

IN VIVO HEAT SHOCK PROTEIN ASSEMBLES WITH SEPTIC LIVER NF- κ B/I- κ B COMPLEX REGULATING NF- κ B ACTIVITY

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Received 12 Apr 2005; first review completed 2 May 2005; accepted in final form 6 Jun 2005

ABSTRACT—This study elucidates the mechanism through which heat shock treatment influences the outcome of sepsis. Post-heat shock sepsis was induced in rats by CLP 24 h after whole-body hyperthermia. Liver cytosolic and nuclear fractions were collected and analyzed in early and late sepsis rats (sacrificed 9 and 18 h after CLP, respectively). During sepsis, levels of I- κ B and nuclear factor- κ B (NF- κ B) declined in the cytosol of liver, whereas NF- κ B increased in nucleus. NF- κ B activity was significantly enhanced during sepsis, and the products of NF- κ B target genes, such as TNF- α and inducible nitric oxide synthase (iNOS), were overexpressed. Heat shock treatment, inducing heat shock protein synthesis, prevented down-regulation of cytosolic I- κ B and decreased translocation of NF- κ B into the nucleus. Therefore, the sepsis-induced acceleration of NF- κ B activation was inhibited. Expression of TNF- α and iNOS mRNA was also down-regulated. Coimmunoprecipitation with anti-NF- κ B (p65) and anti-I- κ B antibodies verified an assembling phenomenon of heat shock protein (HSP) 72 with NF- κ B and I- κ B. We suggest that the mechanism preventing septic activation of NF- κ B is that oversynthesized HSP72 forms a complex with NF- κ B/I- κ B, thus inhibiting nuclear translocation of NF- κ B. HSP72 appears to play a crucial protective role in modulating the gene expression controlled by NF- κ B in sepsis.

KEYWORDS—Sepsis, cecal ligation and puncture, HSP72, coimmunoprecipitation, liver

INTRODUCTION

Nuclear factor- κ B (NF- κ B) was first identified by Sen and Baltimore (1) as sequestered in the cytoplasm with a specific inhibitory protein termed I- κ B. Through stimulation, I- κ B can be phosphorylated and degraded via the proteasome pathway. When NF- κ B is released from I- κ B, it in turn activates and migrates from cytosol to the nucleus (2, 3). NF- κ B plays an essential role in the transcriptional regulation of genes involved in the early onset of immune response, including inflammatory mediators, adhesion molecules, and acute phase proteins.

Clinically, sepsis causes adverse metabolic alterations resulting in the systemic inflammatory response syndrome, perfusion abnormalities, and tissue dysfunction and multiple organ failure (4). Liver functions were known to be seriously compromised in septic shock and experimental endotoxic shock, corresponding to the outcome of sepsis (5, 6). After the disease cascade, tissue inflammation and injury may be induced by local production of cytokines, chemokines, and enzymes. In particular, tumor necrosis factor- α (TNF- α) and nitric oxide (NO), produced by inducible NO synthase (iNOS), have been shown to play a potential role in the induction of liver function failure during sepsis (6, 7). These productions are regulated by the ubiquitous transcription factor complex, NF- κ B (8, 9).

The pathogenesis of sepsis has been extensively investigated over several decades using animal model studies. This lead to the clinical trial of antiendotoxin or anticytokine therapy (7, 10). The effectiveness of this therapy is limited. However, the heat shock response (HSR) has been found to have a protective mechanism. It has become a good model to investigate pathogenesis of sepsis as well as a therapeutic intervention. Heat shock proteins (HSPs) are a family of highly conserved proteins induced in HSR to various stresses. HSPs act as a molecular chaperone to protect cells against subsequent lethal circumstances (11, 12). Our previous work demonstrates that the HSR contributes to maintaining mitochondrial function during sepsis (13) and attenuation of sepsis-induced apoptosis (14), and decreases mortality from sepsis (15). Studies have also shown that the HSR may protect cells from damage by endotoxins, a range of inflammatory cytokines, or free radical species (16, 17). This protective effect is mediated, at least in part, through the member of HSPs, such as HSP72. However, mechanisms by which heat shock and HSP72 exert an anti-septic effect during sepsis are still unclear.

This study was designed to investigate the activation and expression of NF- κ B in septic liver, and its modulation by HSP. A possible mechanism is discussed.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male Sprague-Dawley rats (weighing 270–350 g) obtained from the National Experimental Animal Center (Nan-Kang, Taipei, Taiwan). Experiments conducted in this study were approved by the Animal Care and Treatment Committee of Kaohsiung Medical University, and the authors have adhered to the National Institutes of Health guidelines for use of experimental

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This work was supported by the National Science Council, Taiwan (grant no NSC-90-2320-B-041-016).

DOI: 10.1097/01.shk.0000174020.87439.f2

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animals. Animals were divided into the five groups: sham-operated group (no sepsis, $n = 8$); sham-heated early sepsis group (9 h after CLP operation, $n = 8$); sham-heated late sepsis group (18 h after CLP operation, $n = 8$); preheated early sepsis group (heat shock 24 h before and sacrifice 9 h after CLP operation, $n = 8$), and preheated late sepsis group (heat shock 24 h before and sacrifice 9 h after CLP operation, $n = 8$).

Induction of sepsis

Sepsis was induced by cecal ligation and puncture (CLP) as described previously (18). Animals were permitted nothing but oral intake of water for 6 h before to the operation. Under anesthesia, laparotomy was performed. The cecum was pulled out and ligated just below the ileocecal valve. The ligated cecum was punctured twice at different sites, and was gently compressed until feces were extruded. The bowel was then returned to the abdomen and the incision was closed in two layers. Control animals underwent a sham operation. All animals were given 5 mL/100 g body weight normal saline subcutaneously at the completion of surgery and also at 9 h postoperatively. Animals were deprived of food but had free access to water after surgery.

Heat shock treatment

Rats in the preheated sepsis group had whole-body heating with an electric pad after pentobarbital injection anesthesia (13, 19). When rectal temperature reached 41°C, it was maintained between 41°C and 42°C for 15 min. After the heating pad was removed, the temperature was kept at 37°C until complete consciousness was regained. Airways were free of obstruction throughout. Heated rats were put back in their cages to recover for 24 h. The rats of the sham-heated group were also anesthetized, but were not heated.

Isolation of cytosolic and nuclear fractions of liver

Animals were sacrificed and livers were removed and washed twice with ice-cold phosphate-buffered saline. All subsequent procedures were performed at 4°C. Samples were finely minced and homogenized in cold cytosolic buffer (100 mM Tris-HCl, 70 mM sucrose, 10 mM EDTA, and 210 mM mannitol, pH 7.4), centrifuged twice at 1500g, and then the combined pellet was collected for nucleus isolation. Supernatant was centrifuged again at 100,000g for 1 h and was collected as the cytosolic fraction.

Combined pellets were resuspended and homogenized with 10 volumes of nuclear buffer A (10 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.1 mM EDTA, 3.3 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 2.4 M sucrose) and filtered with four layers of cheesecloth. After centrifugation at 40,000g for 1 h at 4°C, the nuclear pellet was resuspended in nuclear buffer B (1 M sucrose and 1 mM CaCl₂) and was then rehomogenized twice and centrifuged again at 2600g for 20 min at 4°C. Nuclear pellet purity was assured by microscopic observation. To exclude cross-contamination between the nuclear and cytosolic fractions, specific antibodies of Lamin A (Santa Cruz Biotechnology, Santa Cruz, CA) and NF- κ B (p50/p105) (eBioscience, San Diego, CA) were used as individual markers for each fraction. The Lamin A protein served as nuclear contamination marker (20) and the p105, the precursor of NF- κ B (p50), served as a cytoplasmic contamination marker (21).

Assay of NF- κ B DNA-binding activity

NF κ B-binding activity was detected using the TransAM NF- κ B transcription factor assay kit (Active Motif, CA) an enzyme-linked immunoabsorbant assay-based format. This kit has been reported to be more sensitive than electrophoresis-mobility shift assay (22). We followed the manufacturer's instructions. The kit consists of a 96-well plate into which oligonucleotides containing the NF- κ B consensus site (5'-GGGACTTCC-3') are bound. Equal quantities of total nuclear protein (20 μ g) were assayed. The p65 antibody, recognized one subunit of NF- κ B, was used as primary antibody. A secondary horseradish peroxidase-conjugated antibody, providing a sensitive colorimetric readout easily quantified by spectrophotometry at 450 nm, was applied. NF- κ B-binding activity of testing samples, controls, and blanks were measured in duplicate and were averaged for statistical analysis.

Reverse transcription polymerase chain reaction (RT-PCR)

Frozen tissue was pulverized in liquid nitrogen with mortar and pestle. Total RNA was isolated in REzol C&T (PROtech Technologies, Taiwan, Republic of China), an RNA extraction buffer. Aliquots of 0.1 μ g of total RNA were used as template for RT-PCR by a one-step RT-PCR kit (Qiagen, Valencia, CA). The oligonucleotide primers were used for TNF- α (sense 5'-CACGCTCTTCTGTC-TACTGA and antisense 5'-GGACTCCGTGATGTCTAAGT), iNOS (sense 5'-AGCATCACCCCTGTGTTCCACCC and antisense 5'-TGGGGCAGTCTC-CATTGCCA) and I- κ B (sense 5'-AACTCCCTTACTGCCCCATTC and antisense 5'-CCTGGAAAATCTTACAGACTGC). Each reaction also included two oligonucleotide primers (5'-CTACAATGAGCTGCGTGTGG and antisense 5'-TAGCTCTTCTCCAGGGAGGA) to amplify β -actin as an internal control.

The predicted size of the PCR-amplified products was 541 base pairs (bp) for TNF- α , 264 bp for iNOS, 310 bp for I- κ B, and 420 bp for β -actin. Thirty-two cycles of PCR were executed for TNF- α and iNOS individually. Steps involved denaturation (at 95°C for 60 s), annealing (at 60°C for 60 s), and extension (at 72°C for 120 s). Thirty cycles of PCR were executed for I- κ B individually: denaturation (at 95°C for 60 s), annealing (at 63°C for 60 s), and extension (at 72°C for 120 s). RT-PCR products were visualized by electrophoresis on a 1.5% agarose gel containing trace amounts of ethidium bromide, and were quantified by a densitometer and analysis software (Bio-1D V.97 software; Vilber Lourmat, France).

In situ RT-PCR

The *in situ* RT-PCR method detected the mRNA location in tissues, with 100-fold more sensitivity than *in situ* hybridization (23). *In situ* RT-PCR followed previous procedures (24) with minor modifications. The removed liver was cut in pieces and fixed in fresh 4% paraformaldehyde at 4°C. Samples were embedded in OCT compound (Tissue-Tek; Sakura, Tokyo, Japan) and sectioned (6 μ m). Sections were treated with 0.3% Triton X-100 in diethyl pyrocarbonate-treated PBS, deproteinated with 1 μ g/mL proteinase K (Roche Diagnostics, Mannheim, Germany), and DNA was digested overnight (37°C) using RNase-free DNase (Roche Diagnostics). To prevent evaporation, the solution was covered with a plastic seal (Easi Seal; Hybaid, London, UK). After washing in diethyl pyrocarbonate-treated distilled water (1 min) and in 100% ethanol (1 min), RT-PCR was performed as described above. A positive control section was not treated with DNase. A negative control was the cells/tissue DNase-digested section, which had no RT-PCR-reactive enzyme. The absence of signal demonstrates that DNase digestion successfully rendered the native DNA template unavailable for DNA synthesis.

Digoxigenin (DIG) labeled-dUTP was added to the reaction mixture to label RT-PCR products. After RT-PCR, sections were washed in xylene (5 min) and in 100% ethanol (5 min). Products of DIG-labeled nucleotides were detected according to the DIG-detection packet illustration (Roche Diagnostics). After the blocking treatment, the sections were incubated with anti-DIG-AP antibody (antidigoxigenin Fab fragment alkaline phosphatase conjugates; 90 min at room temperature). Colorimetric detection was performed by color solution (0.33 mg/mL 4-nitroblue tetrazolium chloride and 0.16 mg/mL 5-bromo-4-chloro-3-indolyl- phosphate).

Coimmunoprecipitation (IP)

Immunoprecipitation was executed with p65, I- κ B, or HSP72 antibodies. First, the selected antibody was absorbed with protein A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in IP binding buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 μ g/ μ L leupeptin, 1 μ g/ μ L pepstatin, and 1 mM phenylmethylsulfonyl fluoride; 90 min at room temperature). The pellet was washed twice with IP binding buffer, centrifuged (10,000g for 1 min), and cytosol protein extract (1 mg) was added. It was incubated overnight with gentle rotation at 4°C. Afterward, it was washed three times with IP binding buffer and centrifuged at 10,000g for 1 min. Immunoprecipitated complexes were isolated from protein A Sepharose by adding 50 μ L of 0.1 M citric acid (pH 2), then neutralized with 10 μ L of 1 M Tris buffer (pH 9). Immunoprecipitated complexes were analyzed by Western blot analysis.

Western blot analysis

Equal amounts (10 μ g) of protein extract were loaded and separated by SDS-PAGE. After electrophoresis, the gel proteins were transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA). Blocked with 10% nonfat dry milk, the polyvinylidene difluoride membrane was incubated with the selected primary antibody, HSP72 (StressGen Biotechnologies, Victoria, British Columbia, Canada), NF- κ B (p65), or I- κ B (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as a second antibody. Blots were stripped and reincubated with a monoclonal antibody against β -actin (Chemicon, Temecula, CA) to confirm equal protein load. The target was then detected by enhanced chemiluminescence and exposed to x-ray film for the appropriate time.

Statistical analysis

Protein bands were quantified by densitometer and analysis software (Bio-1D V.97 software; Vilber Lourmat). All data are expressed as mean \pm SEM. The statistical analysis of the data was performed by using one-way analysis of variance followed by Newman-Keuls test, and a 95% confidence limit was accepted as statistically significant.

RESULTS

Heat shock treatment regulates the NF-κB activity during sepsis

As shown in Figure 1, the NF-κB-binding activity of liver was raised in early sepsis, and continued in late sepsis compared with the sham group ($P < 0.05$). Although the NF-κB-binding activity was also upregulated in preheated early and preheated late groups compared with the sham-operated group, they were less significant than those of the sham-heated early and late group ($P < 0.05$). This implies that heat shock treatment might

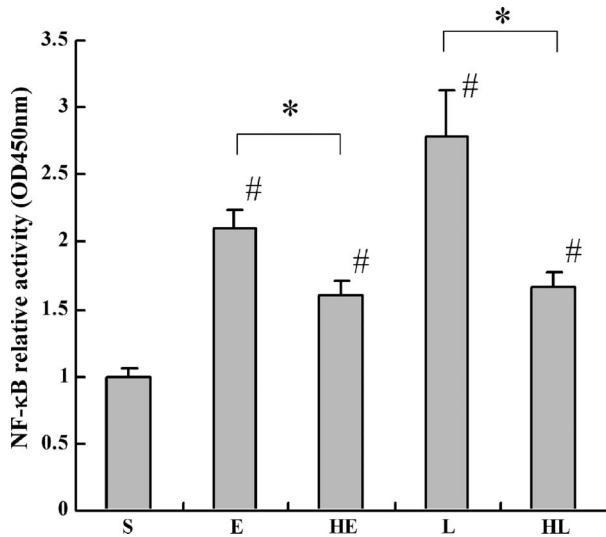


FIG. 1. Influences of heat shock treatment in NF-κB activity in septic liver. NF-κB activity detected by an enzyme-linked immunoabsorbant assay-based kit and spectrophotometer at 450 nm. Data are presented as means ± SEM; eight samples per group. S, Sham-operated control group; E, nonheated, early stage sepsis group; L, nonheated, late stage sepsis group; HE, preheated, early stage sepsis group; HL, preheated, late stage sepsis group. # $P < 0.05$ versus sham-operated control group; * $P < 0.05$ versus each other as indicated.

contribute to modulating the NF-κB activity and preventing the NF-κB activation from being highly induced by sepsis.

Expression of I-κB and NF-κB in the cytosol was detected by Western blot analysis, with simultaneous detection of β-actin as an internal standard. As shown in Figure 2, expression of NF-κB declined significantly during early and late sepsis compared with the sham-operated sepsis group ($P < 0.05$), and heat shock treatment prevented the decrease of NF-κB expression induced by sepsis in the cytoplasm ($P < 0.05$). Expression of I-κB, with or without heat shock treatment, was similar to NF-κB expression. Regarding the ratio of NF-κB and I-κB in the cytosol of liver, there was no significant difference with or without heat shock during sepsis (Fig. 2D). These results imply that heat shock treatment contributed to maintaining NF-κB and I-κB in the cytosol of liver during sepsis.

However, there was an increase in the amount of NF-κB in the nuclear fraction of liver during early and late sepsis compared with the sham-operated group ($P < 0.05$; Fig. 3). Heat shock treatment, compatible with the result of NF-κB-binding activity, prohibited NF-κB translocation. These data indicate that although NF-κB was activated during sepsis, it was sequestered in the cytoplasm after heat shock treatment. To exclude cross-contamination between the nuclear and cytosolic fractions, the Lamin A and the p105, the precursor of p50, were used as individual markers for each fraction. The Lamin A marker was undetected in the cytosol fraction, confirming that no contamination of the cytosolic fraction occurred in the nuclear fraction (Fig. 2). Likewise, p105 was detected only in the cytosolic fraction, confirming that no contamination occurred (Fig. 3).

Heat shock treatment influences the mRNA expression of TNF-α and iNOS in liver during sepsis

Fragments of 264 and 541 bp were amplified corresponding to iNOS and TNF-α mRNA, respectively, by 30 reaction cycles

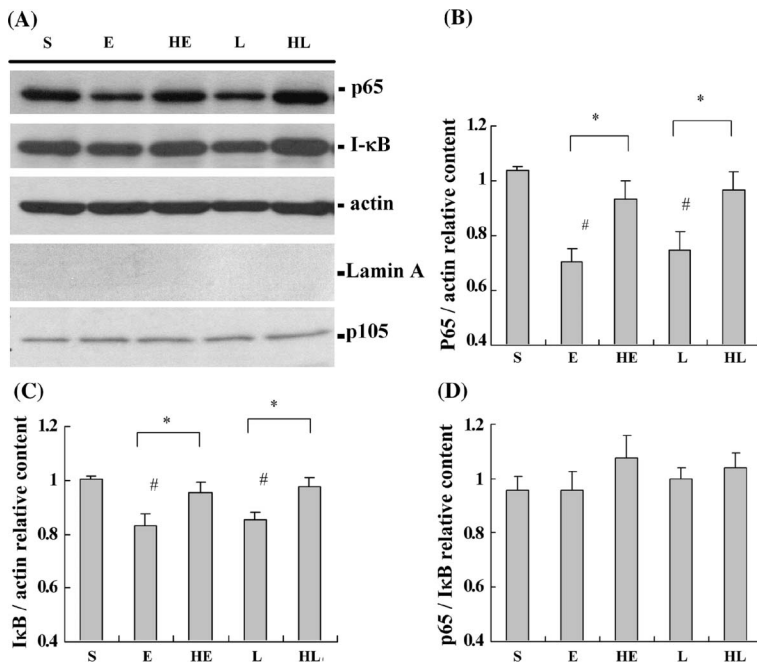


FIG. 2. Expression of NF-κB and I-κB in cytoplasm of septic liver and heat shock treatment effect. Expression of NF-κB (p65) and I-κB in cytoplasm was detected by Western blot and immunochemical analysis. Actin was detected simultaneously and acts as the internal standard. One representative expression of six is shown (A). The detection of Lamin A and the p105 were used as individual markers to exclude the cross-contamination between the nuclear and cytosolic fractions. The statistical analysis of relative content of NF-κB (p65) (OD ratio of p65/actin), I-κB (OD ratio of I-κB/actin), and ratio of NF-κB/I-κB are shown in B, C, and D, respectively. Data are presented as means ± SEM; six samples per group. S, Sham-operated control group; E, nonheated, early stage sepsis group; L, nonheated, late stage sepsis group; HE, preheated, early stage sepsis group; HL, preheated, late stage sepsis group. # $P < 0.05$ versus sham-operated control group; * $P < 0.05$ versus each other as indicated.

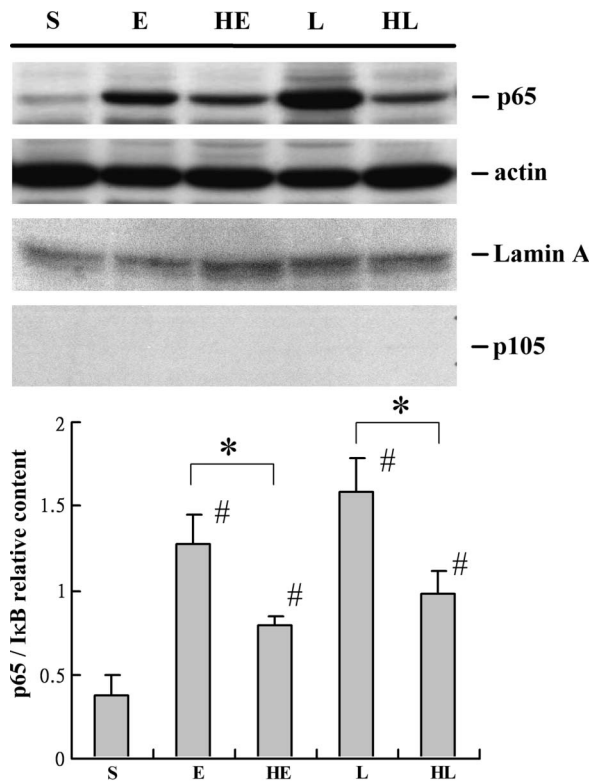


FIG. 3. Alteration of NF- κ B (p65) in nucleus of heat shock-influenced septic liver. Nuclear expression of NF- κ B (p65) detected by Western blot and immunochemical analysis (top panel). One representative expression of six is shown. The statistical analysis of relative content of NF- κ B (p65) (OD ratio of p65/actin) is shown in the lower panel. Lamin A and p105 were individual markers to verify lack of cross-contamination between nuclear and cytosolic fractions. Data are presented as means \pm SEM; six samples per group (bottom panel). S, sham-operated control group; E, nonheated, early stage sepsis group; L, nonheated, late stage sepsis group; HE, preheated, early stage sepsis group; HL, preheated, late stage sepsis group. # P < 0.05 versus sham-operated control group; * P < 0.05 versus each other as indicated.

of RT-PCR from total RNA. As shown in Figure 4A, the RT-PCR product of TNF- α mRNA increased in the early and late stages of sepsis compared with the sham-operated group (P < 0.05). Heat shock treatment prevented sepsis-induced TNF- α mRNA accumulation in liver. Early and late stage the mRNA expression of iNOS was greater than that in the sham-operated group (P < 0.05; Fig. 4B). This effect was also attenuated by heat shock treatment (P < 0.05).

During sepsis, the TNF- α mRNA was detected in liver specimens by an *in situ* RT-PCR method. The signal was more condensed in early and late sepsis than in the sham-operated group (Fig. 5A). Heat shock treatment contributed to preventing the accumulation of TNF- α mRNA in the hepatocyte during sepsis. Moreover, a similar result was found with iNOS (Fig. 5B). These results were compatible with those detected by RT-PCR analysis.

Interaction of HSP72 with cytosolic NF- κ B and I- κ B in the septic liver

Expression of HSP72 in the liver was detected by Western blot analysis. β -Actin was simultaneously detected as the internal standard. In the cascade of sepsis, as shown in Figure 6A, there was no detectable expression of HSP72 after CLP operation in

either stage of sepsis. However, the heat shock group showed significant expression of HSP72 synthesis in both stages, indicating the successful induction of HSR.

The association of HSP72 with NF- κ B and I- κ B was verified by coimmunoconjugated analysis (Fig. 6B). Three executor antibodies for HSP72, NF- κ B (p65), and I- κ B were used to interact with the cytosolic fraction. With NF- κ B (p65) and I- κ B antibodies, not only the interaction of NF- κ B (p65)/I- κ B or I- κ B/NF- κ B (p65) was detected by Western blot analysis, HSP72 was also detected in the immunoprecipitated complex. Whereas the antibody of HSP72 was used for immunoprecipitation, surprisingly, NF- κ B (p65) and I- κ B could be detected by the Western blot analysis. To exclude the nonspecific binding of HSP72, p65, and I- κ B with protein A Sepharose, the immunoprecipitation was performed with protein A Sepharose alone (Fig. 6C, lane L and L and HL without HSP72 antibody added). The results showed that the nonspecific binding between protein A and HSP72, p65, and I- κ B was negligible. The nonspecific binding of anti-HSP72 antibody with p65 and I- κ B during immunoprecipitation can be clarified by detecting the association of anti-HSP72 antibody with the lysate of the late sepsis group (Fig. 6C, lane L and L and HL with HSP72 antibody added). The results showed that the anti-HSP72 can associate with p65 and I- κ B only through the HSP72 interaction detected in the lysate of heat shock treatment. The nonspecific binding was negligible compared with the specific target binding.

In addition, the associated form of NF- κ B (p65)/I- κ B in the cytosolic fraction of the various groups was detected by coimmunoconjugated analysis with anti-p65 antibody. The results showed the ratios of NF- κ B (p65)/I- κ B without significant difference (Fig. 6D). The result was consistent with Figure 2D. It shows that it makes no change with/without heat shock treatment in sepsis about the NF- κ B retained in the cytoplasm by interaction with I- κ B, not alone in free form. It is clear that HSP72, induced by previous heat shock treatment, can interact with the NF- κ B/I- κ B complex in the liver and retain the NF- κ B and I- κ B in the cytosol concomitantly, rather than the free form of NF- κ B or I- κ B.

DISCUSSION

Sepsis has been defined as the systemic inflammatory response to infection (25). The pathogenesis is highly correlated with the overexpression of inflammatory cytokines (4–6). NF- κ B plays a central role in regulating a broad range of genes involved in the inflammatory response or sepsis, such as TNF- α and iNOS (8). The role of NF- κ B and its correlation in the cascade of sepsis has been researched enthusiastically in the past decade (8). However, most previous studies were executed *in vitro* in various cultured cell lines (26, 27). In the present study, we showed *in vivo* that HSR, inducing HSP72 oversynthesis, contributes in modulating the NF- κ B activity through attenuating the activation of NF- κ B DNA-binding ability in the liver of experimentally induced sepsis. Moreover, HSP72 is demonstrated for the first time to associate with NF- κ B/I- κ B complex *in vivo*. We suggest that HSP72 plays a crucial role in exerting protective effects through its molecular chaperone

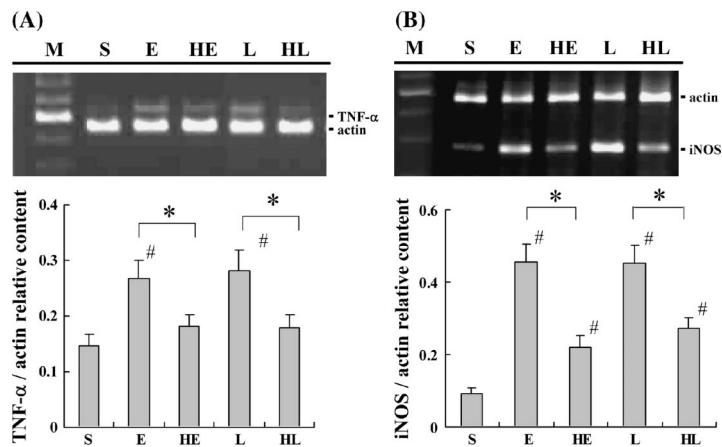


FIG. 4. Detection of the mRNA expressions of TNF- α and iNOS by RT-PCR. Expression of TNF- α mRNA and iNOS in A and B, respectively. Data are presented as means \pm SEM; eight samples per group. S, sham-operated control group; E, nonheated, early stage sepsis group; L, nonheated, late stage sepsis group; HE, preheated, early stage sepsis group; HL, preheated, late stage sepsis group. [#] $P < 0.05$ versus sham-operated control group; * $P < 0.05$ versus each other as indicated.

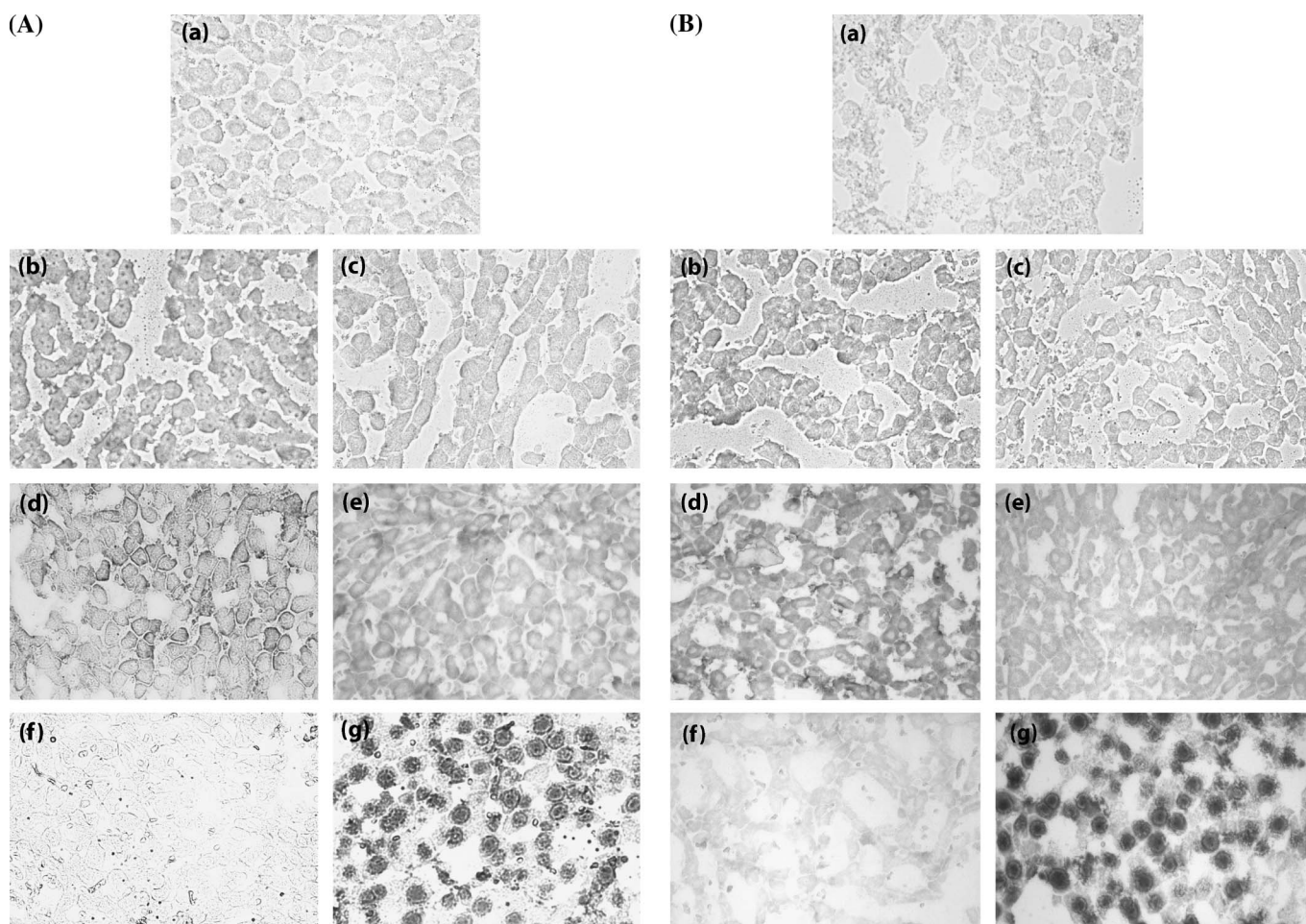


FIG. 5. Localization of TNF- α and iNOS mRNA expressions by *in situ* RT-PCR (original $\times 200$). Results of TNF- α and iNOS shown in A and B, respectively. (a) Sham-operated control group; (b) nonheated, early stage sepsis group; (c) preheated, early stage sepsis group; (d) nonheated, late stage sepsis group; (e) preheated, late stage sepsis group; (f) negative control; (g) positive control.

character to modulate the gene expression controlled by NF- κ B in sepsis.

NF- κ B is retained in the cytoplasm in an inactive form by assembling with I- κ B, which masks the nuclear translocation sequence of NF- κ B. After stimulation, I- κ B is phosphorylated, ubiquitinated, and degraded by proteasome. NF- κ B then escapes from the I- κ B squatting and translocates to the nucleus inducing transcription of target genes (3, 28). TNF- α and

iNOS are potential mediators leading to the hepatic failure in sepsis (5, 7). Our results found that in sepsis, the cytosolic amount of I- κ B decreased, and then the declination of cytosolic NF- κ B is detected simultaneously. Hence, the final ratio of I- κ B and NF- κ B in the cytoplasm is not altered in sepsis. Additionally, in the septic liver, the amount of nuclear NF- κ B is significantly increased and the DNA-binding activity of NF- κ B is obviously enhanced. It implies that in sepsis, after I- κ B

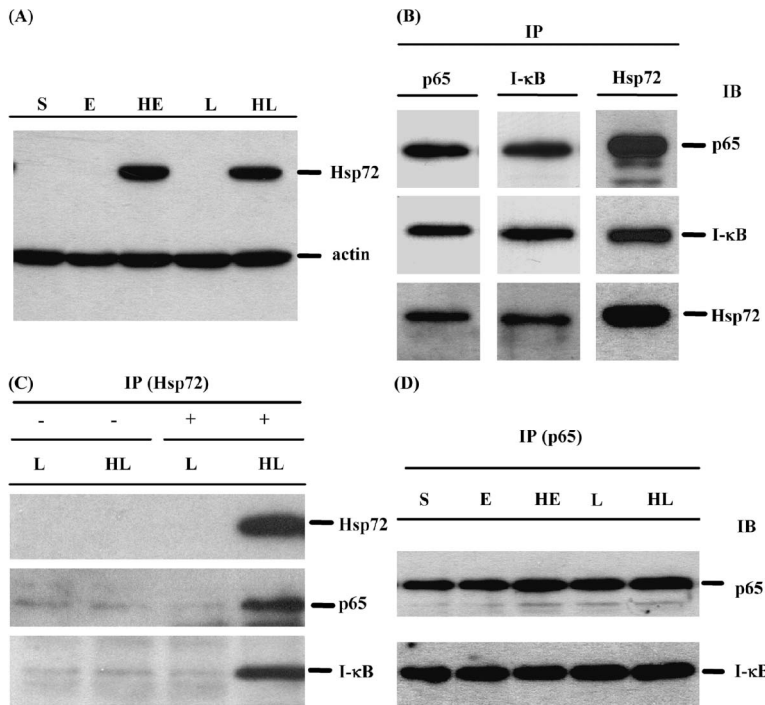


FIG. 6. Expression of HSP72 and its interaction with NF-κB and I-κB in the cytosol of liver. (A) Expression of HSP72 in various groups by Western blot and immunochemical analysis. Actin was also detected simultaneously as the internal standard. (B) Detection of HSP72, NF-κB, and I-κB interaction in preheated late sepsis specimen by IP with anti-HSP72, anti-NF-κB (p65), and anti-I-κB antibodies, followed by Western blot analysis. (C) Specificity of antigen-antibody reaction of HSP72 in immunoprecipitation assay. (D) Detection of the interaction between NF-κB and I-κB in various groups by IP. S, Sham-operated control group; E, nonheated, early stage sepsis group; L, nonheated, late stage sepsis group; HE, preheated, early stage sepsis group; HL, preheated, late stage sepsis group.

dissociated from NF-κB and degraded, the free form NF-κB translocates into nucleus soon, leading the cytosolic I-κB/NF-κB ratio was constant. Subsequently, RT-PCR analysis demonstrates that the mRNA levels of TNF-α and iNOS are increased significantly in the liver. Although previous studies show that macrophages are the major secretor of TNF-α and iNOS in the liver under cellular stresses (29), we additionally prove by *in situ* RT-PCR that hepatocytes, the major cell type of liver, can also induce the synthesis of TNF-α and iNOS during sepsis.

Heat shock induces various metabolic and biochemical alterations in the body, whereas the overexpression of HSPs is regarded as one of the major characteristics of the HSR (11). Heat shock proteins can be classified according to their molecular weight. The HSP72, one member of the HSP70 family, is the most inducible form in mammalian cells, and is typically selected as the marker of a successful HSR. Overexpression of HSP72 can persist more than 42 h after heat shock (30), which covers the whole experimental period of sepsis in this study. Here, HSP72 was only expressed in the preheated group and its presence is a sufficient indication of a HSR.

Reports have shown that HSR, inducing a HSP expression, can prevent cytokine-induced cellular damage, primarily through attenuation of cytokine synthesis (17). Increased HSP70 is also known to prevent LPS-treated cellular damage through the modulation of iNOS activation and the subsequent synthesis of NO (31). TNF-α and iNOS, the major proinflammatory mediators, are NF-κB-mediated target genes. In this study, we showed that heat shock treatment, inducing HSP72 synthesis, decreased the mRNA expression of TNF-α and iNOS. Simultaneously, translocation of NF-κB from cytosol to nucleus was inhibited, and the DNA-binding activity of NF-κB was also attenuated. The relationship between HSP72 and NF-κB no doubt contributes to decreasing sepsis-induced

expression of TNF-α and iNOS. Similar reports have documented that HSP72 overexpression, induced by chemical treatment or transferred by liposome, can also prevent NF-κB activation in endotoxin- or cytokine-stimulated cultured cells (2, 32). However, the mechanism is still obscure.

I-κB is regarded as a director in associating or dissociating with NF-κB. When released, NF-κB translocates to the nucleus. Maintenance of the NF-κB/I-κB complex may be key to the inertness of the NF-κB activation. Growing evidence demonstrates that activation of NF-κB is primarily affected by preservation of I-κB levels in the cytosol. Two major mechanisms are believed to regulate cytosolic I-κB levels. One is the prevention of I-κB degradation, the other is enhancement of I-κB synthesis (27, 33). Wong et al. (34) have shown that the HSR, inducing the heat shock protein overexpression, induces the I-κB expression through increasing the I-κB-α mRNA expression by activating its promoter, and then inhibiting the NF-κB activity. Moreover, heat shock is now known to inhibit NF-κB translocation by stabilizing I-κB without association of phosphorylation (26, 27). Upregulation of HSP, by heat shock or sodium arsenite, prevents I-κB phosphorylation by decreasing the activation of I-κB kinase, which subsequently inhibits NF-κB activation (26, 27). Significantly, in this study, we found that I-κB mRNA levels were the same in all groups (data not shown), whereas heat shock significantly preserved the down-regulation of cytosolic I-κB in sepsis. This implies that inhibition of I-κB degradation, rather than increased synthesis, may play a more important role in the protective mechanism of HSR in sepsis.

Activation of the NF-κB/I-κB complex can be regulated by many ways, including upstream I-κB kinase activity, interaction of NF-κB/I-κB, NF-κB translocation, and DNA-binding activity. I-κB phosphorylated through the NIK/IKK pathway is one of the earliest events in the common NF-κB activation

pathway, which consequently results in polyubiquitinylation. This modification then targets I- κ B for rapid degradation by the 26S proteasome. HSR, by heat shock treatment or after exposure to sodium arsenite, leads to inhibition of I- κ B phosphorylation via inhibition of IKK activity and prevents NF- κ B activation (35). Regardless of the upstream mechanism, dissociation of I- κ B determines the fate of NF- κ B. The present study shows that both detected directly in the cytosolic lysate and detected the p65 antibody coimmunoprecipitate the ratio of p65 to I- κ B was the same in each group, indicating a consistent translocation course of free form NF- κ B in the cytosol. We suggest that activated and free NF- κ B can easily translocate from the cytosol to the nucleus without marked influenced by heat shock treatment. Most interestingly, we found that p65 and I- κ B could be coprecipitated by using anti-HSP72 antibody. A similar phenomenon was obtained with anti-p65 and anti-I κ B antibodies. We suggest that oversynthesized HSP72, the most abundant HSR protein, can bracket together with NF- κ B/I- κ B complexes and subsequently attenuate the activation and translocation of NF- κ B. This process eventually decreases the expression NF- κ B-mediated mediators and minimizes hepatic damage induced by sepsis. Moreover, we suggest that the association between NF- κ B, I- κ B, and HSP70 might be reversible. The evidence comes from the fact that most thermotolerance or cross-tolerance is reversible, whereas HSPs are degraded several days after the heat shock (36). Limited to the current results, we cannot point out the precise binding site of HSP72 clearly, whereas two possible sites are considered. One is that HSP72 may bind with the NF- κ B/I- κ B complex nearly the phosphorylated and/or ubiquitinylation sites of I- κ B and prevent phosphorylation and ubiquitinylation of I- κ B by masking the phosphorylated and/or ubiquitinylation sites. The other one is that HSP72 may bind across the NF- κ B and I- κ B macromolecule to stabilize the interaction of NF- κ B and I- κ B, and prevent the I- κ B dissociation or degradation, even though I- κ B was phosphorylated. The related investigations need further study.

In conclusion, HSP72 can prevent dissociation and degradation of I- κ B, restraining cytosolic NF- κ B and attenuating the expression of NF- κ B-targeted genes in the septic liver. This appears to lead to an improved outcome for rats with sepsis exposed to heat shock treatment. These findings provide a novel clue in supporting the therapeutic potential of heat shock protein in treating patients with sepsis.

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