases as time passes reflects that blocking GR may induce the production NF-kB in liver, thus resulting in secondum liver damage. Meanwhile, in reinjury after blocking NF- kB, pathological manifestations became less severe, such as less liver cell degeneration, less liver congestion and swelling, less inflammatory cell infiltration in hepatic sinusoids, recovery in conspicuous liver damage, and decrease in serum TNF - , IL-6 levels. All the facts above reflect that blocking NF-kB may increase GR expression in liver. This research demonstrates that after serious injury shock, the deficiency of GR may increase the activity of NF-kB, while normal GR can inhibit NF-kB expression apparently. The fact that GR expression increases after inhibition of NF-Kb demonstrates that there is reciprocal inhibition between GR and NF-kB.

Studies have shown that in the regulation of immune response and inflammatory, GR and NF-kB are a pair of transcriptional regulation factors that have complete contrary function. NF-kB can induce the transcription and expression of multiple immune- and inflammation-related cytokines and other related genes, while GR can inhibit the NF- kB-induced expression of them, thus exerting immune suppression and anti-inflammatory effects (Adcock and Caramori, 2001). The mechanism of the reciprocal inhibition between GR and NF- kB remains still unclear. The possible mechanism may be as follows: GR and NF-kB may have functional antagonism (De et al., 2000). GR can induce the expression of IkB. GR may complete the same transcription cofactor with NF-kB. GR may affect the acetylation of histone. NF-kB may induce the expression GR. The interaction between GR and NF-kB is of great physiological significance. Some stimulating factors such as bacterial or viral infection and oxygen free radical could active NF-kB and could also stimulate the hypothalamus-pituitary gland-suprarenal cortex, simultaneously. Therefore, the GC level in serum was increased, thus activating GR immunosuppression pathway and decreasing the damage of immune reaction and inflammatory reaction on the body, indicating that the antagonistic effect between GR and NF-kB is an important regulatory mechanism maintaining the homeostasis during the activation of immune defence system (Adcock and Caramori, 2001).

Our results suggested that GR deficiencies or functional inhibition could cause substantial release of systemic inflammatory mediators, resulting in weak anti-inflammatory activity, which led to an aggravated liver injury due to the traumatic hemorrhagic shock of rats. However, if the activity of NF-kB was inhibited, the liver injury was significantly reduced. It has been shown that the activity of NF-kB may be regulated through the inhibition of HSP70 (Yin et al., 2008). HSP70 is a protective protein under stress condition, which has protective effect on the organism, indicating that GR and NF-kB play an important role during the process of insecondary liver injury following the traumatic hemorrhagic shock. The simultaneous increased GR content (Zhang et al., 2005) and increased NF-κB activity (Shen et al., 2008) are of great significance in attenuating liver injury after traumatic shock and delaying the hepatic failure. However, its mechanism still needs to be further studied.

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