Heat-shock Pre-treatment Reduces Liver Injury and Aids Liver Recovery After Partial Hepatectomy in Mice

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Abstract. *Background: Heat-shock proteins (HSPs) are members of a chaperone protein family reported to modify stress responses. The aim of this study was to clarify the hypothesis of whether pre-treatment with heat shock reduces liver damage and influences liver regeneration after partial hepatectomy. Materials and Methods: Mice (N=6) were divided into two groups: the control group underwent partial hepatectomy without heat shock pre-treatment, the heat shock (HS) group underwent partial hepatectomy 12 hours after pre-treatment with heat shock. Mice were sacrificed at different time points after hepatectomy, remnant liver and blood were collected for further analyses in blood samples and liver tissues. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin-6 (IL6), and tumor necrosis factor-alpha (TNFα) were measured using enzyme-linked immunosorbent assay (ELISA). We used tissue samples for several experiments: staining by 5-bromo-2-deoxyuridine (BrdU), evaluation of cytokines, transcription factors and signal-transduction associated proteins. Results: HSP70 levels in the liver were clearly increased from 6 h to 72 h after heat shock treatment. Serum ALT and AST levels were significantly reduced in the HS group compared to the control group after partial hepatectomy. Liver regeneration rate and BrdU labeling index were significantly higher in the HS group than in the control group after partial hepatectomy. IL6 and TNFα in serum and liver tissues were significantly reduced in the HS group compared to the control group after hepatectomy. We did not detect phosphorylation of signal transducer and activator of transcription-3 (STAT3) protein by western blotting. Binding activities of transcription factors: nuclear factor-interleukin-6 (NF-IL6) and nuclear factor-kappa B (NF-kB) were significantly lower in the HS group than in the control group after hepatectomy. Conclusion: Pre-treatment with heat shock appears to reduce liver injury and promote liver regeneration, as HSP70 can reduce the inflammatory response and up-regulate liver regeneration without IL6 STAT signaling pathway in the liver after partial hepatectomy.*

In recent years, several strategies have been established to maximize hepatic liver regeneration, to maintain the capability and capacity of residual liver before liver resection against hepatocellular carcinoma and metastatic liver tumors. Heat shock proteins (HSPs) are members of a chaperone protein family reported to modify stress responses. Under heat stress conditions, synthesis of several proteins is reduced, whereas HSPs continue to exist (1).

Recently, Nagata *et al*. reported that HSPs were similarly induced under stress, for example in response to surgical stress (2). Functions of HSPs, found in the cytoplasm, include quality control of proteins. HSPs also regulate the heat-shock transcription factor (HSF) family, which is composed of four members in mammals. HSF1 is activated by accumulation of de-naturized proteins in the cytosol. Heat-shock elements (HSEs) exist at the promoter region of stress proteins and are induced by heat-shock stress. If an HSE exists in the promoter region, HSPs are removed from the cytoplasm to the cytosol by stress responses. HSFs form trimmers, activating their binding activity, and are transferred into the nucleus. After HSE binds with HSF trimers in the nucleus, the HSE activates target genes of HSPs (3). Recently, HSPs were found to protect against inflammatory response, tissue damage and severe stress responses. Heat-shock treatment induces HSP70 expression in several organs, which was found to reduce mortality after lipopolysaccharide (LPS) challenge in mice (4). Similarly, heat-stress pre-treatment mitigated arachidonic acid accumulation after ischemia and/or reperfusion in rats (5). Under the experimental acute respiratory distress syndrome in peritonitis models, HSP70 are transfected into pulmonary epithelium using an adenoviral method was found to improve the respiratory condition in rats (6).

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Key Words: Heat-shock protein, heat-shock pre-treatment, liver regeneration, mouse model, hepatectomy.

In the present study, we investigated whether HSPs reduce liver damage and can be useful in liver regeneration after hepatectomy.

Materials and Methods

Animals. We used male C57BL/6 mice aged from 6 to 8 weeks. Mice were maintained in a 12-hour light-dark cycle under standard conditions and food and water were provided.

Heat shock pre-treatment. An empty mouse cage and a tray filled with water were placed in an incubator for at least two hours at 42˚C. These conditions should provide a relative humidity of 75% during the experiment. Mice were placed in the cage for 20 minutes at 42˚C, after that they were transferred to a clean cage at room temperature (19). Mice were sacrificed at different times (1, 3, 6, 12, 24, 48, 72 and 96 hours).

Experimental groups. For Heat-Shock pre-treatment, mice (C57BL/6) were placed in an incubator for 20 minutes at 42˚C. Mice were divided into two groups: the control group underwent partial hepatectomy without pre-treatment, and the heat-shock (HS) group underwent same operation 12 hours after with HS. Mice were sacrificed at different times after surgery. We used the remnant liver and blood from mice for feather analyses (15).

Partial hepatectomy model. Anesthesia was performed with intraperitonial injection of 50 mg/kg thiopental. Partial hepatectomy was performed as previously described (8). Briefly, 4-0 nylon suture ligatures were secured around the base of the median and left lateral hepatic lobes and the lobes were performed, and that were measured and weighed. It was decided that hepatectomy would be 70% of the liver weight, and the remnant liver weight was these estimated as 30% of the liver weight. At different time points, we removed the remnant liver and measured the remnant liver weight. The rate of liver regeneration was calculated as a ratio of the measured remnant liver weight to the initial estimated remnant liver weight.

Measurement of hepatocyte DNA synthesis. Animals were injected intraperitoneally with 50 mg/kg BrdU (AAT Bioquest, Inc, CA, USA) two hours before being sacrificed. Animals were then sacrificed and liver specimens were obtained at 36 hours after hepatectomy (8). Liver tissues were fixed in 70% ethanol for 24 hours, them used for histological analysis.

Three mice were used from each treatment group. Histology was assessed using five separate low-power fields per sample per animal. The number of cells staining positively for BrdU per low-power field were counted and data were expressed as the mean±SEM for each group.

Biochemical assay. Blood samples were collected *via* cardiac puncture. Serum aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activity were determined by hydroxyphenylpyruvate oxidase-toluidine (POP-TOOS) colorimetric method (Wako, Osaka, Japan). Samples from six different mice per group were evaluated at each time point.

Enzyme-linked immuno sorbent assay (ELISA). We measured the levels of interleukin-6 (IL6) and tumor necrosis factor α (TNFα) in the serum samples and the homogenated liver tissues with 100 mM HEPES buffer using an ELISA Kit (Kit No. #KMC3011 and #KMC0061 BioSource international, CA, USA). Samples were evaluated from six different mice per group at each time point. Each assay was performed in triplicate with murine recombinant cytokine as standard.

Extraction of protein. In brief, tissue samples were homogenized in four volumes of HEPES buffer (10 mM HEPES, (pH 7.9), 0.25 M sucrose, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.16 mM spermidine), with 1 mM dichlorodi-phenyltrichloroethane (DTT), protease inhibitor [0.5 mM 4 2-Aminoethyl benzene sulfonic acid (AEBSF), 0.4 μM aprotinin, 1 μM leupeptin] (Sigma-Aldrich, Inc, MI, USA) and phosphatase inhibitor (10 mM NaF, 1 mM $NaVO_4$, 1.5 mM Na_2MoO_4)(Sigma-Aldrich, Inc.) using a Tissumizer (Tekmar, Cincinnati, OH, USA). Lysates were centrifuged at 13,000 ×*g* for 60 minutes and supernatants were stored at –80˚C until used. These samples were used for ELISA and western blotting.

Western blot analysis. After measuring the protein concentration, protein samples were denatured by boiling in a sample buffer containing 1% sodium dodecyl sulfate (SDS) and loading buffer and were separated in 5-20% ReadyGel (Bio-Rad, Laboratories, Inc, CA, USA), allowing a 32.6-kDa band of a marker (Bio-Rad) to reach the bottom of the gel. Membranes were blocked in a Blotto solution containing 1X TBS [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl], 5% milk, and 0.1% Tween-20 for 1 hour at room temperature. Membranes were then incubated with primary antibodies [diluted 1: 2000 (HSP70), 1:500 (STAT3)] in Blotto for 12 hours at 4˚C. Mouse polyclonal antibodies against HSP70 (SPA-810, Stressgen, Inc, BC, Canada) and phosphorylated form of STAT3 (sc-8059, Santa Cruz, TE, USA) were used for blotting. After extensive washings in 1X TBS with 0.1 Tween-20, proteins were detected with the ECL Plus Western Blotting Detection System (Amersham Biosciences, VA, England). All data was analyzed by NIH Image 1.60 (Microsoft, WA, USA).

Preparation of the nuclear extracts. A modified nuclear extraction was performed as previously described (25). Tissue samples were homogenized four volumes of HEPES buffer (as above). The supernatants were removed, and the pellets were washed twice in four volumes of HEPES buffer without 0.25 M sucrose and centrifuged at 4,000 ×*g* for 10 minutes. Pellets were resuspended in four volumes of buffer [25% glycerol, 20 mM HEPES (pH7.9), 1.5 mM MgCl 2, 0.42 M NaCl, 1 mM EDTA], and were kept on ice for 30 minutes. Nuclear extracts were obtained by centrifugation at 14,000 ×*g* for 20 minutes, and aliquots of the supernatant were stored at –80˚Cuntil used. These samples were used for colorimetric transcription factor measurement.

Colorimetric transcription factor measurement. We measured binding activities of (NF-IL6 and NF-κB) nuclear protein from liver using Trans AM kit (kit no. #ACV44198 and #ACV0061 Active Motif, CA, USA). The kits contain a 96-well plate on which has been immobilized a specific oligonucleotide that contains NF-IL6 or NF-κB consensus binding sites respectively. Nuclear proteins (10 μg per well) were added and plates were incubated for one to two hours, specifically binding to these oligonucleotides. The primary antibodies used the accessible epitopes on NF-IL6 or NF-κB protein upon DNA binding. Addition of secondary horseradish peroxidase (HRP) conjugated antibodies provided sensitive colorimetric readout quantified by spectrophotometry.

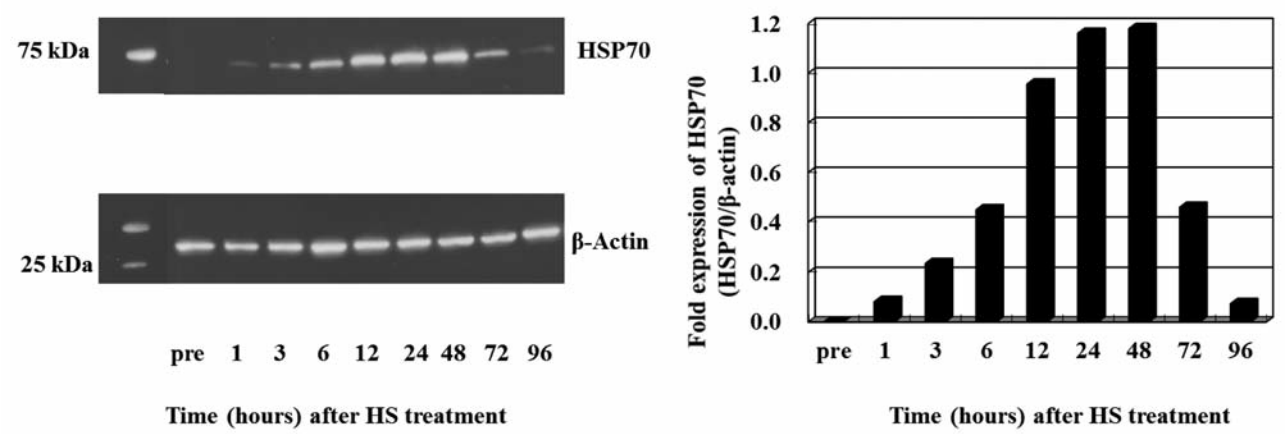

Figure 1. *Western blot analysis of HSP70 expressed in the liver. HSP70 levels in the liver were clearly increased from 6 hours to 72 hours after HS treatment. The expression was maximal between 12 and 48 hours following HS.*

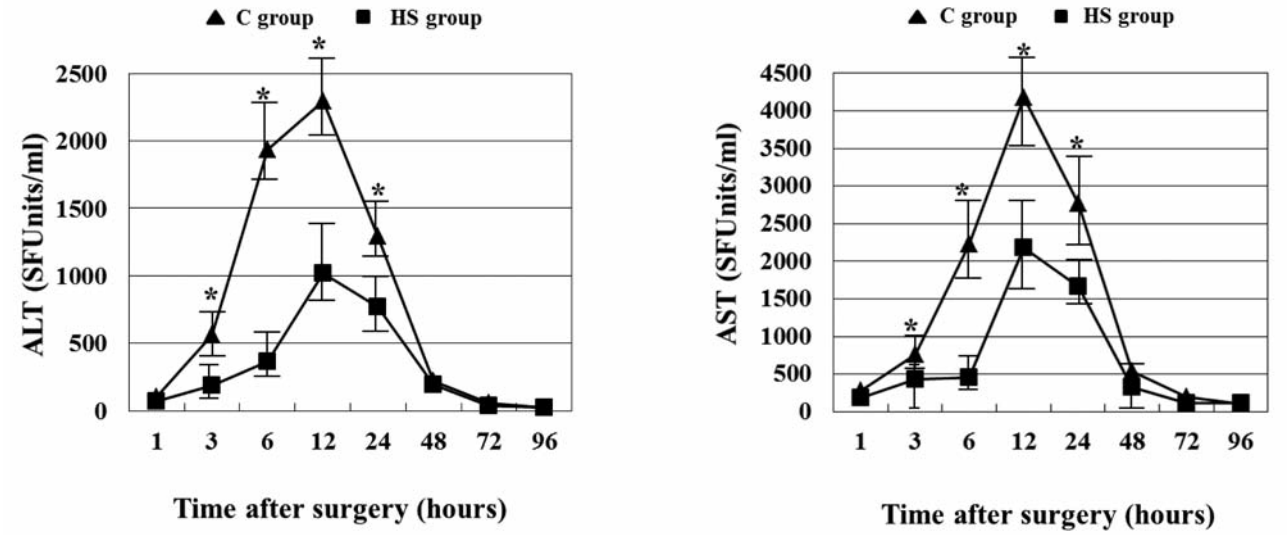

Figure 2. *Effect of HS pre-treatment on serum AST and ALT activity after partial hepatectomy in the HS or control (C) group. Serum ALT and AST levels were significantly reduced in the HS Group compared to the control group at 3, 6, 12 and 24 hours after partial hepatectomy. *p<0.05.*

Statistical analysis. All data were analyzed by ANOVA followed by Fisher's protected least significant difference (PLSD) method with the Stat View program (Macintosh, USA). Differences between the means were considered significant at a *p*-value of less than 0.05.

Results

Induction of HSP70. We confirmed that the heat stress conditions used led to a strong induction of HSP70 in this model. Mice were treated with heat-shock for 20 minutes, 1, 3, 6, 12, 24, 48, 72 and 96 hours thereafter, mice were killed. Liver was removed and homogenized in HEPES buffer. The homogenates were analyzed by western blot for HSP70 presence detection (Figure 1). HSP70 levels in the liver were clearly increased from 6 hours to 72 hours after heat stress treatment. The expression was maximal between 12 and 48 hours following heat stress. In control mice, no detectable HSP70 was present.

Influence of heat stress on Liver damage after partial hepatectomy. We examined the effect on the function of the remnant liver in the HS group and control group, we measured serum biochemical markers after hepatectomy. Figure 2 shows that the time course of hepatic transaminases levels of AST and ALT were higher after surgery, reaching maximum values 12 hours after the operation. The levels then declined at 24 hours, and returned to near baseline levels 72 hours after hepatectomy.

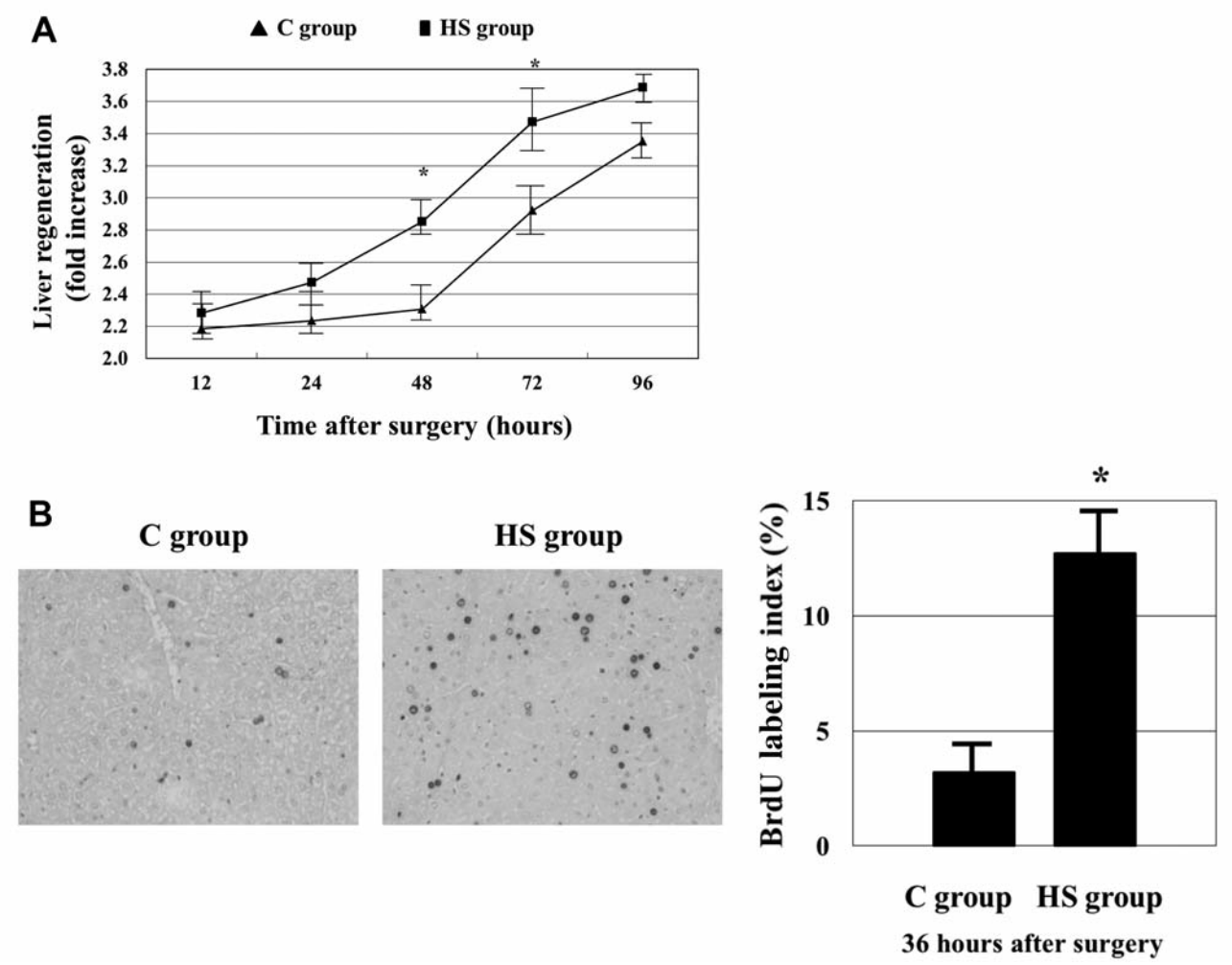

Figure 3. *A: Comparison of the rate of liver regeneration after partial hepatectomy in the HS group and the control group. Rates of the liver regeneration were significantly higher in the HS group than in the control group at 48 and 72 hours after partial hepatectomy. *p<0.05. B: BrdU labeling index was determined using liver after partial hepatectomy. BrdU labeling index was significantly higher in the HS group than in the control group at 36 hours after hepatectomy. *p<0.05.*

Serum ALT and AST levels were significantly reduced in the HS group compared to the control group at 3, 6, 12 and 24 hours after partial hepatectomy (Figure 2).

Effect of HS treatment on liver regeneration. We investigated and compared the influence of the preconditioning heat stress on liver regeneration in a murine hepatectomy model. Rates of liver regeneration were significantly higher in the HS group than in the control group at 48 and 72 hours after partial hepatectomy (Figure 3A). The BrdU labeling index was also significantly higher in the HS group than in the control group at 36 hours after hepatectomy. The HS group had early induction of DNA synthesis and liver regeneration (Figure 3B). Because these data suggest that heat shock treatment both modified liver damage and promoted liver regeneration after hepatectomy, we decided to measure levels of inflammatory cytokines after hepatectomy.

Induction of inflammatory cytokines after partial hepatectomy. We measured IL6 and TNFα levels in both the serum and the remnant liver in mice at each time point. Serum IL6 levels were significantly reduced in the HS group compared to the control group at 3, 6 and 12 hours after hepatectomy. IL6 levels in the liver were also significantly reduced in the HS group compared to the control group at three hours after hepatectomy (Figure 4A). Serum TNFα levels were also significantly reduced in the HS group compared to the control group at 6, 12 and 24 hours after hepatectomy. TNFα levels in the liver were also significantly reduced in the HS group compared to the

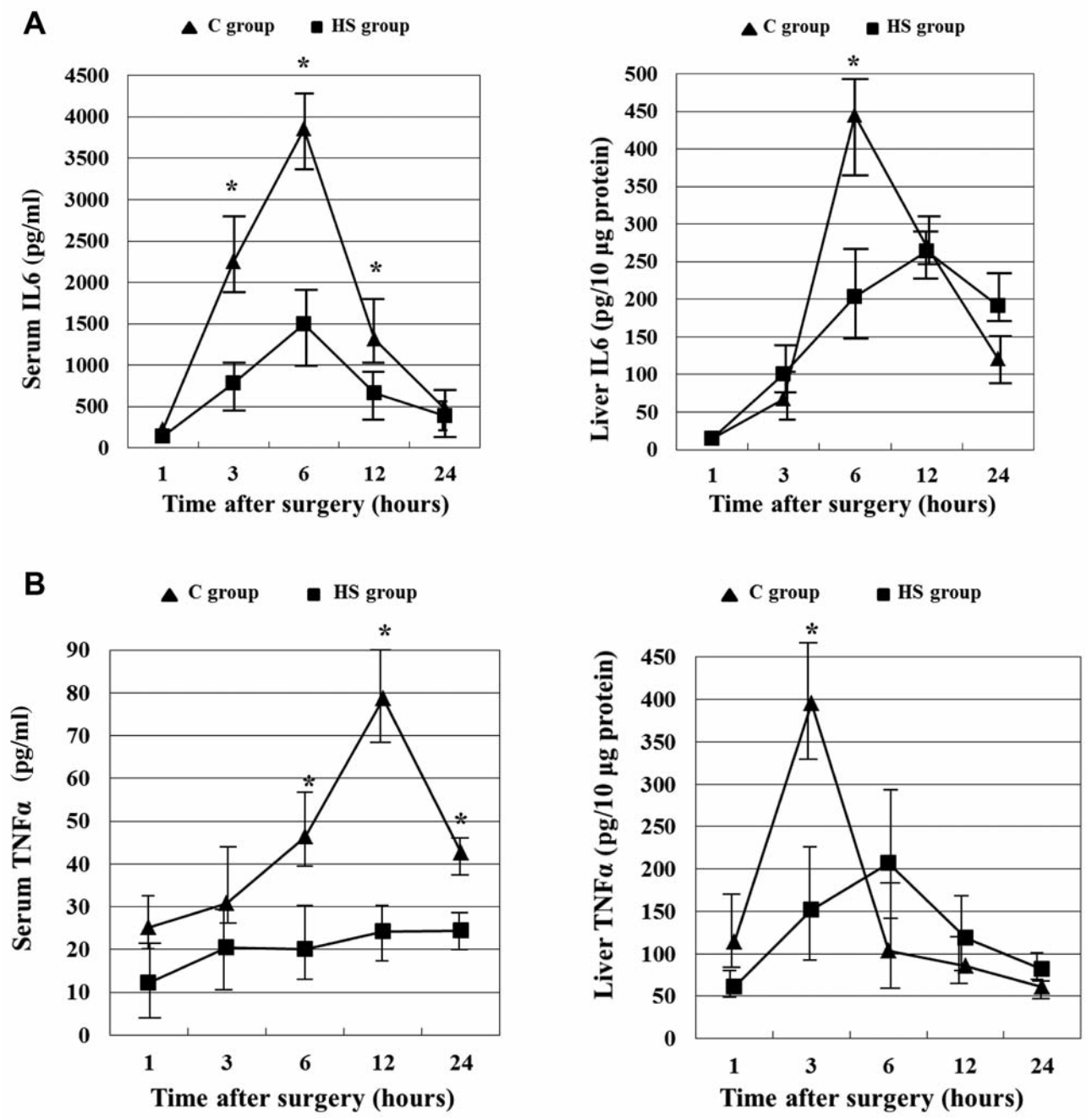

Figure 4. *Effect of HS preconditioning IL6 levels in serum and liver tissue after partial hepatectomy using ELISA. A: Serum IL6 levels were significantly reduced in the HS group compared to the control group at 3, 6 and 12 hours after hepatectomy. IL6 levels in the liver were also significantly reduced in the HS group compared to the control group at 3 hours after hepatectomy. *p<0.05. B: Effect of HS preconditioning on TNFα levels in serum and the liver after partial hepatectomy using ELISA. Serum TNFα levels were significantly reduced in the HS group compared to the control group after hepatectomy. The TNFα levels in the liver were also significantly reduced in the HS group compared to the control group after hepatectomy. *p<0.05.*

control group at three hours after hepatectomy (Figure 4B). From these results, we hypothesized that protection of liver tissue was caused by a reduction of inflammatory cytokine response by pre-treatment with heat shock.

Induction of liver regenerative factors by treatment with heat shock. There are some reports that heat shock treatment could induce IL6 expression (11,12). We made our hypothesis that heat shock induces IL6 expression, which then induces

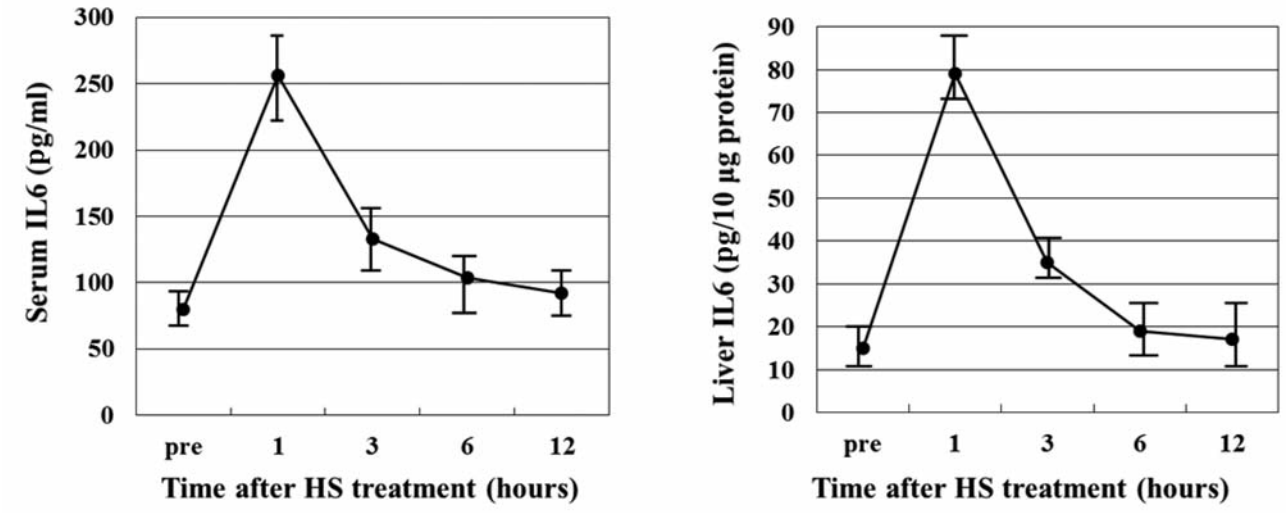

Figure 5. *IL6 levels in the serum and the liver were increased 1 hour after HS treatment, but decreased to baseline levels 12 hours after HS treatment.*

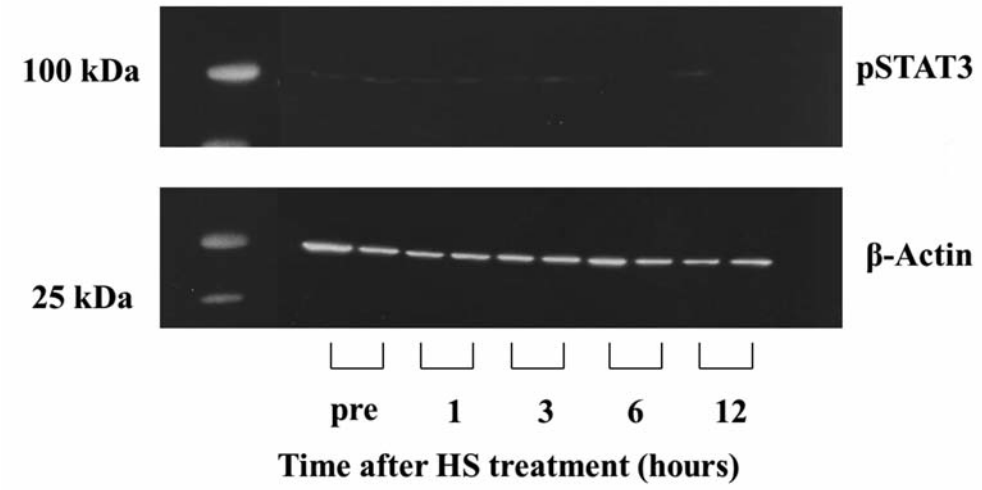

Figure 6. *Western blot analysis of phosphorylated STAT3 expressed in the liver. It can be seen that there is no phosphorylated STAT3 after HS treatment.*

phosphorylation of the STAT3 protein. IL6 levels in the serum and the liver were increased one hour after heat shock treatment, but decreased to baseline at 12 hours after treatment (Figure 5). We also evaluated phosphorylated STAT3 protein status using western blotting. It can be seen that there is no phosphorylated STAT3 after heat shock treatment (Figure 6). These data suggest that heat shock treatment induces IL6 expression, but did not activate the IL6/STAT3 signaling pathway in our study.

Modulation of transcription factor binding activity in liver by heat shock treatment. IL6 and TNFα levels were significantly reduced in the HS group compared to the control group. To investigate the mechanism of inhibition of cytokine expression by heat shock, we analyzed the activation of NF-IL6 and NF-κB. NF-IL6 and NF-κB binding activity were significantly lower in the HS group than in the control group at three hours after hepatectomy (Figure 7).

Discussion

Preconditioning by heat shock was found here to reduce liver injury and promote liver regeneration without induction of the IL6 STAT signaling pathway in the liver after partial hepatectomy.

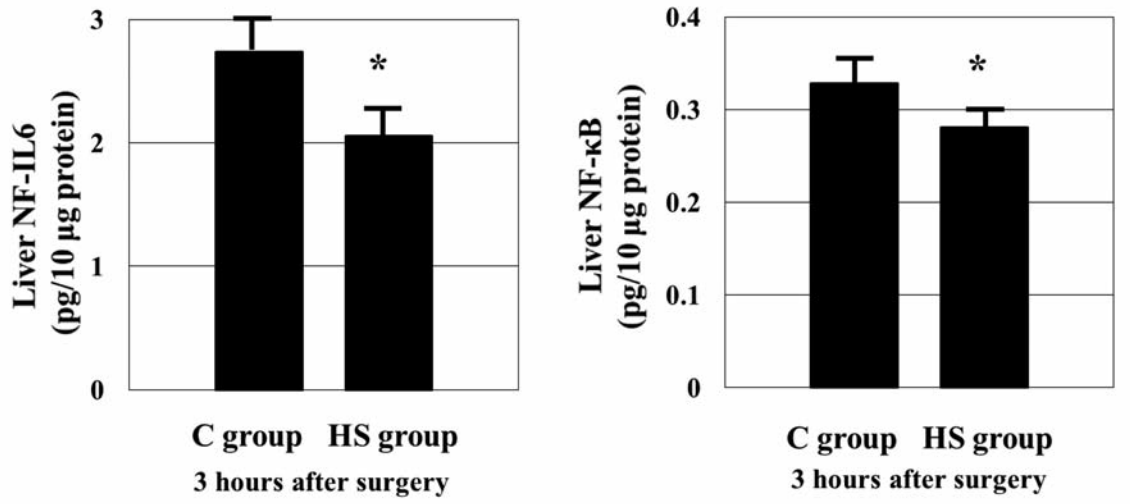

Figure 7. *Comparison of transcription factor binding activity in the liver after HS treatment. NF-IL6 and NF-κB binding activities were significantly lower in the HS group than in the control group at 3 hours after partial hepatectomy. *p<0.05.*

Molle *et al*. reported that 12 hours after heat shock, mice had reduced mortality in an LPS challenge model after whole-body heat shock, compared to the control group (7). Our study shows that HSP70 expression was induced strongly from 12 hours after whole-body preconditioning with heat shock. Our study also showed that serum ALT and AST levels were significantly reduced in the HS group compared to the control group, and that heat shock reduced liver damage after partial hepatectomy. Under the same conditions, we found that IL6 and TNFα expressions after hepatectomy were suppressed under heat shock, therefore, heat shock pre-treatment reduced inflammatory cytokine expression. This is one of the reasons why the HS group exhibited low levels of liver damage.

Some studies report that DNA synthesis is down-regulated after hepatectomy in IL6-knockout mice (9) or in tumor necrosis factor receptor-1 (*TNFR-1*)-knockout mice (10). Yamada *et al*. also reported that DNA synthesis had accelerated after hepatectomy in a mouse model where mice which were injected with recombinant IL6, whereas expression of tyrosine receptor family [hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ) and vascular growth factor (VGF)] were down-regulated or did not change under the same condition (10). Therefore, IL6 and TNFα were recognized to be important factors in liver regeneration.

In the HS group, DNA synthesis and liver regeneration were up-regulated early after hepatectomy compared to the control group. Some studies report that heat shock induced IL6 expression (11, 12) and IL6/STAT3, which is an important pathway, induced early liver regeneration in the HS group (16).

We hypothesized that heat stress induces IL6 expression, which induces phosphorylation of the STAT3 protein. Unfortunately, our results did not confirm this hypothesis because we detected only IL6 and not phosphorylated STAT3 protein in serum and liver tissues after heat shock. From these results, we suggest that the IL6/STAT3 pathway does not mediate liver regeneration in this model.

Peters *et al*. reported a strong IL6-dependent activation of STAT3 before hepatectomy, using a hyperstimulation IL6 model. Specifically, human soluble IL6 receptor/gp80 was overexpressed in hepatocytes, with delay and inhibition of cell-cycle progression which correlated with cyclin A and E expression after hepatectomy (16). We suggest that IL6 and TNFα have several functions, but both cytokines are needed in optimal concentrations to exercise their capabilities for liver regeneration in this model.

We also considered the binding activity of NF-IL6 and NF-κB, which are transcription factors of IL6 and TNFα, under heat shock pre-treatment. Figure 7 shows that binding activities of NF-IL6 and NF-κB of the liver were significantly lower in the HS group than the control group at three hours after hepatectomy. Heat shock preconditioning protected liver against ischemia/reperfusion injury by suppressing the activation of NF-κB and the subsequent expression of proinflamtory mediators through the stabilization of IκB proteins (19). Our results showed that activation of NF-IL6 and NF-κB were inhibited by heat shock preconditioning. Consensus sequence of HSE overlaps both NF-IL6 and NF-κB, and could inhibit binding of these transcription factors. This might be one reason why heat shock preconditioning could reduce induction of both cytokines.

In this study, we only detected HSP70 induction. Heat shock preconditioning can induce HSP90, HSP27 and αB-crystallin protein too. Under normal conditions, expression of the HSP90 protein is high in cytosol. HSP90 functions not only in quality control of proteins but also in various modulations of cell functions, and causes optimal activation of mitogen-activated protein kinase (MAPK) family proteins (20-22). HSP27 and αB-crystallin are so-called small HSPs. HSP27 in transgenic cells blocked Fas-induced apoptosis, and phosphorylated αB-crystallin, through activation of p-38MAP kinase, and had important roles in tolerance of myocardium ischemia (23, 24). We suggest these chaperone proteins may also be induced by heat shock preconditioning, and may participate in reducing liver damage and up-regulating liver regeneration after hepatectomy.

Our findings show that whole-body heat shock treatment induced expression of HSP70 in the liver and reduced expression of inflammatory cytokines in serum and liver tissues. We believe this is one reason why the HS group had low levels of liver damage. We conclude that heat shock preconditioning may be a useful tool for hepatectomy, because it reduces inflammatory response and promotes liver regeneration.

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