Heat Shock Pretreatment 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 chaperone protein family which had been reported to modify stress responses. The aim of this study is to clarify the hypothesis whether heat shock pretreatment could reduce liver damage and could influence liver regeneration after partial hepatectomy.

Materials and Methods- Mice were divided into two groups, Control (C) group were received partial hepatectomy without Heat Shock (HS) pretreatment and Heat Shock (HS) group were received partial hepatectomy with 12hours after HS pretreatment. Mice were sacrificed at different time point after hepatectomy, remnant liver and blood were collected for farther experiments. Blood samples and liver tissues were measured AST, ALT, IL-6, and TNF-α. using ELISA. We used tissue samples for several experiments; staining BrdU, evaluated for cytokine, measured transcription factors and signal transduction associated proteins.

Results- HSP70 levels in the liver were clearly increased from 6hrs to 72hrs after HS treatment. Serum ALT and AST levels were significantly reduced in HS Group compared to C Group after partial hepatectomy. Rates of the liver regeneration and BrdU labeling index were significantly higher in HS Group than in C Group after partial hepatectomy. IL-6 and TNF-α in serum and liver tissues were significantly reduced in HS Group compared to C Group after hepatectomy. We could not detect

phosphorylated STAT3 protein using Western blotting. We analyzed activation of transcription factors (NF-IL6 and NF-kB) which binding activities were significantly lower in HS group than in C group after hepatectomy.

Conclusion- HS pretreatment could reduce liver injury and promote liver regeneration because HSP 70 could reduce inflammatory response and up-regulated liver regeneration without IL-6 / STAT signaling pathway in the liver after partial hepatectomy.

Key words

Heat shock protein, Heat shock pretreatment, Liver regeneration

In recent years, several strategies have been established about residual hepatic liver regeneration, to maintain the capability and capacity of residual liver treatment before liver resection against hepatocellular carcinoma and metastatic liver tumors. Heat shock proteins (HSPs) are members of chaperone protein family which had been reported to modify stress responses. Under the heat stress condition, syntheses of several proteins are reduced, whereas HSPs were synthesized in same condition (1). Recently, Nagata et al. reported that HSPs had been induced under the several stress responses, for example surgical stress condition as same as heat stress shock condition (2). Functions of HSPs, which are existence in cytoplasm, are quality control of proteins. HSPs also regulate to induce of heat shock transcription factor (HSF) which family is composed in four members in mammal. HSF1 is activated by accumulation of denaturized proteins in cytosol. Heat shock element (HSE), site arrange, exists on the promoter area of stress proteins which are induced by heat shock stress . If HSE exists on the promoter area, HSPs are removed from cytoplasm to cytosol by stress responses. HSF form trimers formation, which is getting binding activity, are transferred into the nucleus. After HSE is binding with trimers HSF in nucleus, HES activates target gene for HSPs proteins (3). Recently, HSPs can be protecting against inflammatory response, tissue damage and severe stress responses. Heat shock treatment induces HSP70 product in several organs which reduced

mortality after LPS challenge in mice (4). Similarly, heat stress pretreatment mitigates arachidonic acid accumulation after ischemia and/or reperfusion in rat (5). Under the experimental acute respiratory distress syndrome is caused by peritonitis models, HSP70 which are transfected into pulmonary epithelium using adenoviral method can improve the respiratory condition in rat (6). In the present study, we investigated whether HSPs reduce the liver damage and can be useful liver regeneration after hepatectomy.

Materials and Methods

Animals

We used male C57BL/6 mice at the age from 6 to 8 weeks. Mice were maintained in a 12-hours light-dark cycle under standard condition and provided food and water.

Heat Shock (HS) pretreatment

An empty mouse cage and a tray filled with water were placed in an incubator for at least 2 hours at 42 ℃. These conditions should provide a relative humidity of 75% during the experiment. Mice were placed in the cage for 20 min at 42 ℃, 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, 96 hours).

Experimental groups

Heat Shock pretreatment was that mice (C57BL/6) were placed on an incubator for 20 min at 42 ℃. Mice were divided into two groups; control (C) group were received partial hepatectomy without pretreatment, and Heat Shock (HS) group were received same operation with HS pretreatment before 12 hours. We used the remnant liver and blood from mice for feather experiment (15).

Partial hepatectomy model

Anesthesia was performed with 50mg/kg thiopental intraperitonial injection. Partial hepatectomy was performed as previously described (8). Briefly, 4-0Nylon suture ligatures were secured around the base of the median and left lateral hepatic lobes and the lobes were performed, and that were measured weight. We decided that this hepatectomy was 70% of the liver weight, and remnant liver weight was estimated 30% as liver weight. After several time points, we removed remnant liver and measured remnant liver weight. Rate of liver regeneration was calculated as a ratio of remnant liver weight to estimated remnant liver weight.

Measurement of hepatocyte DNA synthesis

Animals were injected intraperitoneally with 50mg/kg BrdU (Wako) two hours before 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 used for histological analysis etc. Every treatment group was three mice.

Histological were assessed by five separate low-power fields per animal. The number of cells staining positively for BrdU per low-power field were counted and expressed as mean ±SEM for each group.

Biochemical assay

Blood samples were collected from the cardiac puncture. Serum aspartate aminotransferase (AST) activity and alanin aminotransferase (ALT) activity were determined by POP・TOOS colorimetric method (WAKO). Samples from 6 different mice per group were evaluated at each time point.

Enzyme Linked Immuno Sorbent Assay (ELISA)

We measured the levels of Interleukin-6 (IL-6) and Tumor necrosis factor α (TNFα) in the serum samples and the homogenated liver tissues with 100mM HEPES buffer using ELISA Kit (BioSource international, Inc. Kit No. #KMC3011 and #KMC0061). Samples were evaluated from 6 different mice per group at each time point. Each assay was performed in triplicate with murinerecombinant cytokine as standard.

Extraction of protein

In brief, tissue samples were homogenized in 4 vol of Hepes buffer (10mM Hepes, pH 7.9, 0.25M sucrose, 15mM NaCl, 5mM EDTA, 1mM EGTA, 0.15mM spermine, 0.16mM spermidine), with 1mM DTT, protease inhibitor(0.5mM AEBSF, 0.4μM Aprotinin, 1μM Leupeptin) (Sigma-Aldrich, Inc. Product No. P

8340) and phosphatase inhibitor (10mM NaF, 1mM NaVO4, 1.5mM Na2MoO4) (Sigma-Aldrich, Inc. Product No. P 5726) using a Tissumizer (Tekmar, Cincinnati, OH). Lysates were centrifuged at 13,000 ×g for 60min and supernatants were stored in -80℃ until used. These samples were used for ELISA and Western blotting.

Western blot analysis

After measuring the protein concentration, protein samples were denatured in a boiling bath in a sample buffer containing 1%SDS and loading buffer and were separated in 5-20% Ready Gel (BIORAD), allowing a 32.6-kDa band of a marker (BIORAD) to reach the bottom of the gel. After electrophoresis, gels were transferred onto a PVDM. Membranes were blocked in a Blotto solution containing 1x TBS (10mM Tris-HCl (pH 8.0) and 150mM NaCl), 5% milk, and 0.1% Tween-20 for 1 hour at room temperature. Membranes were then incubated with primary Abs diluted (HSP70/ 1: 2000, STAT3/ 1:500) in Blotto for 12 hours at 4℃. Mouse polyclonal Abs against HSP70 (SPA-810, Stressgen, Inc.) and phosphorelated form of STAT3 (sc-8059, Santa Cruz) 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). All data was analyzed by NIH Image 1.60.

Preparation of the nuclear extracts

A modified nuclear extract was performed as previously described (Schreiber et al). Tissue samples were homogenized in 4 vol of Hepes buffer (same as above). The supernatants were removed, and the pellets were washed two times in 4 vol of buffer B (Hepes buffer without 0.25M sucrose) and that were centrifuged at 4,000g for10 minutes. Pellets were resuspended in 4 volumes of buffer C (25% glycerol, 20mM Hepes,pH7.9, 1.5mM MgCl2, 0.42M NaCl, 1mM EDTA), and were kept on ice for 30 minutes. Then these were obtained by centrifugation at 14,000g for 20 minutes, and aliquots of the supernatant were stored in at -80℃until used. These samples were used for Colorimetric transcription factor measurement.

Colorimetric transcription factor measurement

We measured binding activities of NF-IL6 and NF-kB in nuclear protein of the liver using Trans AM kit (Active Motif. Kit No. #ACV44198 and #ACV0061). This kits contain a 96-well plate on which has been immobilized a specific oligonucleotide that contains NF-IL6 or NF-kB consensus binding sites respectively. Nuclear proteins were added 10μg of samples per well, incubated for 1-2 hours, specifically binding to these

oligonucleotides. The primary antibodies used the accessible epitopes on NF-IL6 or NF-kB protein upon DNA binding. Addition of the secondary HRP-conjugated antibodies provided sensitive colorimetric readout quantified by spectrophotometry.

Statistical analysis

All data was analyzed by ANOVA followed by Fisher's PLSD method with the Stat View program. Differences between the means are considered significant at value of $p < 0.05$.

Results

Induction of HSP70

We confirmed the HS conditions used had led to a strong HSP70 induction in this model. Mice were HS treated for 20 min； 1, 3, 6, 12, 24, 48, 72, 96 hours thereafter, mice were killed. Liver was removed and homogenized in HEPES buffer. The homogenates were analyzed by Western Blot for HSP70 presence (Figure 1). HSP70 levels in the liver were clearly increased from 6hours to 72hours after HS treatment. The expression was maximal between 12 and 48 hours following HS. In control mice, no detectable HSP70 was present.

Influence of HS treatment for Liver damage after partial hepatectomy

We examined the effect of HS group and C group on the function of the remnant liver, we measured serum biochemical markers after hepatecyomy. Figure 2 shows the time course of hepatic transaminase AST and ALT leveles increased 3 hours after hepatectomy in both groups. The levels were inceesed 12 hours after the operation,

declined at 24 hours, and returned to near baseline levels 72hours after hepatectomy. But serum ALT and AST levels were significantly reduced in HS Group compared to C Group at 3, 6, 12, 24 hours after partial hepatectomy (Figure 2).

Effect of HS treatment on liver regeneration

We investigated and compared the influence of the HS preconditioning on liver regeneration in a murine hepatectomy model. Rates of the liver regeneration were significantly higher in HS Group than in C Group at 48 and 72 hours after partial hepatectomy (Figure 3A). And BrdU labeling index were significant higher in HS group than in C group at 36 hour after hepatectomy. HS group had early induction of DNA synthesis and liver regeneration (Figure 3B). Because those data suggest that Heat Shock treatment could modify both liver damage and promote liver regeneration after hepatectomy, we decided to measure levels of inflammatory cytokines after hepatectomy.

Induction of inflamatory cytokines after partial hepatectomy

We tried to measure IL-6 and TNFα levels both the serum and the remnant liver in mice at each time points. Serum IL-6 levels were significantly reduced in HS Group compared to C Group at 3, 6, 12 hours after hepatectomy. IL-6 levels in the liver were also significantly reduced in HS Group compared to C Group at 3 hours after hepatectomy (Figure 4A). Serum TNF-α levels were also significantly reduced in HS

Group compared to C Group at 6, 12, 24 hours after hepatectomy. TNF-α levels in the liver were also significantly reduced in HS Group compared to C Group at 3 hours after hepatectomy (Figure 4B). From these results, we hypothesized that protection of liver tissue was caused by reduction of inflammatory cytokine response by HS pretreatment.

Induction of liver regenerative factors by HS treatment

There were some reports that HS treatment could induce IL-6 expression (11.12). We made our hypothesis that HS induces Il-6 expression and which induces physporylation of STAT 3 protein too. IL-6 levels in the serum and the liver were increased 1hr after HS treatment, but they were decreasing to baseline until 12hours after HS treatment (Figure 5). We also evaluated phosphorylated STAT3 protein using Western blotting. It can be seen that there is no phosphorylated STAT3 after HS treatment (Figure 6). Those data suggest that actually HS treatment could induce IL-6 expression, but that phenomenon could not activate IL-6/STAS3 signaling pathway in our study.

Modulation of Transcription factor binding activity in liver by HS treatment

IL-6 and TNFα levels were significantly reduced in HS Group compared to C Group. To investigate the mechanism of inhibition of cytokine expression by HS, we analyzed activation of NF-IL6 and NF-kB. NF-IL6 and NF-kB binding activity were

significantly lower in HS group than in C group at 3 hours after hepatectomy (Figure 7).

Discussion

HS preconditioning could reduce liver injury and promote liver regeneration without induction of IL-6 / STAT signaling pathway in the liver after partial hepatectomy.

Molle et.al reported that 12 hours after HS group mice had reduced mortality about LPS challenge model after whole body HS compared to control group (7). Our study showed that HSP70 induced strongly from 12hours after whole body HS preconditioning too. Therefore, we used the mice which were 12 hours after HS in the present study. Our study also showed that serum ALT and AST levels were significantly reduced in HS Group compared to C Group, and HS treatment could reduced liver damage after partial hepatectomy. Under same condition, we calculated inflammatory cytokines in serum, especially IL-6 and TNF-α. In HS group IL-6 and TNF-α after hepatectomy were suppressed compared to C group, therefore, HS pretreatment reduced inflammatory cytokine expression. This is one of the reasons why HS group shows low levels of liver damage.

Some studies were reported that DNA synthesis had down-regulated after hepatectomy in IL-6 knockout mice (9) or in TNFR-1 knockout mice (10). Yamada

et al also reported that DNA synthesis had accelerated after hepatectomy in the model of mice which were injected recombinant IL-6, whereas expression of tyrosin receptor family (HGF, TGF-β and VGF) had down-regulation and/or no change under same situation (10). Therefore, IL-6 and TNF-α were recognized to important factor of liver regeneration.

HS group had up-regulated early stage DNA synthesis and liver regeneration compared to C group after hepatectomy. Some studies were reported that HS treatment could induce IL-6expression (11.12) and IL-6/STAT3, which is an important pathway for life, had early induced of liver regeneration in HS treatment group (16).

We made hypothesis that HS induces IL-6 expression and which induces physporylation of STAT 3 protein. Unfortunately, our results were differenced with hypothesis because we could detect only evaluated IL-6 and could not phosphorylated STAT3 protein after HS in serum and liver tissues. Our results showed IL-6 levels in the serum and the liver tissue were increased 1hr after HS treatment, but they were decreasing to baseline until 12hrs after HS treatment. And we could not detect phosphorylated STAT3 after HS treatment. From these results, we suggested that IL-6/STAT3 pathway does not mediate liver regeneration in this study.

Peters et al reported that strong IL-6-dependent activation of STAT3 before

hepatectomy, using hyperstimulation IL-6 model, which were overexpressing the human soluble IL-6 receptor/gp80 in hepatocyto, made delayed and inhibited of cell cycle progression correlated with cyclin A and E expression after hepatectomy (16). We suggested that IL-6 and TNFα have several functions, but both cytokines have needed optimal concentration to exercise their capabilities for liver regeneration in this model.

In the meantime, we considered that binding activity of NF-IL6 and NF-kB, which were transcription factor of IL-6and TNFα, under HS pretreatment. Figure 7 showed binding activities of NF-IL6 and NF-kB of the liver were significantly lower in HS group than C group at 3 hours after hepatectomy. HS preconditioning was protected liver against I/R injury by suppressing the activation of NF-kB and the subsequent expression of proinflamtory mediators through the stabilization of I-kB proteins (19). Our results showed that transport of activated NF-IL6 and NF-kB were inhibited by HS preconditioning, and which participated induction of IL-6 and TNFα. HSE exists on the promoter area of stress proteins which are induced by heat shock. Consensus sequence of HSE was overlapped both NF-IL6 and NF-kB, and which could inhibit to bind of these transcription factors. That might be one of the reasons why HS preconditioning could reduce induction of both cytokines.

In this study, we can detect just HSP70 induction. Usually, HS preconditioning

can induce other HSP90, HSP27 and α-Bcrystarin protein too. Under normal condition, expression of HSP90 protein is high level in cytosol which functions are not only quality control of proteins but also various modulation of cell function, and that protein caused to optimal activation of MAP kinase family (20, 21, 22). HSP27 and αB-crystalin were so called Small HSPs. Transgenic cell of HSP27could block Fas-induced apoptosis, and phosphorelated αB-crystalin, through activation of p-38MAP kinase, had important roles to be taken tolerance of myocardium ischemia (23, 24). We suggested these chaperon proteins were also induced by HS preconditioning, and which participated to reduce liver damage and up-regulate liver regeneration after hepatectomy.

Taken together, our finding support that whole body HS treatment induced expression of HSP70 in the liver and its condition could reduce expression of inflammatory cytokines in serum and liver tissues. This is a one of the reasons why HS group showed low levels of liver damage. We conclude that HS preconditioning is useful tool for hepatectomy, because it could reduce inflammatory response and promote liver regeneration.

In late years the efficacy of the thermotherapy in the cancer therapy was known, and a cancer cell was the therapy that kept characteristic to be weak alive for a fever as compared with normal cell (25, 26). There were total-body hyperthermia (27) and

but localized hyperthermia for a method, and it warmed the local site using supersonic waves in a thing of localized hyperthermia (28). It was in future prospective treatment, independent was rarely treated, and combination with the chemotherapy, radiation

therapy and immune therapy (29, 30). In our study, we examined a liver damage and liver reproduction after hepatectomy by the whole-body thermotherapy, but it seemed to

have possibilities to be as one of the in future treatment that was effective for recurrences of remnant liver after hepatecty it in the primary and metastatic liver cancer.

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FIGURE LEGENDS

Figure 1 .

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

Figure 2 .

Effect of HS pretreatment on serum AST and ALT activity after partial hepatectomy in HS or C group. Serum ALT and AST levels were significantly reduced in HS Group compared to C Group at 3, 6, 12, 24 hours after partial hepatectomy. *P < 0.05.

Figure 3 .

(A) Comparison of the rates of the liver regeneration after partial hepatectomy in HS or C group. Rates of the liver regeneration were significantly higher in HS Group than in C Group at 48 and 72 hours after partial hepatectomy. *P < 0.05.

(B) BrdU labeling index were performed using liver after partial hepatectomy. BrdU labeling index were significant higher in HS group than in C group at 36hour after hepatectomy. *P < 0.05.

Figure 4 .

(A) Effect of HS preconditioning IL-6 levels in serum and the liver tissue after partial hepatectomy using ELISA. Serum IL-6 levels were significantly reduced in HS

Group compared to C Group at 3, 6, 12 hours after hepatectomy. IL-6 levels in the liver were also significantly reduced in HS Group compared to C Group at 3 hours after hepatectomy. *P < 0.05.

(B) Effect of HS preconditioning TNF-α level in serum and the liver after partial hepatectomy using ELISA. Serum TNF-α levels were significantly reduced in HS Group compared to C Group after hepatectomy. TNF-α level in the liver were also significantly reduced in HS Group compared to C Group after hepatectomy. *P < 0.05.

Figure 5.

IL-6 levels in the serum and the liver were increased 1hr after HS treatment, but they were decreasing to baseline until 12hours after HS treatment.

Figure 6.

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

Figure 7.

Comparison of transcription factor binding activity in the liver by HS treatment. NF-IL6 and NF-kB binding activity were significantly lower in HS group than in C group at 3 hr after partial hepatectomy. *P < 0.05.