

Original Article

Effects of simulated weightlessness on liver Hsp70 and Hsp70mRNA expression in rats

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Abstract: Space flight is known to induce a number of hepatic physiological alterations. In this study, we investigated Hsp70 expressing features of rat liver under simulated weightlessness. Tail-suspension was used to simulate the weightlessness animal model. Forty-eight male Wistar rats were randomly assigned to 6 experimental groups and Hsp70 protein and mRNA expressions in the liver were detected by Western blot and RT-PCR respectively. The tail-suspension significantly increased Hsp70mRNA expression levels in rat liver ($P<0.05$). The semi-quantitative PCR showed that Hsp70mRNA was upregulated as early as 6 hours of suspension. Western blot analysis indicated that Hsp70 protein was significantly upregulated in the early stage of suspension as compared with controls ($P<0.05$). The results suggest that simulated weightlessness acts as a kind of stress to elevate liver Hsp70 expression both at protein and mRNA levels. This may be meaningful in astronaut's trainings by preadaptation to non-damaging stress exposures or other environmental factors to foster the astronaut's ability of weightless tolerance.

Keywords: Hsp70, simulated weightlessness, rats, liver, RT-PCR, Western blotting

Introduction

Gravity has been a constant physical factor during the evolution and development of life on Earth. Although the absence of gravity can not accurately be simulated on the ground, several kinds of models could mimic some responses observed after exposure to microgravity. Antirothostatic bed rest in humans and tail-suspension or hindlimb unloading in rodents are two of the most commonly used models which can mimic many of the physiological alterations in various organ systems caused by actual spaceflight including fluid shifts, muscle atrophy, bone demineralization, and depressed cellular immunity [1,2]. Although there were research reports on the pathophysiological alterations of the digestive system under microgravity in the literature, the effects of the simulated weightlessness on liver Hsp70 and Hsp70mRNA expression have not been elucidated. Accordingly, the present study was designed to document Hsp70 expressing features of rat liver under simulated weightlessness and to determine

whether there is associated stress response that microgravity imposed upon liver.

Materials and methods

Animals

The experiments were performed after approval by the local Ethics Committee and conducted on Male Wistar rats (Laboratory Animals Center, China Agriculture University). Animals were fed standard laboratory chow, given free access to water, and maintained on a 12:12-h light-dark cycle at $23 \pm 2^\circ\text{C}$ in pathogen-free conditions.

Hindlimb suspension

Forty eight male Wistar rats weighted 300 ± 20 g were randomly assigned to 6 experimental groups: suspended for 6 hrs, 12 hrs, 24 hrs, 48 hrs, 96 hrs and 0 hr (control).

The procedure of tail-suspension described by Chen *et al* [3] was adopted in the present study.

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Animals were suspended in a 30° headward tilt position, and the forelimbs maintained contact with the floor, allowing a 360° range of movement and access to food and water freely. The suspension height was adjusted to prevent the animal's hindlimb from touching any supportive surface. The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages.

At the end of the experiment, animals were anesthetized with pentobarbital (45 mg/kg) and laparotomy via middle line was made. The right lobes of the livers obtained from control and suspended animals were immediately frozen in liquid nitrogen at -80°C until analysis, and the left lobes of the rat livers were preserved in zinc-buffered formalin.

RT-PCR Analysis

Total RNA was extracted from the liver tissues of both suspended and control animals with Tri-Reagent kit (Invitrogen, Carlsbad, CA, USA). The total RNA was used for analysis of Hsp70 messenger RNA by RT-PCR. The primer pairs designed from sequences published in GenBank were as follows: Hsp70 upper primer: 5'-TGA GCA GCC CAT CCT TAG TG-3'; Hsp70 lower primer: 5'-ATA GGC ATC CGT CCC TTT GT-3'; GAPDH upper primer: 5'-AAA CCC ATC ACC ATC TTC CAG-3'; GAPDH lower primer: 5'-AGG GGC CAT CCA CAG TCT TCT-3'. The amplified fragments of Hsp70 and GAPDH were 324bp and 360bp respectively.

After sample denaturation at 94°C for 5 min, PCR was done for 35 cycles consisting of denaturation at 94°C for 50 s, annealing at 50°C, 53°C, 56°C, 60°C and extension at 72°C for 10 s. The amplification was terminated by a 10 min final extension step at 72°C. The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining and gene sequence analysis. The separated PCR products were visualized under ultraviolet (UV) light and the integrated optical density (IOD) was determined for each PCR product using Alphamager 2200 and AlphaEase FC software package (Version 3.2.1, Alpha Innotech Corporation, CA, USA).

Western blot analysis

The frozen tissues were pulverized by freeze-fracturing in liquid nitrogen 3-5 times and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 10 mM Na pyrophosphate, 50 mM Na fluoride, 5 mM Na orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 10 g/ml leupeptin, and 10 g/ml pepstatinA). Homogenates were centrifuged (12000 rpm) at 4°C for 80 min to remove cellular debris. After 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis, all samples were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding sites were blocked with 5% (w/v) BSA and 0.01% (v/v) Tween-20 in Tris-buffered saline (TBS, pH 7.6) for 3 h at room temperature. The membrane was washed for 10 min with three changes of TBS, and the proteins on the membrane were incubated with 1:200 dilution of monoclonal antibody to Hsp70 (Santa Cruz, USA) overnight at 4°C. Membranes were washed for 10 min with three changes of TBS, and then incubated with secondary antibodies conjugated to horseradish peroxidase (GAR-AP, Zymed, USA) at room temperature for 3 h. Immunoreactive proteins were detected by enhanced chemiluminescence. The relative levels of intensity of each blot was densitometrically scanned and analyzed.

Data analysis

Statistical analyses were performed using the SPSS 11.0 for Windows software package. For each parameter tested, data are expressed as means ± SE. Differences were considered significant at $P < 0.05$.

Results

RT-PCR analysis

We first studied whether Hsp70mRNA is expressed in rat liver under simulated weightlessness using RT-PCR. The results are shown in **Figure 1**. PCR products around the predicted size of 324 bp were detected in the liver tissues of both suspended and control animals. Compared with non-suspended animals, however, the tail-suspension significantly increased Hsp70mRNA expression levels in rat liver ($P < 0.05$). The semi-quantitative PCR showed that Hsp70mRNA was upregulated as early as 6

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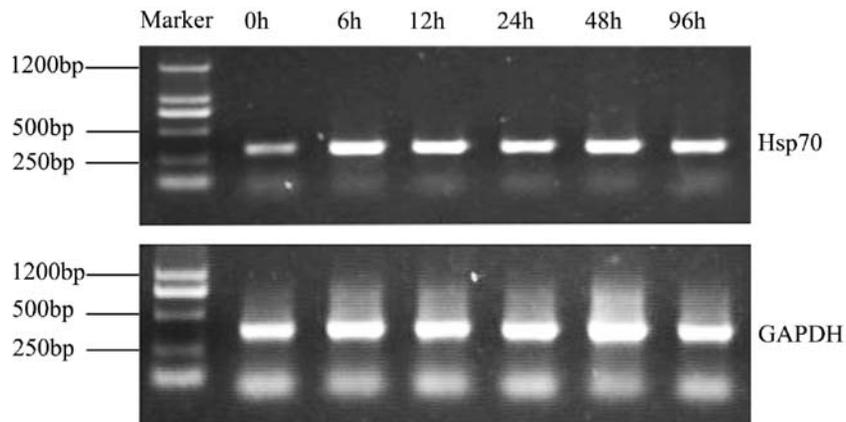


Figure 1. Hsp70mRNA (324 bp) was detected in the liver tissues of both suspended and control animals by RT-PCR analysis.

hours of suspension, with peak value at 12 hours, and persisted above the control level in all suspension subgroups. A fluctuation of Hsp70mRNA expression existed among suspended groups but without significant difference (**Figure 2**).

Western blot analysis

Western blot analysis indicated that Hsp70 pro-

tein was expressed in livers of all suspension subgroups as well as control group animals. The form of Hsp70 protein in rat liver was approximately 70 kDa (**Figure 3**). The expression of Hsp70 in rat liver was significantly upregulated in the early stage of suspension as compared with controls ($P < 0.05$). The level of Hsp70 expression appeared peak at 6 hours, followed by a declining tendency, and decreased to below the level of the control group at 96 hours sus-

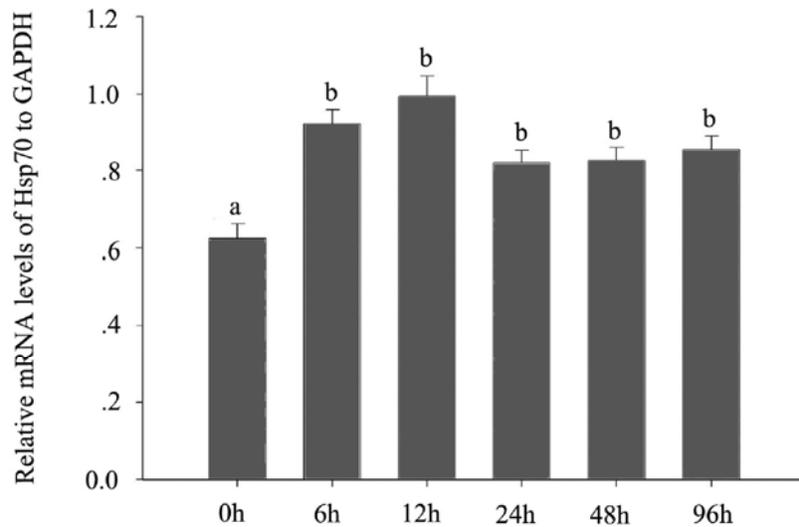


Figure 2. The semi-quantitative PCR showed that Hsp70mRNA was upregulated as early as 6 hours of suspension, with peak value at 12 hours, and persisted above the control level in all suspension subgroups. Values are expressed as means \pm SE. Significant differences ($P < 0.05$) are indicated by different letters.

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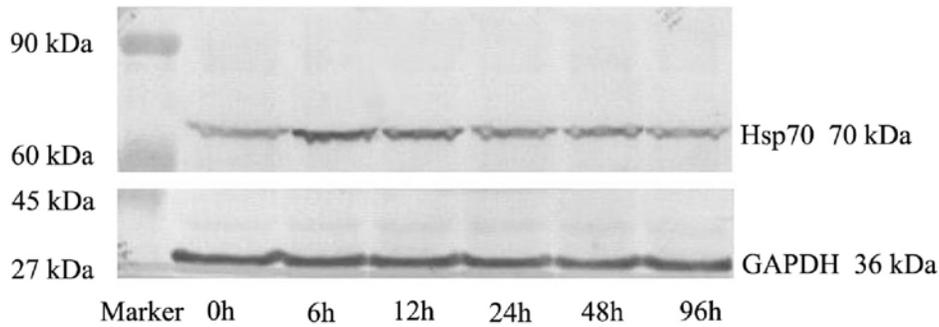


Figure 3. Western blot analysis indicated that 70 kDa Hsp70 protein was expressed in liver samples of all suspension subgroups and control group animals.

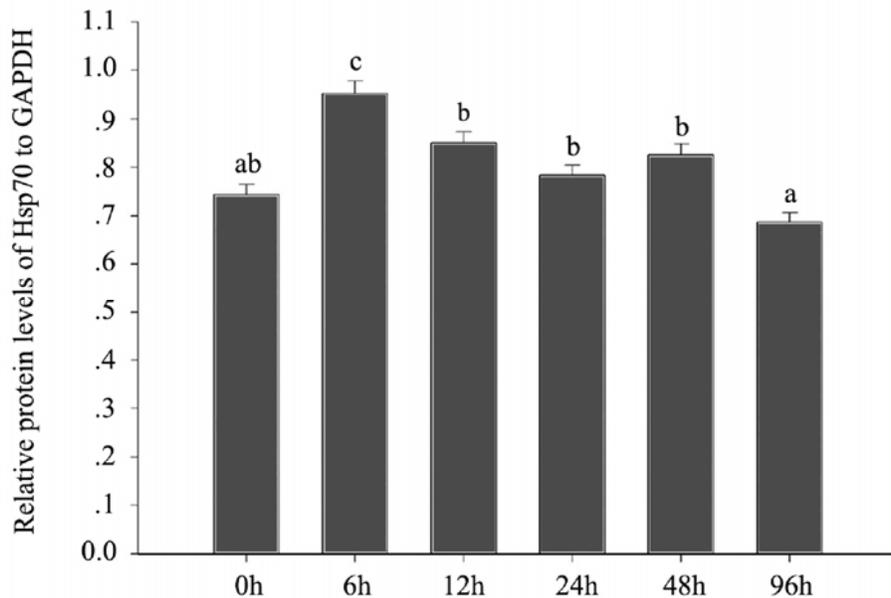


Figure 4. The expression of liver Hsp70 in rats was significantly upregulated in the early stage of suspension as compared with controls ($P < 0.05$). The peak hour of Hsp70 expression appeared at 6 hours suspension, earlier than that of Hsp70mRNA, followed by a declining tendency with more gradient than that of Hsp70mRNA. Values are expressed as means \pm SE. Significant differences ($P < 0.05$) are indicated by different letters.

pension with no significant difference (**Figure 4**).

Discussion

Space flight is known to induce a number of hepatic physiological alterations. Previous studies indicate that certain constituents and enzymes of liver metabolism are indeed altered by exposure to microgravity. Abraham *et al* [4] investigated hepatic biochemical changes in rats

flown aboard the Cosmos 690, 936, and 1129 biosatellite missions. At landing, hepatic glycogen content increased by 30-70% above ground-based controls; accumulation of fat droplets in hepatocytes was noted; and increases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were also reported. The findings were later supported by data collected from rats flown aboard the Cosmos 2044 and 1887 as well as the Spacelab 3

missions [5]. Rivera *et al* [6] studied the effects of 4 wk hindlimb unloading on the liver in Wistar rats and two mouse strains: endotoxin-sensitive C57BL/6 mice and endotoxin-resistant C3H/HEJ mice, and found that endotoxin was elevated by >50% after hindlimb unloading in portal blood samples. The portal endotoxemia was associated with hepatic injury in rats and endotoxin-sensitive mice as indicated by inflammation and elevated serum transaminase activities. Clement *et al* [7] applied DNA microarray technology in time-course experiments for genome-wide search of genes whose expression are altered by microgravity, as part of the effort in the identification of major space genes. The global gene expression profiles for a human liver cell line exposed to a ground-based modeled microgravity system for 1, 3, and 4 days were analyzed using the rotary cell culture system (RCCS) and the Agilent 22k human oligo DNA microarrays. Some of these identified genes were further verified by Northern analysis. The results showed that 139 genes' mRNA levels were significantly ($P < \text{or} = 0.01$) altered by the microgravity exposures. Cui and colleagues [8] investigated NF- κ B expression in rat liver under simulated weightlessness. Tail-suspension was used to simulate the weightlessness animal model. The expressions of NF- κ B p65 were detected by using Western blot analysis and immunohistochemistry PV-6001 respectively. The results showed that tail-suspension significantly increased liver NF- κ B expression in the rats, with peak expression in 1- and 2-day suspension subgroups, followed by the gradually declining to near the normal level in 5- to 7-day subgroups. NF- κ B expression in rat liver stained as brown particles were detected in the infiltrated cells and kupffer cells as well as hepatocytes. Three types of intracellular NF- κ B expression, i.e. cytoplasm, nucleus, and cytoplasm plus nucleus, were found uniformly or co-existent in rat liver according to the location of positive NF- κ B particles. This study showed that the simulated weightlessness acts as a kind of stress to induce the activation of NF- κ B in liver especially in the early stage of microgravity, and suggested that NF- κ B plays important roles in the cascade reaction and adaptation of the liver to the weightlessness stress.

The hepatic pathophysiological alterations are supposed to be affected by the comprehensive factors under weightlessness condition, directly or indirectly. Microgravity may be considered as

a stressor for astronauts or experimental animals. However, little data is available about whether there is associated stress proteins response that microgravity imposed upon liver, though there are certain reports on the expression of stress proteins in other organs of those exposed to microgravity environment. Ohnishi *et al* [9] studied the levels of Hsp72 in several organs from goldfish which were taken into space on the NASA space shuttle. They found that a remarkable accumulation of Hsp 72 was detected in muscle and spleen of those fish taken into space as compared with controls, and considered that the microgravity may be a stressor for goldfish as a possible mechanism for the accumulation of HSP72 in flight samples. The experimental studies showed that the expression of stress proteins is a kind of stress response at the molecular level induced by the space environment consisting of microgravity and/or cosmic radiation as stressors. Cotrupi and Maier [10] tried to verify that Hsp70 is upregulation crucial for cellular proliferative response in simulated microgravity. They characterized the behaviors of endothelial cells and of the human monocytic cell line U937 cultured in the NASA-developed bioreactor to simulate microgravity. It was noteworthy that all the cells which maintained the capability to proliferate in microgravity upregulated the stress protein Hsp70. Liu and his colleagues [11] examined the effect of simulated weightlessness on inducible Hsp70 expression in the heart and vessel tissues of rats, a tail-suspension rat model was used to simulate weightlessness. The results showed that the levels of inducible Hsp70 expression in the vessels were related with their anatomical locations, for that the expression of both Hsp72mRNA and Hsp72 significantly increased in basilar arteries, whereas it showed a slight decline in femoral arteries. The blunted Hsp70 expression in myocardium suggests that simulated weightlessness may induce myocardial changes similar to those in aging, and Hsp70 expression changes in arteries are in accord with the trend of differential adaptation changes in vessels to simulated weightlessness. Amann *et al* [12] studied the effects of microgravity or simulated launch on testicular function in rats. Testes from flight rats on Cosmos 2044 and simulated-launch, vivarium, or caudal-elevation control rats were analyzed by subjective and quantitative methods. They found that there was no evidence for production of stress-inducible transcripts of the Hsp70 or Hsp90

genes in rat testicular tissues.

It is well established that the heat shock proteins (Hsps) are a family of highly conserved and abundantly expressed proteins. While acting as molecular chaperones in unstressed cells, Hsps protect cells against a wide variety of environmental stresses and promote cell survival during periods of both acute and chronic stress. Within the Hsp families, Hsp70 is a 70-kDa protein originally described as a molecular chaperone and is intimately involved in trans-mitochondrial protein transport. In addition to its properties as a chaperone, Hsp70 also functions as an important cytoprotectant. The over-expression of liver Hsp70 can be induced by many adverse conditions such as heat including thermotherapies, certain drugs and metabolic poisons, ischemia-reperfusion, oxidative stress, apoptosis and oncogenic activity, etc [13-16].

Our study showed that the tail-suspension significantly increased Hsp70mRNA expression levels in rat liver ($P<0.05$). The semi-quantitative PCR indicated that Hsp70mRNA was upregulated as early as 6 hours of suspension, with peak value at 12 hours, and persisted above the control level in all suspension subgroups. The expression of Hsp70 in rat liver was also significantly upregulated in the early stage of suspension as compared with controls ($P<0.05$). The peak hour of Hsp70 expression appeared at 6 hours suspension, earlier than that of Hsp70mRNA, followed by a declining tendency with more gradient than that of Hsp70mRNA. The results showed that Hsp70mRNA had more stability than HSP70 in rat liver under simulated weightlessness. It has been suggested that Hsp70mRNA is stabilized after the exposure to some physical stresses, although its half-life is relatively short under nonstress conditions. Hsp70 is involved in repair and protection after the insult, and the prolonged presence of this protein is detrimental. Consequently, Hsp70 expression must be tightly regulated. A possible hypothesis is that Hsp70 mRNA is targeted for degradation when Hsp70 exceeds denatured substrates. Thus, there is a rapid increase in Hsp70 transcription when cells are exposed to stress. Immediately after the insult, Hsp70 protein in the cell is largely associated with denatured polypeptide substrates, in accordance with the chaperone function of this stress protein. Over time, the number of polypeptide substrates is reduced due to refolding

of these polypeptides, and the proportion of free vs. substrate-bound Hsp70 is increased. This free Hsp70 protein is then able to affect further Hsp70 production by attenuating transcription or accelerating the degradation of its own mRNA. Prior studies have shown that binding of Hsp70 to a target polypeptide causes a conformational change in the protein, reducing its RNA binding capacity. The binding of Hsp70, directly or indirectly, to its own message during recovery after stress may be related to the decay process. This event may be part of the self-limiting mechanism for Hsp70 expression previously proposed [17]. However, it needs to be interpreted with caution because the stress conditions are quite different and complicated. The molecular mechanisms of interaction between Hsp70 and Hsp70mRNA in cells after stress remain to be explored.

Interestingly, although its name suggests that expression is exclusively induced during cellular hyperthermia, the Hsp70 and Hsp70mRNA in liver can be induced and/or constitutively expressed by another cellular insult or stressor—microgravity. The findings suggest that liver is susceptible to the microgravity or simulated weightlessness stress, and the expression of the Hsp70 gene is regulated not only at the transcription level but also at post-transcriptional levels, including mRNA stabilization and translation. It was at the early stage of suspension that the expression of both Hsp70mRNA and Hsp70 in the liver were upregulated, which is consistent with the features of inducible Hsp70. The ability of cells to develop thermotolerance or stress tolerance, a state of transient and nonspecific resistance to severe stress, after a mild heat shock or stress exposure is thought to be largely the responsibility of Hsp70, which is proposed to be due to stabilization of basic cellular processes, such as translation [17]. This may be meaningful in astronaut's trainings by preadaptation to non-damaging stress exposures or other environmental factors to foster the astronauts' ability of weightless tolerance.

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