

Dual effects of heat stress on tumor necrosis factor- α -induced hepatocyte apoptosis in mice

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The major heat shock protein, HSP70, plays a critical role in cell survival in response to stress, possibly by inhibiting a number of antisurvival pathways. However, heat stress (HS) and HSPs also sensitize cells to certain apoptotic stimuli, such as TNF- α . To clarify the relations between HS and apoptosis, we examined the differential effects of the intensity of HS on liver injury and apoptosis induced by TNF- α in mice. TNF- α was injected into D-galactosamine (GalN)-sensitized mice that were pretreated with or without HS. Liver injury was assessed biochemically and histologically. In GalN-sensitized mice, application of HS for 7 days led to significant enhancement of TNF- α -induced hepatotoxicity, despite upregulation of HSP70 in the liver. In contrast, application of HS for 1 day led to attenuation of TNF- α -induced liver injury. Repeated HS decreased the levels of the FLICE inhibitory protein short (FLIP_S) and activated caspase-8 in the liver. The caspase-8 inhibitor Z-IETD-FMK effectively protected both the nontreated and HS-pretreated mice from the hepatotoxicity induced by GalN/TNF- α . HS shows dual effects on TNF- α -induced hepatocyte apoptosis. Exposure to repeated HS, but not to single HS, leads to enhancement of TNF- α -induced hepatocyte apoptosis via the interaction of FLIP and caspase-8.

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Exposure of cells to any of a wide variety of stressors, such as heat stress (HS), hypoxia, hydrogen peroxide, inflammation, and ischemia, results in similar accumulation of the stress proteins known as heat shock proteins (HSPs).^{1,2} The main function of HSPs is to operate as intracellular chaperones for aberrantly folded or mutated proteins and to provide cytoprotection against the stress conditions. The HSP70 protein family is the abundant group in eukaryotic cells, and contains both constitutive and inducible forms that are localized to different intracellular organelles.¹ The heat-inducible form of HSP70 is expressed at low basal levels but is rapidly induced by heat or other stresses. HSP70 can be induced in liver, cardiac muscle, renal cells, skeletal muscle, and brain tissue. A number of studies have indicated that HSP70 protects cells and tissues from injury under various pathological conditions via inhibition of procaspase-9 to the Apaf-1/apoptosome complex,^{3,4} protection of mitochondrial membranes,⁵ inhibition of the activation of c-Jun N-terminal kinase (JNK),^{6–8} or inhibition of

BID cleavage.⁹ In addition to these *in vitro* studies, there are several reports clearly demonstrating that HSP70 protects cells *in vivo*. HS has been shown to prevent lethality induced by lipopolysaccharide (LPS) or tumor necrosis factor- α (TNF- α) in rats and mice.^{10–12} This protection has also been correlated with the upregulation of HSP70 in several organs.¹⁰

TNF- α and the Fas ligand are cell-death mediators that act by binding to their responsive receptors, two TNF receptors (TNFR) TNFR1 and TNFR2, and Fas, respectively, and induce apoptosis in a variety of cell types. Both TNFR1 and Fas share a related C-terminal intracellular domain referred to as the 'death domain', and use similar types of associated molecules in the signal transduction pathway leading to apoptosis.¹³ Fas recruits the Fas-associated death domain protein (FADD), and TNFR-1 recruits the TNFR-associated death domain protein (TRADD), which in turn binds FADD. FADD is then associated with procaspase-8, resulting in its activation. Caspase-8 then functions as an apical caspase, leading to the processing of effector caspases such as caspase-3, -6, and -7 (extrinsic pathway). The other caspase cascade, which is known to be activated by genotoxic stressors such as UV irradiation and anticancer drugs, is triggered by the release of cytochrome *c*, which binds to both the cytoplasmic scaffold, known as Apaf-1, and to procaspase-9. The

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binding to procaspase-9 leads to activation of caspase-9, which in turn activates downstream caspase-3, -6, and -7 (intrinsic pathway).

Even though it has been well established that HSPs inhibit various types of apoptosis, some studies have demonstrated that HS or elevated HSP70 potentiates cell death following various stimuli. Elevated HSP70 or overexpression of heat shock transcription factor 1 enhances Fas-mediated cell apoptosis.^{14,15} HS also sensitizes AML cells and endothelial cells to apoptosis.^{16,17} However, the precise mechanism by which HS promotes cell death rather than protects cells from apoptosis remains unclear. Recently, Tran *et al*¹⁸ have demonstrated that HS sensitizes cells to Fas-mediated apoptosis through downregulation of the FLICE inhibitory protein (FLIP), which is a cytoplasmic protein that has sequence homology with caspase-8 and is capable of inhibiting apoptosis by blocking the recruitment and processing of caspase-8. In further support of the idea that HSP70 promotes apoptosis, it has been reported that HSP70 enhances TNF-mediated apoptosis by binding I κ B kinase γ and impairing NF- κ B survival signaling.¹⁹

In the present study, to clarify the relations between HS and apoptosis, we examined the effect of different levels of HS exposure on liver injury and apoptosis induced by TNF- α in mice. We demonstrate that application of HS for 7 days in mice induces HSP70 in the liver and leads to significant enhancement of TNF- α -induced hepatotoxicity, but application of HS for 1 day leads to attenuation of TNF- α -induced liver injury. We further show that the repeated exposure to HS is able to rapidly down-regulate the levels of FLIP_s and activate caspase-8, and thereby enhance TNFR-mediated apoptosis.

Materials and methods

Mice

Pathogen-free, male BALB/c mice (age, 7–8 weeks; weight, 20–25 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained for at least 1 week under a 12-h day/night cycle with free access to food and water until the day of the experiment.

Reagents

D-Galactosamine (GalN) hydrochloride was obtained from Nacalai Tesque (Tokyo, Japan). Recombinant mouse TNF- α was purchased from Genzyme (Cambridge, MA, USA). The fluorogenic substrate Ac-IETD-AMC (Ac-IETD-7-amino-4-methylcoumarin) was obtained from BIOMOL (Philadelphia, PA, USA), and Ac-DEVD-AMC (Ac-DEVD-7-amino-4-trifluoro-methylcoumarin) was obtained from Alexis (San Diego, CA, USA). The caspase-8 inhibitor Z-IETD-FMK was obtained from Genzyme. Antibodies for Western blotting were obtained as follows:

procaspase-8 and the cleaved product of procaspase-8 (p20), FLIP short/long (FLIP_{S/L}), cytochrome c, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Akt (Ser-473) and Phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA, USA), HSP70 was from Stressgen Biotechnologies Co. (Victoria, Canada), and anti-rabbit IgG HRP-coupled secondary antibody was from Amersham-Pharmacia Biotech (Buckinghamshire, UK).

Experimental Treatment

The experiments were conducted in accordance with the institutional guidelines of the Gifu University School of Medicine. Mice were placed in a specially designed cage that enabled roughly two-thirds of mouse body surface area to be immersed in a hot water bath controlled to $42.0 \pm 0.5^\circ\text{C}$. They received HS for 15 min once a day for 1 day, 3 days or 7 days. GalN was dissolved in sterile, pyrogen-free saline and was administered to mice intravenously (16 mg/mouse). Recombinant mouse TNF- α was diluted with pyrogen-free saline to the required concentrations and intravenously injected into mice (0.5 $\mu\text{g}/\text{mouse}$). Some mice were intraperitoneally treated with 900 $\mu\text{g}/\text{mouse}$ of the caspase-8 inhibitor Z-IEHD-FMK 30 min after GalN/TNF- α administration. Control mice received 2% DMSO in saline at the same time. The mice were anesthetized with ether and killed by drawing blood from the inferior vena cava with heparin-coated syringes. The liver was immediately removed and washed with ice-cold phosphate-buffered saline (PBS). A part of the dissected liver tissue was frozen in liquid nitrogen for measurements of caspase activity, and for Western blot analysis.

Transaminase Activity

Serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured with a standard clinical automatic analyzer.

Caspase Activity

Frozen liver tissues were homogenized in lysis buffer (10 mM HEPES pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 20 $\mu\text{g}/\text{ml}$ leupeptin). The homogenates were incubated on ice for 1 h and centrifuged at $14\,000 \times g$ at 4°C for 20 min, and then the supernatants were collected. Seventy-five μl aliquots of the extracts (200 μg protein) were incubated with fluorogenic peptide substrate Ac-IETD-AMC or Ac-DEVD-AMC at a final concentration of 50 μM in 20 $\mu\text{l} \times 5$ reaction buffer (50 mM PIPES pH 7.4, 10 mM EDTA, 0.5% CHAPS, 25 mM dithiothreitol) for 2 h at 37°C . The

change in the fluorescence of released AMC was monitored at 1-h intervals using a Fluoroskan Ascent FL fluorometer (Thermobioanalysis Co., Helsinki, Finland) using an excitation wavelength of 355 nm and emission wavelength of 460 nm.

Western Blot Analysis

For Western blot analysis of caspase-8, HSP70, FLIP_s, FLIP_L, Akt, phosphorylated Akt, cytochrome *c*, and actin, frozen liver tissues from mice killed at the indicated time points were homogenized in lysis buffer. Equal amounts of the liver lysates, quantified using a commercially available Bio-Rad protein assay kit (Richmond, CA, USA), were loaded onto each lane of a 12.5% SDS-PAGE gel. Nitrocellulose membranes (Bio-Rad) were used to transfer proteins and were blocked with 5% bovine serum albumin buffer overnight. The membranes were probed with Abs against procaspase-8, HSP70, FLIP short/long (FLIP_{s/L}), Akt, phosphorylated Akt, cytochrome *c*, and actin, and then incubated with the anti-rabbit or mouse IgG HRP-coupled secondary antibody. Detection was performed with an ECL system (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Analysis of the processed form of caspase-8 (p20) band was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available over the Internet from an anonymous FTP at zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, Part No. PB95500195 GI).

Preparation of Cytosolic and Membrane Fractions

Livers were washed in PBS and disintegrated by seven strokes with a Dounce-type homogenizer in A-buffer (five times volume against liver) (220 nmol/l mannitol, 70 mmol/l sucrose, 50 mmol/l HEPES pH 7.0, 5 mmol/l EGTA, 2 mmol/l MgCl₂, and protease inhibitors, 0.3 mmol/l PMSF, and 30 µg/ml E64).

Debris was pelleted by centrifugation at 1000 × *g* for 5 min. The protein concentration of the resulting postnuclear fraction (supernatant) was adjusted to 8 mg/ml and then samples were further centrifuged at 100 000 × *g* for 20 min. The resulting supernatant was used as the cytosolic fraction and the pellet as the membrane fraction. The membrane fraction was resuspended and homogenized in the original volume of A-buffer.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

The liver was excised and immediately fixed with 10% buffered formalin. A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, which relies on incorporation of labeled dUTP at the sites of DNA breaks, was also carried out using 5 µm-thick sections of the mouse livers. All reagents and buffers for the TUNEL assay were included in an Apop Tag kit (Intergen, Purchase, NY, USA). The procedures were performed according to the manufacturer's instructions. TUNEL-positive hepatocytes are given as a percentage relative to the total number of hepatocytes in each visual field.

Data Analysis

All values in the figures and text are expressed as the mean ± s.e. The significance of differences among the mean values was evaluated according to the Mann-Whitney *U*-test.

Results

Dual Effects of HS on Liver Injury and Hepatocyte Apoptosis Induced by GalN/TNF-α

Intravenous injections of 16 mg of GalN and 0.5 µg of TNF-α into BALB/c mice caused an increase in plasma aminotransferase activity after 6 h (Table 1).

Table 1 Effects of HS on TNF-α-induced liver injury in mice

Pretreatment	TNF-α (µg/Mouse)	GalN (mg/mouse)	ALT (IU/L)	AST (IU/L)
—	—	—	43.3 ± 1.3	86.6 ± 3.3
—	—	20	46.6 ± 8.8	120.0 ± 14.1
—	0.5	—	130.0 ± 36.5	176.6 ± 19.9
HS (1)	—	—	55.0	105.0
HS (7)	—	—	45 ± 8.6	80 ± 7.0
HS (7)	—	20	56.6 ± 3.3	126.6 ± 12.0
HS (7)	0.5	—	53.3 ± 12.0	106.6 ± 16.6
—	0.5	20	8397.7 ± 1441.8	2054.5 ± 314.0
HS (1)	0.5	20	387.5 ± 33.3*	435 ± 61.3*
HS (7)	0.5	20	20775.8 ± 3067.4*	9672.5 ± 174.0*

Mice were subjected to HS (42.0 ± 0.5°C for 15 min) once a day for 1 day (HS (1)) or 7 days (HS (7)) and injected i.v. with TNF-α and/or GalN 12 h after the last HS exposure. Blood was collected and serum ALT and AST activities were determined as indices of hepatotoxicity 6 h after the administration of TNF-α and/or GalN. Data are expressed as the mean ± s.e. for three to 11 mice.

**P* < 0.05 compared to mice treated with GalN/TNF-α.

In mice that were pretreated with HS for 7 days, plasma ALT and AST activities were significantly increased ($P < 0.05$). On the other hand, pretreatment with HS for 1 day significantly reduced aminotransferase activities after GalN/TNF- α injection ($P < 0.05$). We quantified hepatocyte apoptosis by counting cells positive for TUNEL (Figure 1). After administration of GalN and TNF- α , a marked increase in the number of apoptotic cells was observed in the livers of mice pretreated seven

times with HS as compared to those without HS. However, single treatment with HS significantly reduced the number of TUNEL-positive hepatocytes. These results indicated that the single and repeated HS provided differential effects on liver injury and hepatocyte apoptosis induced by GalN/TNF- α .

Next, to determine whether HS influences the susceptibility of mice to TNF- α , we examined the effects of HS for 7 days on the ALT activities of mice

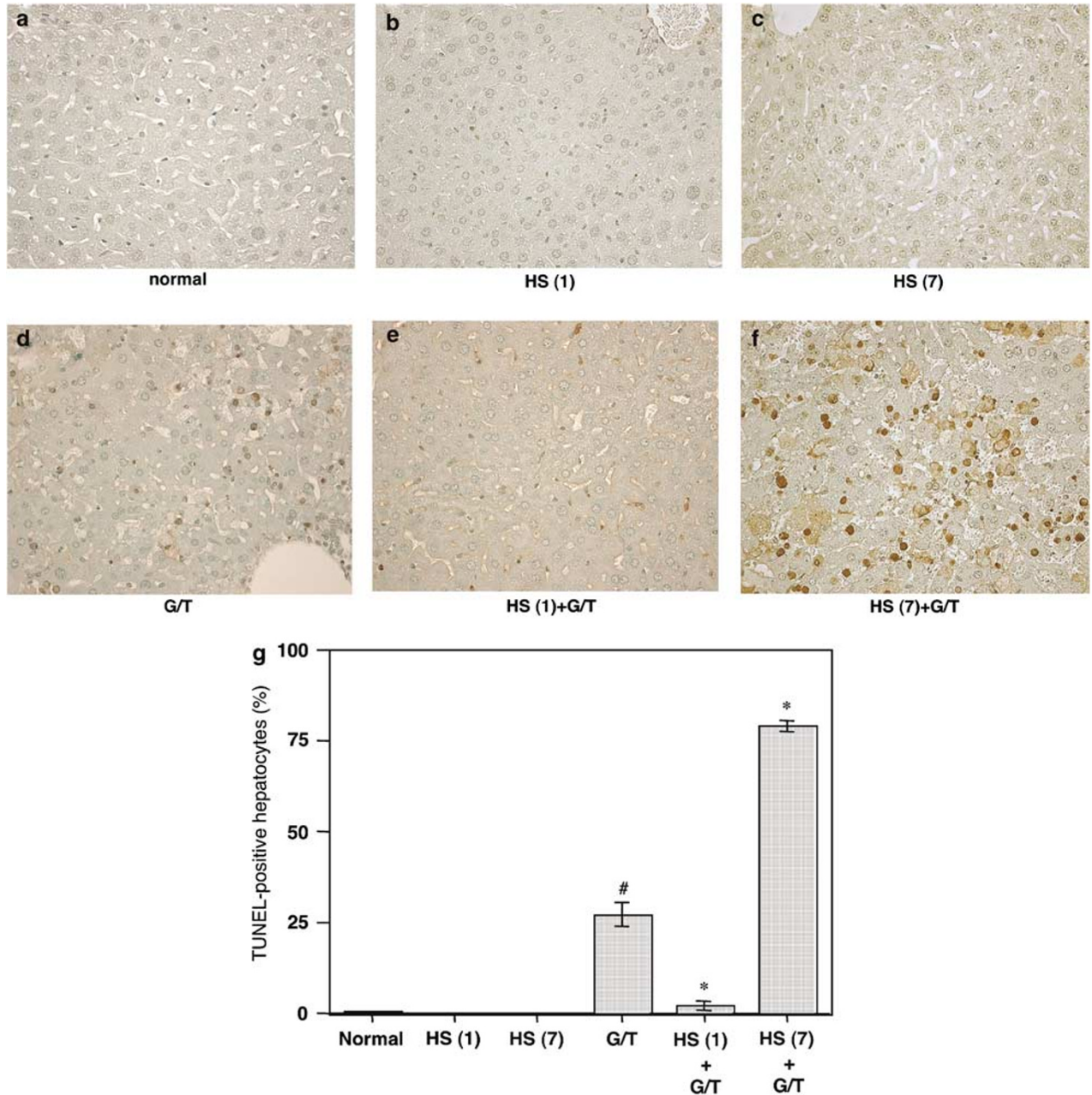


Figure 1 Induction of apoptosis by 16 mg of GalN/0.5 μ g of TNF- α in mouse liver pretreated with HS. Mice were subjected to HS ($42.0 \pm 0.5^\circ\text{C}$ for 15 min) once a day for 1 day (b) or 7 days (c). Control mice were nontreated (a). Mice were injected with GalN/TNF- α 12 h after prior HS for 1 day (e) or for 7 days (f) or after receiving no HS (d). The mice livers underwent an *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to detect apoptotic cells (original magnification $\times 800$). (g) Percentages of TUNEL-positive cells among total hepatocytes were determined. Data are expressed as the mean \pm s.e. for 4–14 mice. * $P < 0.05$ compared to mice treated with GalN/TNF- α ; # $P < 0.05$ compared to normal mice.

given TNF- α alone (Table 1). However, unlike GalN, HS did not induce liver injury in TNF- α -treated mice (compare the third and fourth rows from the bottom). These results indicate that HS does not sensitize mice to TNF- α -induced liver injury and apoptosis.

Effects of HS on Caspase Activities

To assess whether caspases are involved in the apoptotic process induced by GalN plus TNF- α in mice pretreated with or without HS, the activities of ALT, caspase-8-like and -3-like proteases were determined (Figure 2). The activities of caspase-8-like and -3-like proteases increased at 4 and 6 h after administration of GalN plus TNF- α . In mice challenged with prior HS for 7 days, the activities of caspase-8-like and -3-like proteases were promoted to a greater extent at 4 h after GalN plus TNF- α than in mice without prior HS. No significant difference was found at 6 h after the administration between HS-pretreated and -nontreated mice. More severe liver damage was observed in mice pretreated with repeated HS at 6 h after GalN plus TNF- α adminis-

tration compared with mice without prior HS (Figure 2a). This finding may indicate that the decreases in caspase-8-like and -3-like activities at 6 h following GalN plus TNF- α treatment in mice pretreated with repeated HS are probably due to the more severe liver damage. We found that the activity of caspase-8-like protease was already upregulated in mice pretreated with HS before the administration of GalN plus TNF- α , indicating that caspase-8-like protease was constitutively activated in repeated HS-challenged mice.

HS Treatment Induces HSP70 and Caspase-8 Activity

To investigate whether the HS conditions used in the present study led to HSP70 induction and caspase activation, mice were killed 12 h after the final HS treatment. The livers were removed and homogenized, and the homogenates were subjected to Western blot analysis for HSP70, procaspase-8, and p20, which is the cleaved product of procaspase-8 (Figure 3a). The level of HSP70 expression in the liver rose with the number of HS treatments. Similarly, cleavage of procaspase-8 in the liver was

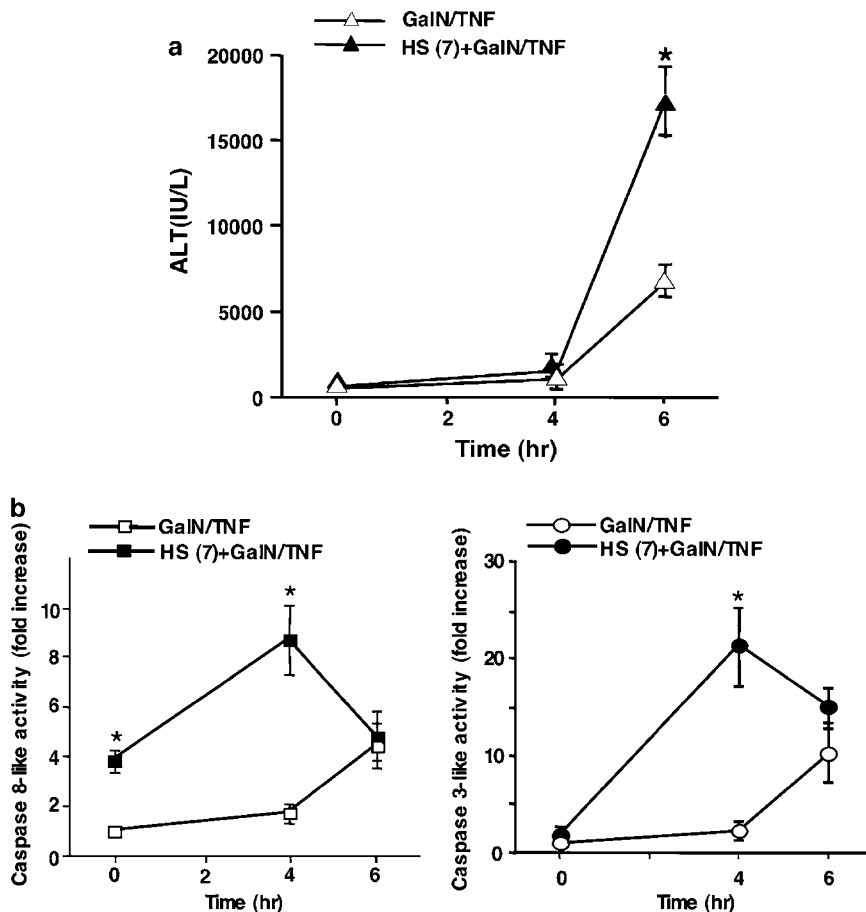


Figure 2 HS promotes activation of liver caspases. Assessment of (a) ALT and (b) caspase-8-like and caspase-3-like activity in the liver collected at the indicated time points after administration with GalN/TNF- α in the presence or absence of HS pretreatment for 7 days. Data are expressed as the mean \pm s.e. for 3–6 mice. * P <0.05 compared to mice treated with GalN/TNF- α .

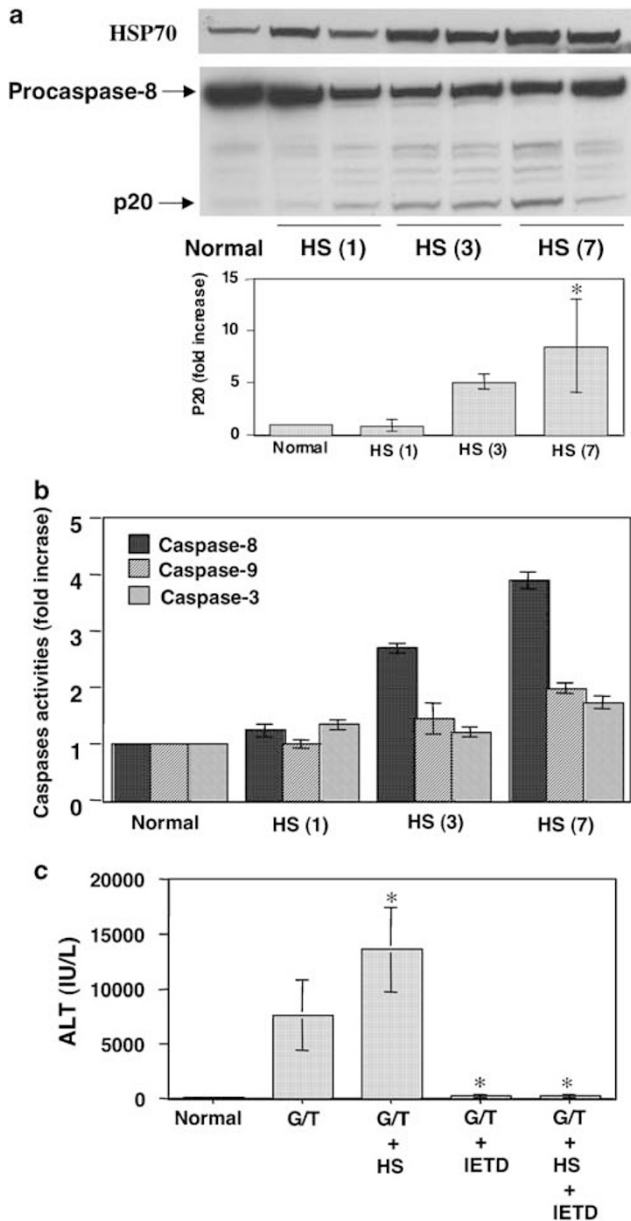


Figure 3 Treatment with HS for 7 days increases HSP70 and caspase-8 activity. (a) Induction of HSP70 and cleavage of procaspase-8 in the liver of mice after whole-body HS. Total liver proteins (50 μ g) from the mice were subjected to SDS-PAGE, and were probed with anti-HSP70 or antiprocaspase-8. The results shown are representative of at least three independent experiments. Analysis of the processed form of the caspase-8 (p20) band was performed on a Macintosh computer using the public domain NIH Image program. Data are expressed as the mean \pm s.e. for 3–4 mice. * $P < 0.05$ compared to mice treated with HS for 1 day. (b) Effects of HS on caspase-8-like, -9-like, and -3-like activity in the mouse liver. For measurement of caspase-8-like, -9-like, and -3-like protease activities, the synthetic fluorogenic substrates, Ac-IETD-AMC, Ac-LEHD-AMC and Ac-DEVD-AMC were used, respectively. Data are expressed as the mean \pm s.e. for three to four mice. (c) Effects of the caspase-8 inhibitor Z-IETD-FMK on the hepatotoxicity of mice induced by GalN/TNF- α with or without prior HS treatment. Blood was collected 6 h after GalN/TNF- α administration, and serum alanine aminotransferase activities were determined as indices of hepatotoxicity. Data are expressed as the mean \pm s.e. for three to four mice. * $P < 0.05$ compared to mice treated with GalN/TNF- α .

induced by HS treatment. The cleavage was maximal in the liver treated with HS for 7 days. Consistent with the Western blot analysis, the activity of caspase-8 (-like) protease drastically increased in the livers of mice treated with HS for 7 days (Figure 3b). However, only a slight increase in the activity of caspase-9 and -3 (-like) proteases occurred in the livers of mice treated with HS. To investigate the role of caspase-8 in the liver injury induced by GalN/TNF- α and HS for 7 days, we evaluated the effect of caspase-8 inhibitor Z-IETD-FMK on the hepatotoxicity (Figure 3c). The caspase-8 inhibitor effectively protected mice from the hepatotoxicity induced by GalN/TNF- α and HS for 7 days and also from that induced by GalN/TNF- α alone. These findings show that TNF- α -induced liver injury associated with HS is caspase-8-dependent.

Effects of HS on the Expressions of FLIP_s and Akt in the Liver

The above results indicate that severe HS may promote TNF- α -induced liver injury through activation of caspase-8. Hence, we determined the expressions of FLIP, a physiological inhibitor of caspase-8, and Akt, which is known to regulate FLIP.²⁰ As shown in Figure 4a, the level of FLIP_s was decreased by single HS treatment, and decreased further by multiple HS treatment, although HS had no effect on the level of FLIP_L. Akt phosphorylation occurred in the normal liver, indicating that Akt is constitutively activated in the liver, and HS decreased the levels of phosphorylated Akt as well as those of Akt. In addition, severe HS decreased the levels of FLIP_s and blocked the activation of Akt in the liver of mice treated with GalN plus TNF- α (Figure 4b). The release of cytochrome *c* to the cytosol was little observed when sequences of HS were given to mice. In contrast, cytochrome *c* content in the cytosolic fraction of the liver dramatically increased with a reciprocal decrease in the membrane fraction of liver in either mice pretreated with or without repeated HS after GalN/TNF- α administration, indicating that hepatocyte apoptosis is induced via mitochondria-dependent pathway in the livers of mice treated with repeated HS and GalN/TNF- α comparably in those of mice treated with GalN/TNF- α alone.

Discussion

This study demonstrated that HS promoted or prevented TNF- α -mediated liver cell death depending on the duration of exposure. We found that repeated exposure to HS led to significant enhancement of TNF- α -induced hepatocyte apoptosis, whereas exposure to single HS protected against such apoptosis. The death-promoting effect of HS observed here is at variance with the commonly

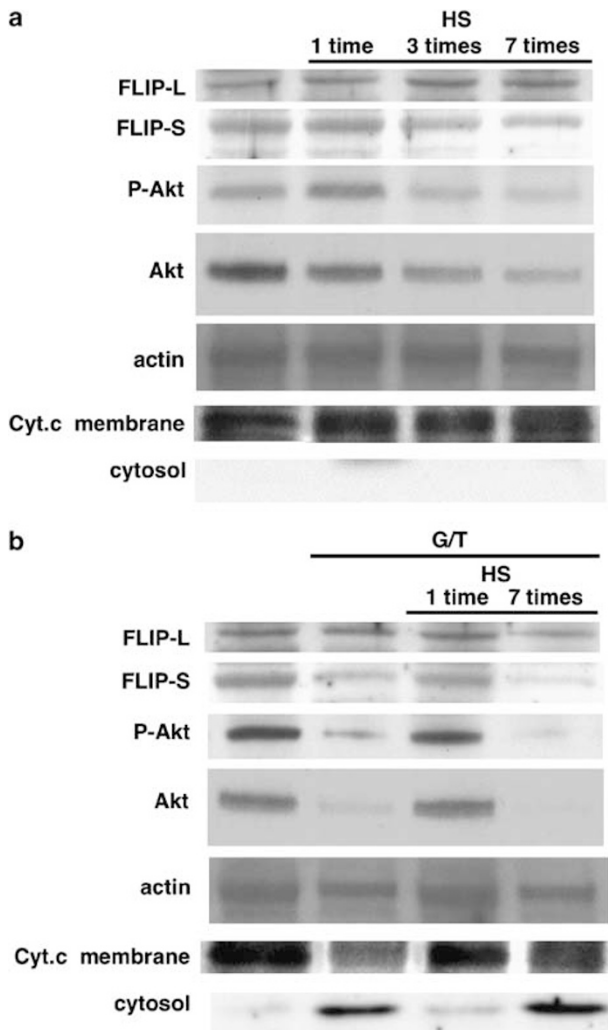


Figure 4 HS effectively blocked FLIP_s and Akt phosphorylation in the liver of untreated mice (a) and mice treated with GalN/TNF- α (b). Total liver proteins (50 μ g) from the mice were subjected to SDS-PAGE, and were probed with anti-FLIP_s, -FLIP_L, anti-Akt, antiphosphorylated-Akt, or *anticytochrome c*. The results shown are representative of at least three independent experiments.

described protective effect of HSP70 induced by HS. Molle *et al*¹⁰ have recently demonstrated that HS treatment of mice leads to a strong induction of HSP70 in several organs and confers significant protection against lethality induced by TNF- α . In addition, they have shown that mice deficient in the inducible *hsp70.1* gene were no longer protected by HS treatment, indicating that HSP70 is at least necessary for the conferred protection induced by whole-body HS of mice. Therefore, it was surprising to observe that HS, with subsequent HSP70 induction, promoted TNF- α -induced hepatocyte apoptosis rather than protecting against such hepatotoxicity. However, this discrepancy could be explained by the differing degrees of HS exposure. We found that single HS suppressed the hepatotoxicity, whereas repeated HS (HS performed for 7 days) promoted it (Table 1). A future experiment should be performed

in HSP70^{-/-} mouse to address whether HSP70 induction *per se* is responsible for exacerbating the liver injury after repeated HS as well as attenuates the injury induced by a single exposure to HS.

Consistent with our results, DeMeester *et al*²⁰ have demonstrated that induction of HS in cells primed by inflammation can precipitate cell death by apoptosis, whereas a prior heat shock stress can protect cell against inflammatory stress both *in vitro* and *in vivo*. They called this seemingly paradoxical ability of HS to induce cytoprotection and cytotoxicity the 'heat shock paradox'. However, Molle *et al*¹⁰ demonstrated that HS treated for 10 consecutive days did not lead to downregulation of HSP 70 expression, and still conferred protection against TNF lethality. It is difficult to precisely explain this discrepancy with our findings. Although they used TNF- α alone and did not show liver injury, we administered to mice TNF- α plus GalN, which is well known to increase the susceptibility of mice especially to liver damage and hepatocyte apoptosis in response to TNF- α .²¹ A sequence of stressful stimuli can produce unexpected responses at the cellular level, and may therefore result in different phenotypic responses in different organs or cells. For example, since caspases are the main executors of apoptosis, any sensitizing stimulus may affect the degree of activation of one or more caspases. In the present study, the activities of caspases-8 and -3 increased to a greater extent in repeated HS-challenged mice than in HS-nonchallenged mice. Moreover, the observation that caspase-8 was already cleaved and activated in the absence of apoptosis in heat-shocked samples of livers indicates that caspase-8 cleavage can be the cause of the acceleration, rather than its consequence.

This hypothesis is supported by the fact that HS specifically activated caspase-8, but not caspase-3 or -9. Moreover, administration of caspase-8 inhibitor prevented GalN/TNF- α -induced liver injuries both in HS-treated and nontreated mice, indicating that caspase-8 is the initial caspase in HS-mediated acceleration of hepatocytes in response to TNF- α -induced apoptosis, and suggesting that the effect of HS on hepatocyte apoptosis was upstream of caspase-8 activation. Many studies have demonstrated that caspase-8 expression acts as a key determinant of sensitivity for apoptosis induced by death-inducing ligands or cytotoxic drugs.²²⁻²⁴ IFN- γ sensitizes tumor cells resistant to apoptosis by upregulating caspase-8 expression through the signal transducer and activator of transcription-1 (STAT1) pathway.²⁵ The LF15-0195 immunosuppressive agent sensitizes Jurkat T cells to apoptosis induced by an anti-CD95 antibody and by the TNF-related apoptosis-inducing ligand by facilitating caspase-8 activation at the death-inducing signaling complex (DISC).²⁶

To further investigate the mechanism of HS-mediated caspase-8 activation, we examined the involvement of the DISC protein in this process,

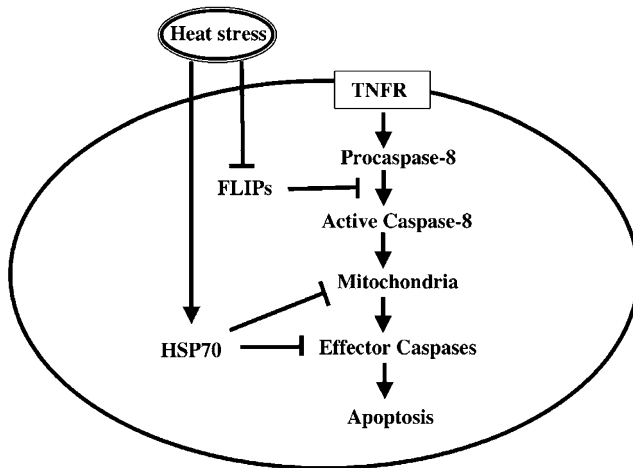


Figure 5 Proposed proapoptotic role of HS in TNF- α -induced hepatocyte apoptosis. Repeated HS simultaneously induces HSP70 and suppresses FLIP_S, resulting in the activation of caspase-8 in cells. These changes in the cells lead to enhancement of TNF- α -induced hepatocyte apoptosis.

since FLIP has been characterized as an endogenous caspase-8 inhibitor.²⁷ FLIP has been shown to correlate with resistance of cells against Fas-mediated apoptosis,²⁸ and downregulation of FLIP sensitizes a variety of endothelial cells and tumor cells to death receptor-mediated apoptosis.^{29–32} Tran *et al*¹⁸ have demonstrated that HS downregulates FLIP and sensitizes Jurkat cells to Fas-mediated apoptosis, which is consistent with our results. In most of the studies, however, FLIP-related sensitization to apoptosis has been shown *in vitro*. We here showed *in vivo* that repeated HS, but not single HS, downregulated FLIP_S and promoted TNF- α -induced liver cell death. FLIP is expressed in two isoforms, FLIP_L and FLIP_S, both of which can inhibit caspase-8 activation.³³ FLIP_L consists of two N-terminal death effector domains and a short C-terminal caspase homology domain devoid of enzymatic activity, whereas FLIP_S is only composed of the N-terminal death effector domains and a short C-terminal stretch of amino acids not found in FLIP_L. Both FLIP_L and FLIP_S can be recruited to the DISC but they function differently.³⁴ It has been reported that each of the FLIP proteins can be involved in susceptibility to death receptor-mediated apoptosis.^{18,24,35} In the present study, FLIP_S was downregulated by moderate HS, and further downregulated by repeated HS although FLIP_L was little changed (Figure 4a). This correlated with caspase-8 activation and apoptosis. These data suggest that downregulation of FLIP_S, rather than FLIP_L, promotes TNF- α -induced hepatocyte apoptosis in repeated HS-treated mice.

The levels of FLIP_S expression were associated with those of Akt activity, which is well known to regulate FLIP expression,^{36,37} but those of FLIP_L were not. It has been reported that FLIP_S and FLIP_L are differently regulated,³⁸ although the difference

in the regulatory mechanisms remains unclear. Our data suggest that Akt activity might regulate only FLIP_S, but not FLIP_L expression, and that this may contribute to the TNF- α -resistance in hepatocytes.

In conclusion, we demonstrated that repeated HS enhanced TNF- α -induced hepatotoxicity in GalN-sensitized mice through the activation of caspase-8 and the suppression of FLIP_S expression (Figure 5), in contradiction to the well established finding that prior heat shock stress protects cells via induction of HSPs. We believe that these results will help to provide an understanding of the mechanism of balance between cell death and viability, and provide important clues regarding the differential stress response interactions between single and sequential stimuli.

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