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RESEARCH ARTICLE

Pre-existent Hsp72 contributes to glutamine-induced hepatic *hsp72* gene activation during heat shock recovery period in rat

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Scope: Functional maintenance of liver is very important at all times for personal health. Hsp induction is associated with the protection of the organ. Glutamine, a nutrient inducer of Hsps, enhances cellular survival via Hsp72 induction in several organs, but not in the liver. The present study showed a novel approach to facilitate glutamine-induced hepatic Hsp72 synthesis and its possible mechanisms were discussed.

Methods and results: Sprague-Dawley rats were used as the experimental animals and the livers were the targets. Glutamine was administered via tail-vein injection, and its effects on hsp72 gene activation, including Hsp72 expression, heat shock factor-1 (HSF-1) phosphorylation and DNA-binding activation, were evaluated. The results showed that Hsp72 itself played a critical role in glutamine-induced hepatic Hsp72 synthesis during HS recovery period in a dose-dependent manner of preexistent Hsp72. The peak value of HSF-1 phosphorylation, HSF-1 DNA-binding activity, hsp72 mRNA accumulation, and Hsp72 synthesis was detected at 8 h after glutamine administration.

Conclusions: Glutamine switched on alteration pathway in inducing hsp72 gene activation. The existence of Hsp72 plays a critical role in the reactivation of hsp72 gene. Glutamine sustained the induction of intracellular Hsp72, which could be beneficial in protecting the liver from various injuries.

Keywords:
Glutamine / HSF-1 / Hsp72 / Liver / Phosphorylation

1 Introduction

Liver is a vital organ playing the role of a chemical factory of our body and its most important function is detoxification. It is very important to keep the liver healthy by eating the right kind of foods and nutrients and to protect and recover the liver from injuries. Heat shock proteins (Hsps) are considered as a series of self-protective proteins that can be induced by a wide variety of stressors and contribute to maintain cellular homeostasis against various forms of stresses [1]. Hsps play an important role in liver regeneration [2]. Hepatocytes probably neither survive nor regenerate without Hsp70 in their nuclei [3]. Hsps induction improved energy metabolism of liver and exhibited protective effect against warm ischemic liver injury and death [4].

In eukaryotic cells, heat shock factor 1(HSF-1) is a crucial transcriptional factor in regulating *hsp* gene expression. Through trimerization and phosphorylation, *HSF-1* is activated and transmitted from cytoplasm to the nucleus. *HSF-1* can bind with heat shock element (HSE) which is composed

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Abbreviations: HS, heat shock; **HSE**, heat shock element; **HSF**, heat shock factor; **Hsp**, heat shock protein

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as adjacent and inverse repeats of the motif 5′ -nGAAn-3′ located in promoters of hsp genes and induce Hsp expressions [5]. Among them, inducible Hsp70, also named as Hsp72, is the most representative one. After Hsps being produced, they can self-repress their expressions through interaction with HSF-1 [6]. The exact mechanism of heat-shock gene repression remains unclear. Recently, the clinical applications of Hsps were extensively described and presented [7, 8]. However, some problematic issues for Hsps induction by inducers, including the optimal dose, cytotoxicity, and induction efficiency in clinical applications, need to be clarified.

Glutamine, a non-essential amino acid, could enhance cellular survival against a variety of stressful stimuli through Hsp70 induction [9–11]. Several clinical trials have demonstrated the efficacy of glutamine as a conditional essential nutrient during extreme stress, serious injury, or illness [12, 13]. Reduced plasma glutamine levels in critically ill patients resulted in increased susceptibility to cell stress, and reduced responsiveness to pro-inflammatory stimuli, which both were associated with decreasing the Hsp70 expression in immune cells [14, 15].

Glutamine supplement, as a pharmacologically acting nutrient, increases serum Hsp70 in critically ill patients, and the magnitude of Hsp70 enhancement in glutamine-treated patients was correlated with the improved health status [16, 17]. Glutamine administration should be easily applied for clinical use to induce Hsp70. Unfortunately, the susceptibility against glutamine treatment is always inconsistent in different tissues and organs [14, 18]. The induction of Hsps is associated with organ protection. It has been documented that glutamine treatment induced Hsp72 overexpression in heart and lung tissues in the unstressed condition, and also in the heart, lung, colon, and kidney tissues of an endotoxin-induced sepsis animal model, but not in the liver [19]. Moreover, pretreatment of glutamine 6 and 24 h prior to hepatic ischemia could not induce Hsp70 in the liver, and the hepatic warm ischemia/reperfusion injury still existed [18].

In the present study, we investigated a feasible approach to induce Hsp72 synthesis by glutamine in the liver. The mechanisms of glutamine-induced hsp72 gene activation were focused on HSF-1 activation. A novel role of existent Hsp72 in regulating the hsp72 gene activation can therefore be highlighted.

2 Materials and methods

2.1 Animals and heat shock treatment

Experiments were performed on adult male Sprague–Dawley rats (weighing 320–350g) obtained from the National Experimental Animal Center (Nan-Kang, Taipei, Taiwan). They were housed under standard environmental conditions with a 12h light-dark cycle, temperature (20-251C), relative humidity (55-65), and had free access to water and feed. The experimental procedure conducted in this study was approved by the Animal Committee of Kaohsiung Medical University, (permission number: 95157), and the authors have adhered to the guidelines for the use of experimental animals laid down by the National Institutes of Health. Heat shock treatment was performed as described previously in [20, 21]. After anesthesia, rats underwent whole-body heating with an electric pad for 15 min in which rectal temperature was maintained between 41 and 42°C. The rats were also anesthetized in the sham-heated group, but were not heated.

2.2 Administration of glutamine

Alanyl-glutamine (Ala-Gln) dipeptide (Sigma, St. Louis, MO, USA) was utilized as a source of glutamine [19, 22]. Ala-Gln is rapidly hydrolyzed into glutamine and alanine; however, alanine is never mentioned in leading to significant enhancement of Hsp72 [23]. Ala-Gln dipeptide solution was prepared freshly as a 37.5% solution dissolved in saline and was administered at a dose of 0.75 g/kg through the lateral tail vein.

2.3 Preparation of hepatic total lysates and nuclear extracts

Preparation of hepatic total lysates and nuclear extracts were performed as described previously [20]. After the animals were sacrificed, the livers were excised, washed, and homogenized on ice in lysis buffer (20 mM 4-(2-hydro-xyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.6), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenehylene-diaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 0.5mM phenylmethylsulfonyl fluoride (PMSF), 2% NP-40, and 25% glycerol) for the total lysate preparation. After centrifugation, the total lysates were collected. Moreover, buffers A and B were used for preparing the nuclear extracts. After homogenising in nuclear buffer A (10 mM Tris-HCl, pH 7.5, 2mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM $CaCl_2$, 0.1 mM PMSF, 0.5 mM DTT, and 2.4 M sucrose), the lysate was filtered, centrifuged, and re-suspended in nuclear buffer B containing 1M sucrose and 1 mM $CaCl_2$. To exclude cytoplasmic contamination, the specific antibody of glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA, USA) were used as the cytoplasmic contamination marker detected in the nuclear fractions.

2.4 Western blot analysis

Equal amount of protein extract was loaded and separated by SDS-polyacrylamide gel electrophoresis, and then blotted on polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA, USA). The monoclonal antibody of Hsp72 and polyclonal antibody of HSF-1 (Stressgene, Victoria, BC, Canada) were utilized as the primary

antibody. The monoclonal antibody of β-actin (Chemicon) was used as the internal control. Anti-mouse and anti-rabbit immu-noglobulin G conjugated with peroxidase was used as the secondary antibody.

2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by an RNA extraction reagent, REzol™ C&T (PROtech Technologies, Taiwan, Republic of China). Aliquots of 2 mg of total RNA were employed as templates for RT-PCR with a QIAGEN One-Step RT-PCR kit (Qiagen, Hilden, Germany). Two pairs of primers, 5′-CTG GGC ACC ACC TAC TCC TG-3′ and 5′-CTC CTT CAT TCT TGG TCA GCA-3′ for Hsp72 [24], and 5′-CTA CAA TGA GCT GCG TGT GG-3′ and 5′-TAG CTC TTC TCC AGG GAG GA-3′ for β-actin, were used to amplify the products with 360 and 420 bp, respectively [25]. β-actin was detected as a reference control. Twenty-six cycles of PCR were executed individually at the following settings: denaturing at 95°C for 60s, annealing at 54°C for 90 s, extension at 72°C for 60s. RT-PCR products were separated on 2% agarose gels containing a trace amount of ethidium bromide.

2.6 Electrophoresis mobility shift assay (EMSA)

The EMSA was performed by LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). Biotin end-labeled double-stranded HSE oligonucleotide was utilized [26]. The nuclear extract (10 μg) added in reaction buffer contained 1μg of poly(dI-dC) and 2nM of biotin-labeled DNA. These mixtures were separated on a 6% non-denaturing gel and transferred onto a nylon membrane by semi-dry transfer. After UV-induced cross-linked process, the shifting of the biotin-labeled DNA was detected by Chemiluminescent nucleic acid detection module (Pierce).

2.7 Statistical analysis

All data were quantified by the densitometer and analysis software, UVP BioImaging system and LabWork4.6 image acquisition (UVP, Upland, CA, USA). The results were presented as mean ± standard deviation (SD). The statistical analysis of the data was performed by using one-way analysis of variance followed by the Scheffe test. The results were considered significant when p-value was < 0.05.

3 Results

3.1 Kinetic changes of *hsp72* gene expression after heat shock treatment in the rat liver

After HS treatment, HSF-1 phosphorylation, as an indicator of activation, was detected in the nuclear fraction of liver at 0.5h after HS. Then, Hsp72 became detectable at 2nd hour, reached a peak at 8th hour, remained at the plateau until 24th hour, then the expression is decreased (Fig. 1A). Following the induction of Hsp72, phosphorylated HSF-1 disappeared 2 h after HS (Fig. 1B). In addition, correlating with the HSF-1 activation, the accumulation of hsp72 mRNA was detected at 0.5h, then waxing and waning following the time course (Fig. 1C). The quantitative results were shown in Fig. 1D.

3.2 Glutamine initiates *hsp72* gene activation in rat liver during HS recovery period

After glutamine treatment, no Hsp72 induction could be found in the liver of non-heated rats (data not shown). Surprisingly, if glutamine was administered in rats 24h after HS, Hsp72 could be re-induced significantly 8 h later (Fig. 2). Not completely similar with the effect of HS, phosphorylation of HSF-1, HSF-DNA binding, and then *hsp72* mRNA expression all became obviously more abundant 8h after glutamine

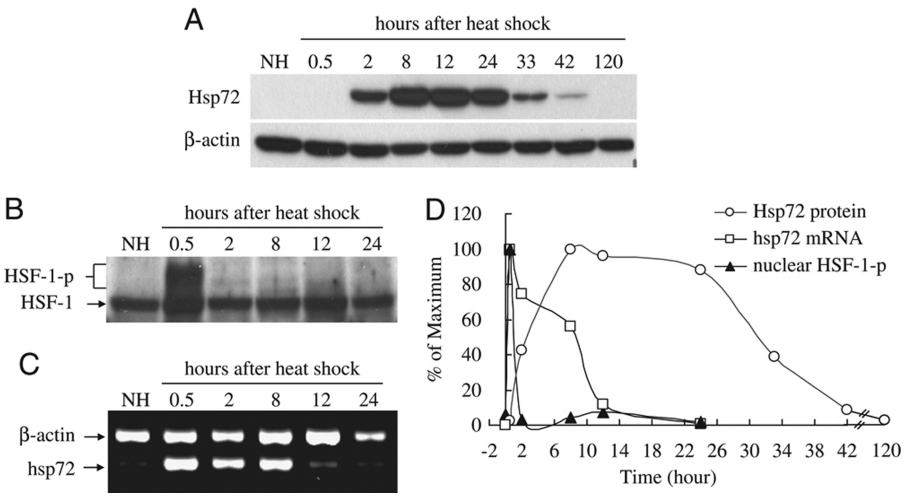

Figure 1. Kinetics of Hsp72 protein expression, HSF-1 phosphorylation and *hsp72* mRNA accumulation of the heat shock response. NH indicated the unheated control rats. Different time points after HS were labeled. (A) Hsp72 expression. (B) HSF-1 expression in nuclear fractions. HSF-1-p indicates phosphorylated HSF-1. (C) Accumulation of *hsp72* mRNA. (D) Quantitative analysis of Hsp72, HSF-1 phos-phorylation and *hsp72* mRNA. The maximum content was set as the 100%. The data are representative of the mean of four independent experiments.

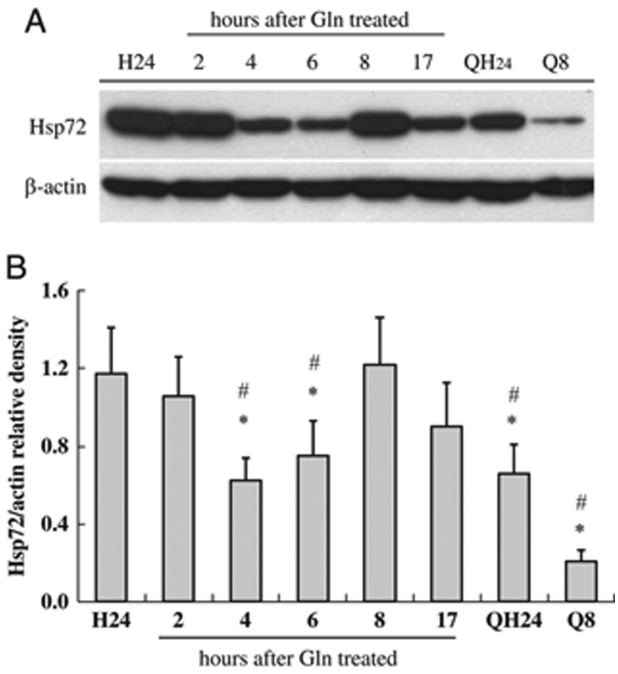

Figure 2. Time course of Hsp72 expression after glutamine administration during HS recovery period. Hsp72 expression. A representative result out of four was shown. H24 indicates at 24 h after HS. 24 h after HS, glutamine was administrated. QH24 and Q8: Quercetin was treated 6 h before HS and samples were collected at 0 and 8 after glutamine treatment. (B) Quantitative analysis. The statistical data are representative of the mean ± SD of four independent experiments. $^{*}p < 0.05$ versus. H24 group; $^{\#}p < 0.05$ versus the group of 8 h after Glutamine (Gln) treatment.

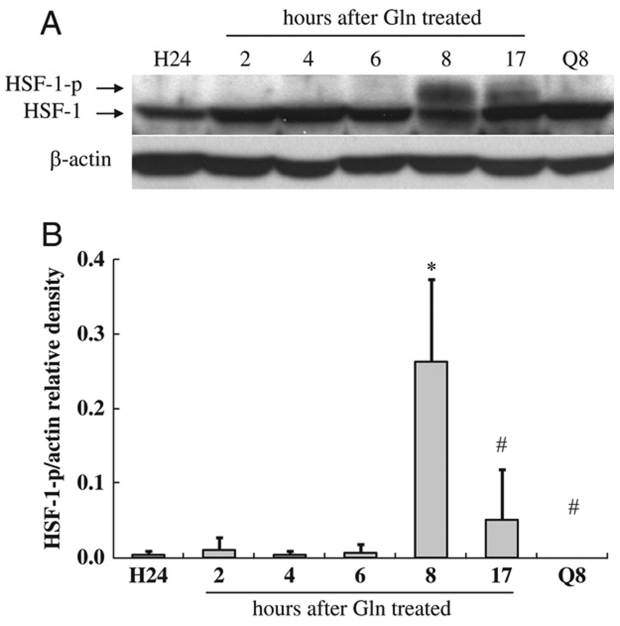

Figure 3. Time course of phosphorylated HSF-1 after glutamine administration during HS recovery period. HSF-1 expression and its phosphorylation. A representative result out of four was shown. H24 indicates at 24 h after HS. 24 h after HS, glutamine was administrated. Q8: Quercetin was treated 6h before HS and samples were collected at 8h after glutamine treatment. Quantitative analysis. The statistical data are representative of the mean ± SD of four independent experiments. $^{*}p < 0.05$ versus H24 group; $^{\#}p < 0.05$ versus the group of 8 h after glutamine (Gln) treatment.

supply (Figs. 3 and 4). Furthermore, this phenomenon was markedly inhibited by quercetin, an inhibitor of Hsp synthesis (Fig. 2A, Lanes QH24, Q8), which was administered to the rats 6h before HS treatment. These results imply that glutamine administration could contribute in vivo to induce Hsp72 synthesis only when the rats were heat-shocked previously.

3.3 Pre-existent Hsp72 is associated with glutamine induced *hsp72* gene activation in a dose-dependent manner

To confirm the correlation between the pre-existent Hsp72 and glutamine-induced hsp72 gene expression, a dose-dependent study of pre-existent Hsp72 was performed. As mentioned above, the Hsp72 expression was attenuated gradually 24h after HS treatment 5(Fig. 6A) and hsp72 mRNA just was detected within 12h after HS (Fig. 1C). We therefore chose a series of recovery time (24, 33, 42, and 120h after HS treatment) of HS rats to evaluate the capacity of Hsp72 induction by glutamine. Eight hours after gluta-mine treatment, hsp72 mRNA was detected. As shown in Fig. 6A, the pre-existent amount of hepatic Hsp72 was different at a series of recovery times. The expression of hsp72 mRNA induced by glutamine was significantly proportional to the amount of hepatic Hsp72 with a dose-dependent manner. In rats of 5 days after HS, while Hsp72 was almost totally disappeared, glutamine failed to induce hsp72 mRNA expression in the liver (Fig. 6B). The results further confirmed that the pre-existent Hsp72 is essential for glutamine-induced hsp72 gene activation in liver of rat.

4 Discussions

For decades, Hsps has been revealed to be a highly preserved protein family in almost all living cells. As shown in the present result, after HS treatment, HSF-1 was phosphorylated within 30min and mRNA of hsp72 was also induced following, and then Hsp72 production was detectable at 2h later and peaking between 12 and 24h in rat liver. Hsp72 synthesis faded away almost completely 5 days after HS treatment. However, Hsp72 was un-detectable in the liver after simple glutamine administration in the intact rat, which is similar with other reports [18–20]. Encouragingly, we found the hsp72 gene activation in rat liver could be induced by glutamine in an Hsp72 dose-dependent manner. Glutamine-enhanced HSF-1 phosphorylation and HSF-1 DNA-binding

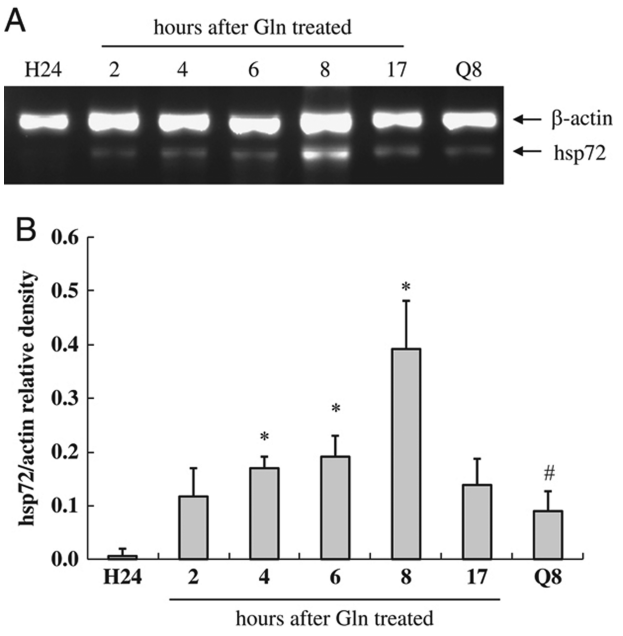

Figure 4. Time course of *hsp72* mRNA after glutamine administration during HS recovery period. Accumulation of *hsp72* mRNA. A representative result out of four was shown. H24 indicates at 24 h after HS. 24 h after HS, glutamine was administrated. Q8: Quercetin was treated 6h before HS and samples were collected at 8h after glutamine treatment. Quantitative analysis. The statistical data are representative of the mean ± SD of four independent experiments. $^{*}p < 0.05$ versus H24 group; $^{\#}p < 0.05$ versus the group of 8h after glutamine (Gln) treatment.

activity in rat liver depended on Hsp72 existence during HS recovery time. The timing of hsp72 gene activation induced by glutamine was more delayed than that induced by HS treatment. The activation of hsp72 gene and Hsp72 re-induction were detectable 8h after glutamine treatment.

More than 300 laboratory enhancers of Hsps have been explored experimentally. However, most of them are clinically unavailable that due to the effects of cytotoxicity and the variation of induction efficiency. Glutamine, a component of nutritional supplement, was reported to have a potential of Hsp induction and contributed to improve clinical outcomes in critically ill patients [8, 16, 17, 27]. Serum Hsp70 was increased by glutamine supplement administered parenterally and its magnitude was correlated with improved clinical outcomes in glutamine-treated patients [8, 27]. However, various organs or tissues had a different responsiveness to glutamine treatment. Liver seemed to be one of the most un-responsive for the gluta-mine treatment [19]. This phenomenon was implying that the susceptibility of liver response to glutamine was weak, so that clinical utilization of glutamine in liver was limited. From our findings, Hsp72 can be also induced in live by glutamine at the proper timing and the special condition. Glutamine-induced *hsp72* gene activation in liver was not only modulated by HSF-1 but was also restricted by intra-cellular Hsp72 expression. The existence of intracellular Hsp72 during recovery time was a key factor. The levels of HSF-1 phosphorylation were correlated closely with the accumulation of intracellular Hsp72 in glutamine-induced-Hsp72 production. We considered that glutamine-induced *hsp72* gene activation in liver occured in a pre-existent Hsp72-dependent manner.

In the general pathway in inducing hsp gene activation, HSF-1 would play as an initial activator. After phosphorylation, trimerization, and transmission, HSF-1 binds to DNA containing inverted repeats of the sequence 3′-nGAAn-5′, such as 5′-nGAAnnTTCnnGAAn-3′, and activates hsp gene. After Hsp72 synthesis, the DNA binding activity of HSF-1 turned to be null and void by Hsp72 negative feedback pathway and degraded by the ubiquitin-proteasome pathway [5, 28]. In the processes, HSF-binding protein 1 (HSBP1) also be associated with HSP–HSF-1 complex that contributes to dissociation of trimeric HSF1 into an inert monomeric form [29]. FILIP-1L interacts with HSF-1 and promotes its ubiquitination and degradation through the ubiquitin-proteasome system and modulates the heat shock response [28]. We considered that HSF-1 re-activation played a pivotal role in Hsp72 induction of liver by glutamine. Hsp72 existence contributed to protect the re-activated function of HSF-1 during HS recovery time and prevented the HSF-1 from ubiquinization and degradation [5, 28]. Glutamine treatment could contribute to

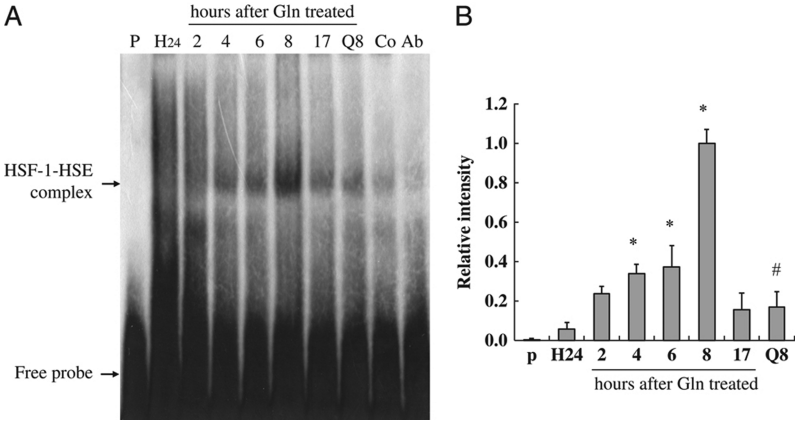

Figure 5. Dynamic changes of HSF-1-HSE binding activity after glutamine administration during HS recovery period. Detection HSF-1-HSE binding activity by EMSA. The position of HSF-1-HSE complex and the free probe are indicated. Selected time points were as described previously. Co: competition reactions with 50-fold excess unlabeled double-stranded HSE oligonucleotide. Ab: supershifting assays by adding of HSF-1 antibody. (B) Quantitative analysis. The data are representative of the mean ± SD of four independent experiments. $^{*}p < 0.05$ versus H24 group; $p < 0.05$ versus the group of 8 h after Glutamine (Gln) treatment.

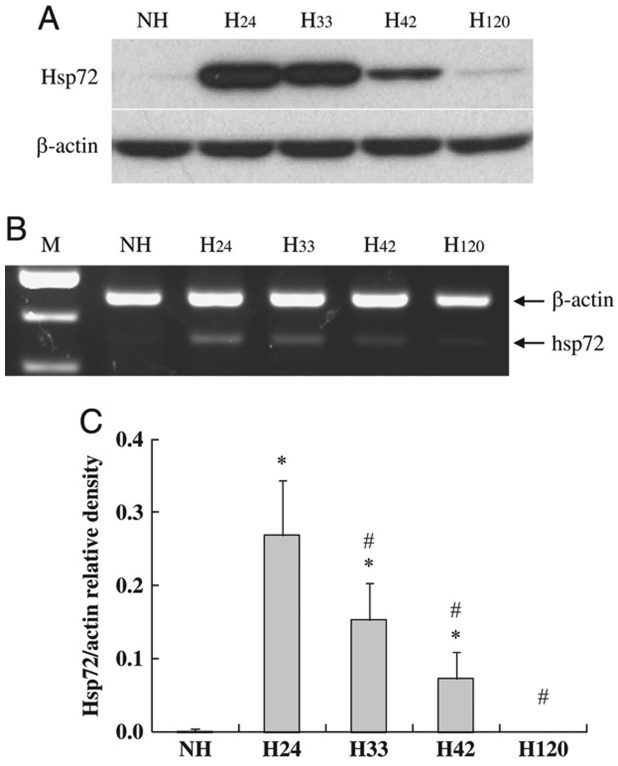

Figure 6. Accumulation of *hsp72* mRNA 8h after glutamine administrated at various timings of HS recovery period. (A) Hsp72 expression was detected by Western blot analysis. β-Actin was detected simultaneously and acted as the internal standard. NH indicates the normal control (NH) rats. H24, H33, H42 and H120 indicate 24, 33, 42 and 120 h after heat shock treatment respectively. (B) Glutamine administrated at various timings of HS recovery period and then mRNA was detected 8h after Glutamine administration. NH indicates the normal control rats. H24, H33, H42 and H120 indicate 24, 33, 42 and 120 h after HS respectively. (C) Quantitative analysis. The statistical data are representative of the mean ± SD of four independent experiments. $^{*}p < 0.05$ versus Glutamine-treated NH group; $^{\#}p < 0.05$ versus Glutamine (Gln)-treated H24 group.

unstrap the complexes. However, it is still a mystery why glutamine could interrupt the negative feedback pathway and promote the processes of Hsp72 releasing HSF-1 and need to be further investigated.

This is totally different from previous reports about that Hsps can regulate Hsps synthesis in a negative feedback mechanism [5, 28]. Besides down-regulation of HSF-1 activity by negative feedback, we suggested that intracellular Hsp72, acting as a chaperon, contributed to protect the deactivated HSF1 from being degraded by the ubiquitin-proteasome pathway. Glutamine treatment contributed to stimulate HSF-1 dissociation from Hsp72 and then re-activation. It could be easier in turning on HSF-1 activation from de-activated form of HSF-1 than from primitive inactivated form of HSF-1. In the processes of HSF-1 re-activation, Hsp72 could be necessary for re-folding and assembling of de-activated form of HSF-1 in the alternative pathway. Despite these, the alternative pathway needs to be illustrated more. However, this phenomenon described a previously undefined glutamine-dependent pathway to induce HSF-1 activation and Hsp induction.

Hsps are known as molecular chaperones that contribute to peptide folding, re-folding, and assembling [30–33]. However, it has been recently suggested that cognate Hsp70 was required in regulating HSF-1 activity during heat stress and required for the subsequent expression of HSF-1 target gene. HSF-1 activation was greatly decreased when trans-fected with siRNAs targeted to cognate Hsp70 [34]. Stable transfection of either Hsp25 or Hsp72 increased endogenous Hsp25 and Hsp72 and then enhanced the HSF-1 activation [35]. These evidences could contribute to support our results that glutamine exhibits its inducibility for Hsp synthesis depending on the pre-existence of Hsp72.

In conclusion, glutamine switched on an alternative pathway in inducing *hsp72* gene activation. The existence of Hsp72 plays a critical role in the reactivation of *hsp72* gene. Glutamine sustained the induction of intracellular Hsp72 which could be beneficial in protecting liver from various injuries.

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The authors have declared no conflict of interest.

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