Aging Effects on the Habitual Expression of HSP70 mRNA in the Hippocampus of Rats

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Abstract

Heat shock proteins are induced by stressful stimuli and have been shown to protect cells and organs from such stresses both *in vitro* and *in vivo*. This study examined the regulation of HSP70 mRNA expression and detected the effect of aging on RNA expression in hippocampus of rats. The stress models were built by using forced-swimming in 25°C and 4°C water, respectively. Two groups of male rats, 2-month-old and 16-month-old, respectively, were randomly divided into three subgroups: acute stress (AS) model, chronic habituation stress (CHS) model and chronic dishabituation stress (CDS) model. Observation of exploratory behavior in an open-field (OF) test indicated stress levels. The expression of HSP70 mRNA in hippocampus was measured by RT-PCR after 0, 30, 60, 180, and 360 min of stress, respectively. Results showed that the number of quadrant crossing in both aged CHS and young CHS groups decreased gradually with the process of stress, reflecting an adaptation to the stress condition. Repeated swimming in warm water resulted in habitual expression of HSP70 mRNA in both young and aged CHS group, indicating an adaptation to the stress. The RNA expression of young CHS group was significantly stronger than that of the aged CHS group at 30, 60, 180, and 360 min after stress ($P < 0.05$). Meanwhile, in an intensive stress level in which the rats swam in 4°C water, a high expression level of HSP70 mRNA was achieved in CDS groups, producing a dishabituation that proved the habitual expression from the other side. These results showed that senescence dramatically affected both exploratory behavior and HSP70 mRNA expression in rats' hippocampus. The results also suggested that chronic stress could lead to the habituational expression of HSP70 mRNA, but high intensive stress could reverse the habituational state and lead to the dishabituational expression. Moreover, the duration of stimuli is one of the important factors that affect the level of HSP70 mRNA expression.

Key Words: chronic stress, HSP70 mRNA, dishabituation, open-field test, hippocampus

Introduction

Heat shock proteins (HSPs) belong to an evolutionarily conserved group of stress proteins that are preferentially synthesized in cells exposed to various stresses such as heat, radiation, anoxia chemicals, ethanol, and viral infection. HSPs function as molecular chaperones facilitating the protein folding and repairing of misfolded and damaged proteins resulting from aging (4, 19, 20). Stress can induce the expression of HSP families (HSP90, HSP70, HSP60, HSP27) in many tissues. HSP70 increases rapidly after stress, and it is very sensitive to temperature change and other kinds of stress (7, 31). So it is recognized as one of the main chaperones associated with cell protection against stress. It was reported that the expression of HSP70 mRNA could be observed for as long as 15 min and maintained at a high level for 6 hours in cerebral,

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cerebellum and brain stem after injury (3, 11). The expression of HSP70 is influenced by many factors, such as models and duration of stress, aging and gender. Some studies suggested that HSP70 was preferentially induced in specific brain region involved in the regulation of hypothalamic-pituitary-adrenal (HPA) axis function that HSP70 was an integral component of the mammalian physiological stress response. *In vitro* experiment, the expression of HSP70 decreased in aging cells. Nitta demonstrated that terminal T-cell expressed HSP70 fewer than early T-cell in heat shock response (HSR), which took place at transcription level (21). There is a little expression of HSP70 mRNA *in vitro* in aged rats' skin, lung and brain. Therefore, with the process of aging, the cellular ability of synthesizing HSP70 decreased obviously (32). The expression of HSPs is mainly regulated at the level of transcription in mammalian cells. It is reported that chronic stress could enhance the level of transcription of HSP70 mRNA in brain and the expression level, those processes are related to the duration of stimuli significantly (5, 8).

Aging is often associated with an increased incidence of infections and general morbidity and mortality (15). Studies in cultured cells and animal models have demonstrated that the stress response is age-dependent (12, 16, 17). An age-related decrease in major HSPs has also been reported in human peripheral blood cells (22), and synthesis of inducible HSP70 is impaired in aged animals following acute stress. Few research has been done to study the regulation of the expression of HSP70 mRNA after stress, which refers to the habituational expression of HSP70 mRNA in our study, and few studies examine the effect of aging on the expression of HSP70 mRNA *in vivo*. In this study, we investigated whether there was an age-related change in habituational expression of HSP70 mRNA in hippocampus of rats aged between 2 and 16 months. Aging is an important factor affecting behavior of animals and human beings. The exploratory behaviors and locomotor activities during open field test can reveal the rat's adaptation to a new environment (5, 9), which indicates the cognitive ability of rats. Therefore, to clarify the change tendency of HSP70 mRNA after chronic stress and the effect of aging on the expression of RNA, we studied the expression of HSP70 mRNA in hippocampus of rats under forced-swimming stress by reverse transcription-polymerase chain reaction (RT-PCR) with an endogenous internal standard.

Materials and Methods

Animals

One hundred and twenty male Wistar rats aged 2 months (n = 60) and 16 months (n = 60) were employed from the Animal Experiment Center of Shandong University, bred on a light/dark (12 h/12 h) cycle at 24°C and given food and water freely except during experiment. Young and middle-aged rats were randomly divided into four subgroups respectively (control group and different stress groups, with 15 rats in each group).

Stress Models

Chronic habituation stress model (CHS): Animals were forced to swim in 25°C warm water in a 70 × 40 × 60 cm aquarium for 8 min at 8:30 am, lasting for 8 days.

Chronic dishabituation stress model (CDS): Animals were forced to swim in 4°C cold water for 8 min on the eighth day after swimming in 25°C water for 8 min of 7 days in the same aquarium.

Acute stress model (AS): Animals were forced to swim in 4°C cold water for 8 min in the same aquarium.

Hippocampus Separation and HSP70 mRNA Assay

Three stressful groups of different aged animals were sacrificed immediately under anaesthesia at each point of 0, 30, 60, 180 and 360 min after stress, and the hippocampus was separated immediately. RNA was isolated with TRIzol solution (Sigma, St. Louis, MO, USA). One microgram of total RNA was reverse transcribed by AMV reverse transciptase (Sigma) at 42°C for 1 h. PCR amplification reaction mixtures contained HSP70 cDNA, HSP70 primers (forward: 5'-CGCGACCTGAACAAGAGCAT-3', reverse: 5'-TCGAAGGTCACCTCGATCTG-3'), β-actin primers (forward: 5'–GTGGGGCGCCC CAGGCACCA–3', reverse: 5'-CTCCTTAATGTCACGCACGATTT3'), TapMan Universal PCR Master Mix. Thermal cycle conditions included holding the reactions at 94°C for 3 min, and cycling for 30 cycles among 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min. The results were identified by gelose electrophoresis. The rate of HSP70 and inter control β-actin represented the relative expression of HSP70 mRNA.

Open Field (OF) Test

OF box is a 90 × 90 × 45 cm wooden box, which is used to study the cognitive and emotional reaction by observing the animal's behaviors (27). Its bottom was divided into 5 × 5 squares, and the square in the middle of the bottom was the center square, while others were peripheral squares. Eight rats selected randomly from each group were observed by OF test, which was performed on the day before stress and right after the stimulus during the period of stress.

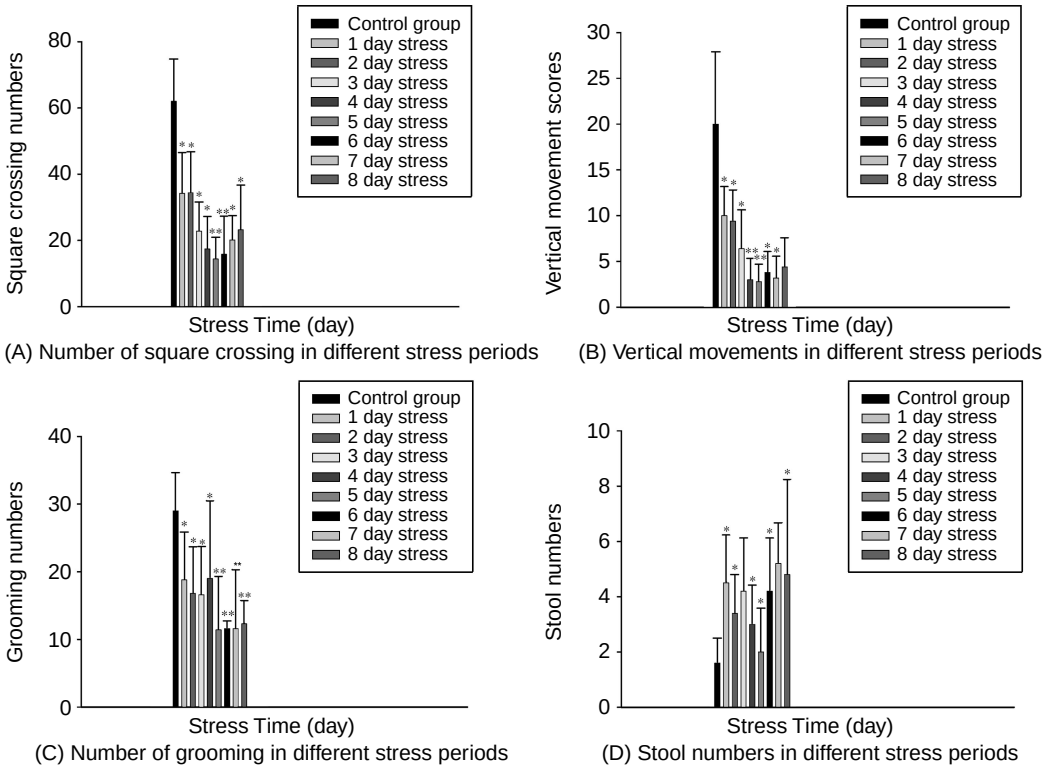


Fig 1. The results of open field test of young CHS group (mean ± SD) (n = 8). $^{**}P < 0.001$, $^{*}P < 0.05$, different from that of the control group (Student's *t*-test).

Each animal was placed in the center of the OF box, observed for 3 min. The indices of test included the number of square crossing, number of grooming, vertical movement scores and numbers of stools. All indices were observed and recorded by two persons who did not know the purpose of this test. OF box was cleaned up after each test session.

Data Analysis

Statistical analysis to test for behavioral differences among different groups was done by using SPSS11.0. Student's *t*-test was used to access differences between individual groups. One-way ANOVA was used to analyze the different expression of HSP70 mRNA between young and aged groups.

Results

Change of OF Test Indices in Different Stress Models

Both young and aged groups represented no significant difference in exploratory behavior before stress. With prolongation of the experiment days, the quadrant crossing, grooming and vertical movement scores of aged and young CHS groups exhibited downtrend obviously. The indices of aged and young CDS groups also had the same trend during the chronic stress, but the trend reversed significantly after the last intense stimulus, reflecting an excitement state. (Figs. 1, 2, 3, 4.)

Expression of HSP70 mRNA in Hippocampus of Different Stress Models

HSP70 mRNA expression was examined by RT-PCR in hippocampus of CHS, CDS and AS groups after 0, 30, 60, 180 and 360 min of stress. There was a significant increase expression of HSP70 mRNA after 0, 30, 60, and 180 min of stress, but a gentle decline at 360 min, which presented an habituation expression tendency in young and aged CHS groups. The aged CHS group resulted in a slighter expression than that of the young CHS group, suggesting that the aging body exhibited weak accommodation to the stressful condition. We found an increase trend of HSP70 mRNA expression as low as 0 min after stress in young and aged AS groups which maintained at a high level for up to 360 min and also noted that HSP70 mRNA of AS groups induced higher than that of the CRS and CDS groups. Special care was taken to examine HSP70 mRNA expression in two CDS groups, which were forced to swim in both 25°C for 7 days and 4°C water for 1 day. The results showed

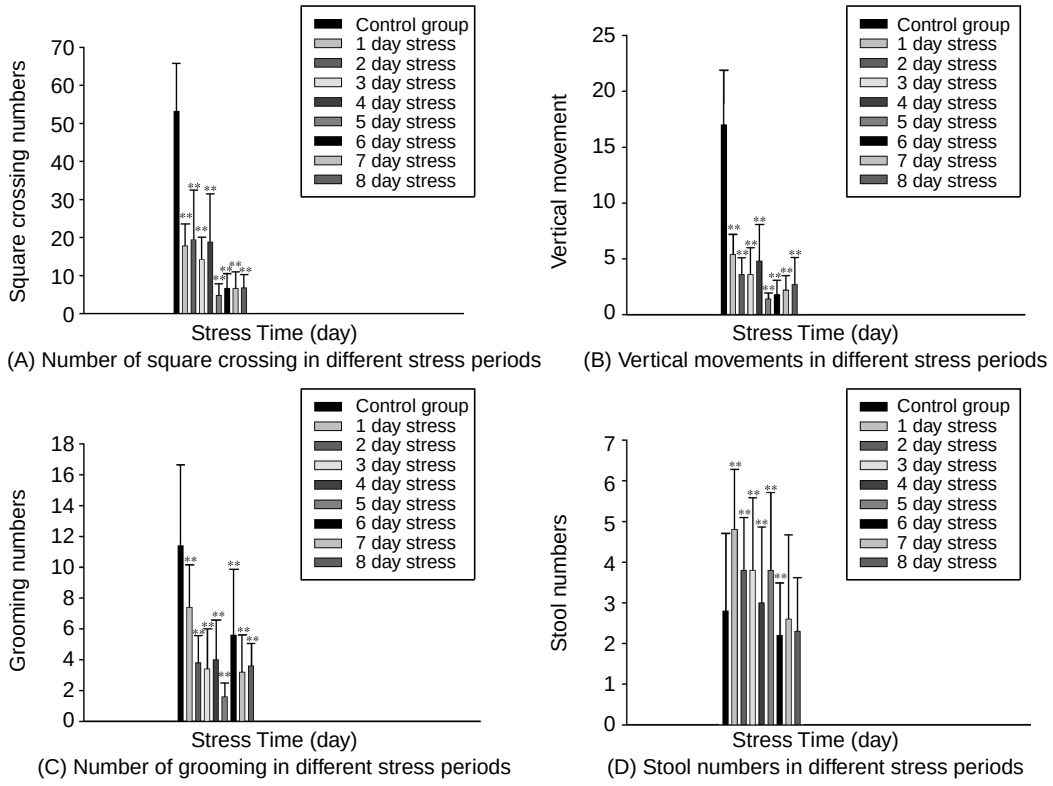


Fig 2. The results of open field test of aged CHS group (mean ±SD) (n = 8). ** $P < 0.001$, *$P < 0.05$, different from control group (Student's *t*- test).

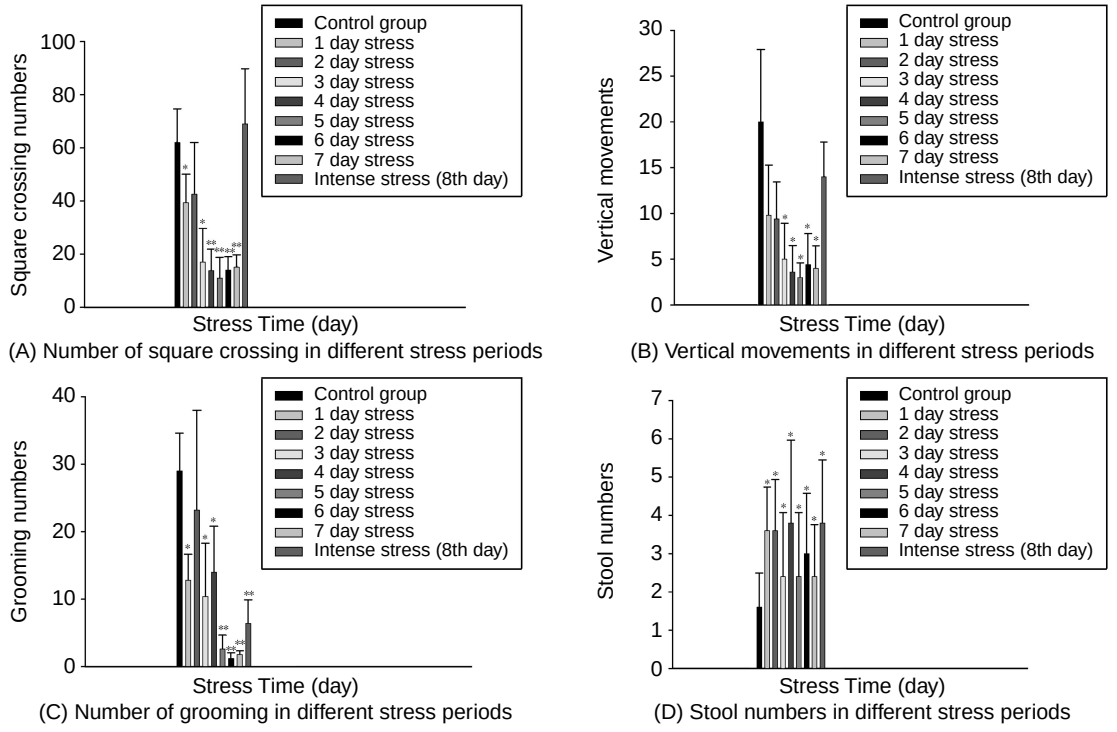


Fig 3. The results of an open field test of young CDS group (mean ± SD) (n = 8). **$P < 0.001$, *$P < 0.05$, different from control group (Student's *t*-test).

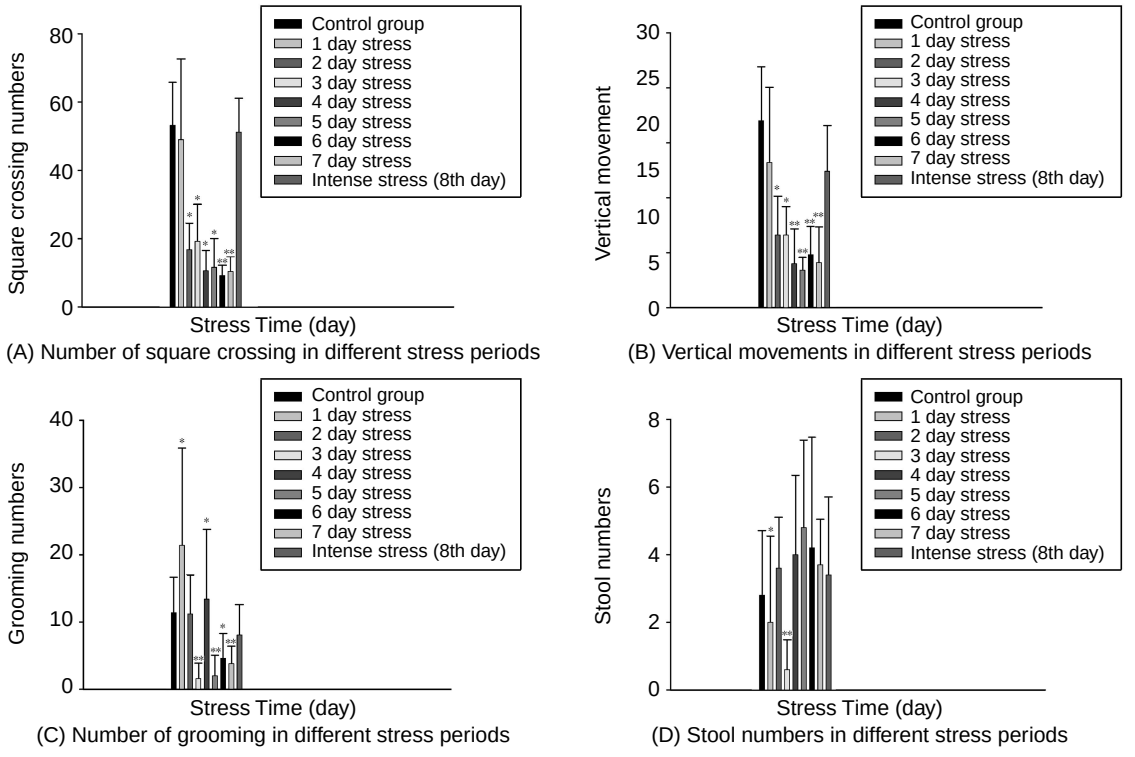


Fig 4. The results of an open field test of aged CDS group (mean ± SD) (n = 8). $^{**}P < 0.001$, $^{*}P < 0.05$, different from control group (Student's *t*-test).

that HSP70 mRNA was induced immediately at the end of stress and increased gently in 360 min. However, it was important to note that the expression level of CDS was higher than that of the CHS but lower than AS (Fig. 5).

Relative HSP70 mRNA Expression of Three Aged Groups

Three aged groups took in different stress models separately and exhibited different expressive tendency (Fig. 6). The elevation in HSP70 mRNA expression occurred rapidly in all three groups compared with control group, but HSP70 expression of aged AS group increased distinctly and maintained at a high level for 6 h expressed RNA is greater than that of chronic stress groups (CHS and CDS). Maximum HSP70 mRNA expression of aged CHS group was achieved after only 30 min but declined with the prolong of the time and hence exhibited a habituational expression, which reflected an adaptation to the stressful condition. HSP70 mRNA expression of the aged CDS group was higher than that of the aged CHS group but lower than that of the aged AS group after as long as 6 h of stress. Those results showed that an intense stimulus could reverse the habitual HSP70 mRNA expression which was based on the adaptation to chronic stress. Meanwhile, the expression mode of the aged CDS group also demonstrated the adaptive response to the stimuli and stressful protection.

Effect of Aging on Relative HSP70 mRNA Expression

To determine whether aging has an effect on HSP70 mRNA expression, hippocampuses from different stress models of both young and aged groups were assayed for the presence of HSP70 (Fig. 7). Tissues from all groups showed induction of HSP70 with different expressive level at different time points. HSP70 of AS group was higher than that of CHS and CDS groups. More importantly, HSP70 expression of aged groups on three stress models was lower than that of young groups at different time points, especially in CHS model, reflecting a poor response for aged animals in chronic stressful condition. It was also important to note that maximum HSP70 expression of aged group achieved after as little as 30 min and declined obviously in CHS model. No same tendency was shown in CDS and AS models. On the contrary, RNA expression of aged CDS and AS groups elevated gradually and achieved as the same level with young groups after 180 min.

Discussion

Animal behaviors including attacking behavior, languishing behavior and excreting behavior change apparently in the stressful conditions (1). In this report, forced-swimming, a relative mild physiological

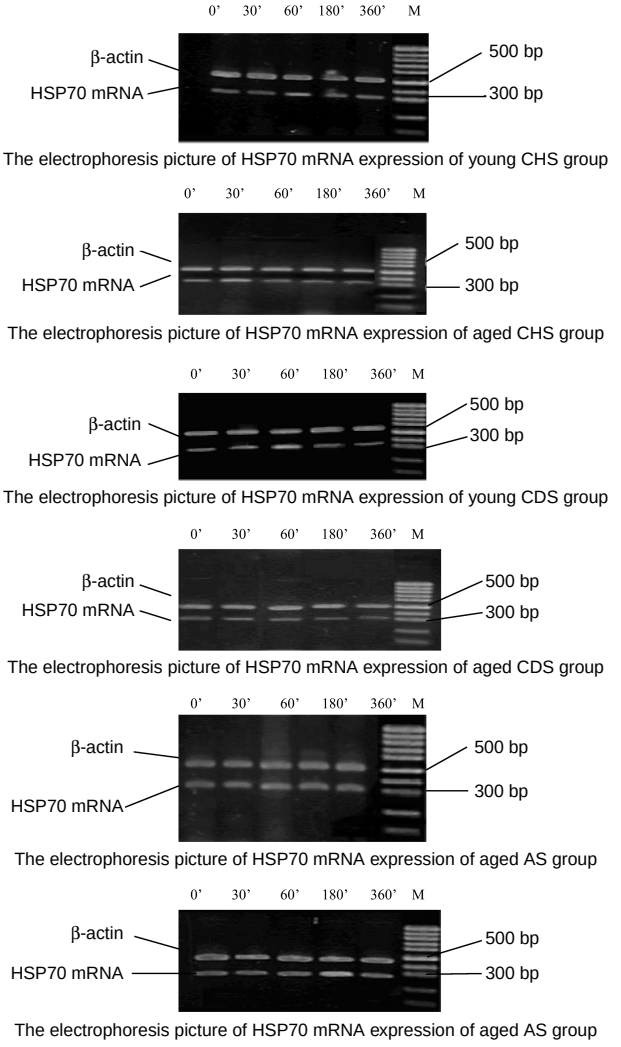


Fig. 5. RT-PCR showing hippocampus HSP70 mRNA expression after a period of different stress. Total RNA was isolated from hippocampus and assayed for HSP70 at 0, 30, 60, 180 and 360 min after stress. All CHS, CDS and AS groups significantly induced HSP70 mRNA expression which exhibited different tendency and expression levels between young and aged rats.

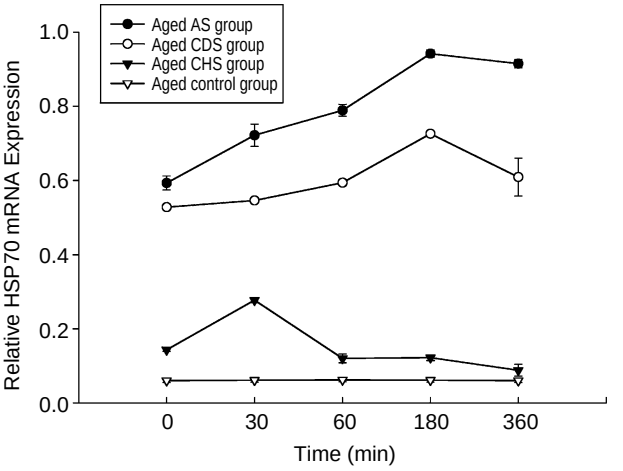
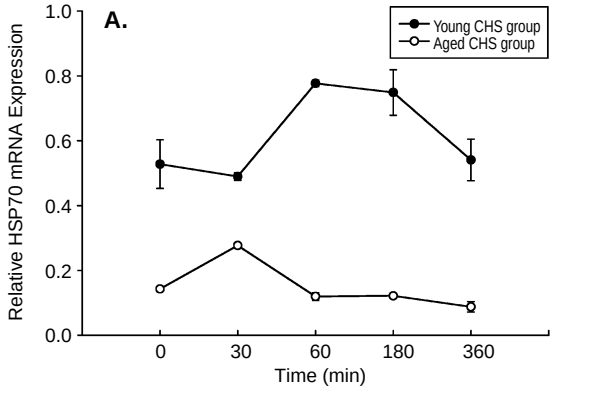


Fig. 6. Dynamic change of HSP70 mRNA relative expression of every aged group (mean ± SD). There were15 rats in each group, rats were killed at 0, 30, 60, 180 and 360 min after stress and RNA was isolated immediately at each time point, which was assayed for HSP70.

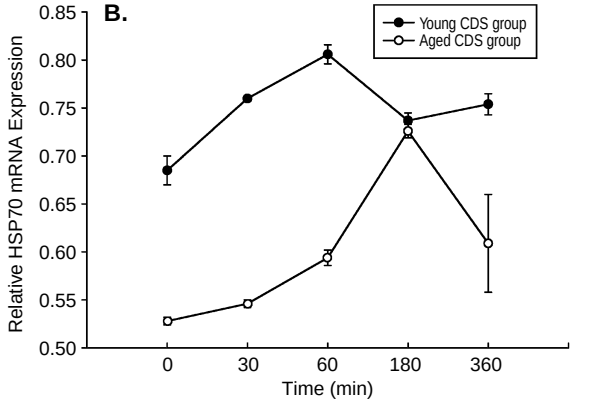
stress that did not cause tissue damage, was used to build an animal model. An open field test was also used to evaluate the rat behavior change during stress. The quadrant crossing was a locomotor activity that showed the rat's movements in open field, grooming, vertical movements and the numbers of stool were emotional responses that indicated the tension of the rats in a new environment. These indices helped us to evaluate the behavioral changes of rats in response to stress (6, 24). Many factors affected the behavior disturbance, including aging and duration of stress which were examined in our experiment. With the aging, normal animal behavior decreased gradually. However, the rats showed an opposite response during acute stress that results in an increase of movements and chronic stress that resulted in an inhibition of activities and emotional disorders (13, 27). Although this change had been recognized by many researchers, the mechanism of the change still remains unknown.

Results showed that with the prolonged stress, both young and aged groups displayed a decrease of square crossing, grooming behavior, vertical movements and stool. Moreover, after 3 days, animal behaviors which were kept at relatively steady state, expressed an adaptation to the stress. Furthermore, aged CHS and CDS groups showed less exploratory activities and grooming behavior, reflecting the weakly responsive level to stress. It was also important to note that in CDS model, animal behavior and emotional response reversed significantly after the last intense stimulus, even in aged CDS group. This suggested that the intensity of stress is one of the important factors that affect behavior.

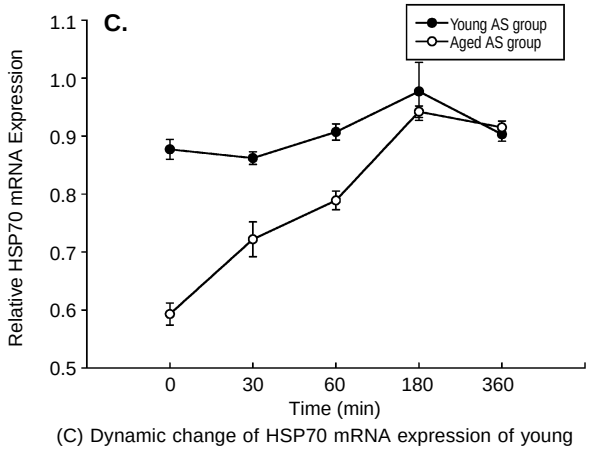
Studies show that when mammalians are exposed to the same stress condition for a period of time, they will gradually decline the response level and show a habituation to the stimuli (29). HSP70 is one of the most important protective factors which is thought to aid in the maintenance of cellular homeostasis and also can serve as biomarkers to evaluate the extent of disease or the degree of environmental stresses (14). Aging is accompanied by a decay of self-defensive mechanisms and by an accumulation of damages at the molecular, cellular, and organic level as a result of a constant exposure to adverse environmental stresses (28, 30). Therefore, we demonstrated that HSP70 mRNA expression induced by chronic forced-swimming resulted in habituation in hippocampus, and aging was



(A) Dynamic change of HSP70 mRNA expression of young and aged CHS groups (mean ± SD)



(B) Dynamic change of HSP70 mRNA expression of young and aged CDS groups (mean ± SD)



(C) Dynamic change of HSP70 mRNA expression of young and aged CDS groups (mean ± SD)

Fig. 7. Dynamic change of HSP70 mRNA expression in different aged rats. There were 15 rats in every group, Animals from different stress models sacrificed after 0, 30, 60, 180, and 360 min of stress and total RNA of three rats isolated at each time point was assayed for HSP70 expression by RT-PCR.

one of the most important factors affecting the expression level.

Results from our study suggested an influencing interrelationship between duration of stress and HSP70 mRNA expression mode. While the rats were forced to swim in 4°C cold water, RNA was expressed rapidly and kept in high level for 6 h. On the contrary, when the rats were forced to swim in warm water for 8 days (CHS model), the RNA expression increased after as little as 0 min of stress but decreased gradually after reaching the maximum. Moreover, all expression levels of CHS model were lower than these of the AS model, indicating that HSP70 mRNA expression mode depended on the duration and intensity of stress. Furthermore, expressive habituation of HSP70 mRNA was induced by mild long-term stress in both young and aged group. Thus, these findings demonstrated that HSP70 induced by forced-swimming was related to stress models and was accustomed to chronic stress, expressing habituation response. Presumably, other mammalians under the same stress conditions would produce similar HSP70 mRNA expression in hippocampus.

HSP70 plays a key role in protection against various kinds of stress (18, 23) and functions as a molecular chaperone in modifying the immune response, which is related to the ability to respond to various kinds of stress and aging processes (2, 25). HSP70 expression is influenced by many factors, including exterior aspects, such as character of stress, and interior aspects. Studies in cultured cells and in animal models have demonstrated that the stress response is age-dependent (12, 26). A subsequent study using cDNA microarray analysis suggested that there were age-related differences in the global expression profile as well as in the translational kinetics of HSPs in the lymphocytes from 15 healthy young donors (aged 19 to 29) and 10 old donors (age from 71 to 85)(10, 26).

Our data on three stress models showed that the induction of HSP70 mRNA was associated with aging that attenuated mammalian stress responsibility. However, it was necessary to note that HSP70 mRNA of aged groups in AS and CDS models elevated obviously and achieved the level as high as that of the young group after 180 min of stress. Though there was habitual HSP70 mRNA expression in aged CHD group, which expressed lower than that of the young group, there was no distinct difference between old and young groups in CDS and AS models. Thus, we concluded that aging could reduce the expressive level of HSP70 mRNA that presented habituation in chronic stress condition. Other reports of age-related changes in HSP70 in human lymphocytes showed that lymphocyte HSP70 was inversely related to the age of their subjects (age between 20 and 80), but this finding was not elaborated afterwards (22, 26). Hence, further investigations on a correlation between different aging phases and HSP70 expression under different stresses condition seems to be necessary. The elucidation of the mechanism in which generated HSP70 occurred

habituation in hippocampus and whether it would happen in other brain areas should be studied to provide insights into why the response decline with age.

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