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HEAT SHOCK PROTEIN72 PROTECTS HIPPOCAMPAL NEURONS FROM APOPTOSIS INDUCED BY CHRONIC PSYCHOLOGICAL STRESS

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When exposed to nonlethal heat stress (i.e., heat shock preconditioning), HSP72 expression increased in the mammalian brain. HSP72 enhance the viability of neurons and decrease TUNEL-positive neurons under several kinds of stress (e.g., ischemic). Chronic psychological stress is a kind of stress that could cause hippocampal neuron apoptosis. But whether overexpression of HSP72 can decrease TUNEL-positive hippocampal neurons caused by chronic psychological stress is unclear. To investigate the possible protective role of HSP72 in decreasing chronic psychological stress–induced hippocampal neuron apoptosis, this study analyzed

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HSP72 expression, apoptotic neurons in the hippocampus of mice. Adult mice were divided into four groups unstressed group; chronic psychological stress group; heat shock stress group; heat shock preconditioning plus psychological stress group; receiving no experimental stress, chronic psychological stress, heat shock stress, heat shock preconditioning plus psychological stress separately. Mice were killed after one month, two months, or three months of stress. A three-way ANOVA (psychological stress × heat shock stress × time) revealed a significant effect of heat shock stress in increasing HSP72 expression, decreasing neuronal apoptosis in hippocampus CA3 region caused by chronic psychological stress, and showed that HSP72 protected hippocampus CA3 neurons from chronic psychological stress.

Keywords apoptosis, heat shock stress, hippocampus, HSP72, psychological stress

Hippocampus is an important part of neuronal circuit in learning, memory, and stress response. Severe and prolonged psychosocial stress, such as chronic restraint stress causes a series of plastic structural changes in the hippocampus, including atrophy in volume, cell loss in CA3 regions (Lucassen et al., 2001; Lucassen et al., 2004), and dendritic atrophy in the apical dendrites of CA3 pyramidal neurons (Magarinos and McEwen, 1995; Magarinos et al., 1996; Uchino et al., 1997; McKittrick et al., 2000). The structural remodeling of hippocampus was accompanied by the impairment of hippocampus-depended function, including declarative memory, spatial memory, and long-term memory (Kitraki et al., 2004; Romeo et al., 2006). Both the structural remodeling and disfunctioning of hippocampus were mediated by glucocorticoid hormone in cooperation with glutamate excitotoxicity (Moghaddam et al., 1994; McEwen, 2000).

In the central nervous system (CNS), transient whole-body hyperthermia can induce a group of proteins expressed in neurons called heat shock protein (HSP), which can protect the neurons from injury caused by stressors, such as ischemia and seizures. HSP have been classified into 5 major families according to their molecular size, of which highly inducible HSP72 of 70kDa family is of particular importance in the CNS. HSP72 overexpression decreases TUNEL-positive neurons caused by glutamate toxicity; this has been previously proved by several reports, that is: Rordorf et al. found that pre-induction of HSP72 by heat shock protected cultured dissociated neurons from glutamate toxicity (Rordorf et al., 1991). Sato and Matsuki (2002) reported that 1 mM glutamate induced significant CA1-selective damage in cultured hippocampal slices, when hippocampal slices were heat-shocked (43.5°C, 30 min) before exposured to glutamate, neuronal damage in CA1 was attenuated. When the expression of HSP72 was suppressed by the antisense oligonucleotide,

the protective effect of the heat shock was completely inhibited. Kwong's result showed that HSP72 overexpression decreased retinal neurons apoptosis caused by intravitreal injected glutamate excitotoxicity *in vivo* (Kwong et al., 2003; Rossler et al., 2004) because chronic psychological stress-induced hippocampal neuron apoptosis (Lee et al., 2002) is related to glutamate excitotoxicity too (Magarinos and McEwen, 1995; Padovan et al., 2000; Mathew et al., 2001). But whether HSP72 overexpression decreases TUNEL-positive neurons caused by glutamate excitotoxicity induced by prolonged psychological stress is unclear. To answer this question, this study established heat shock stress and chronic psychological stress animal model, detected HSP72 expression and TUNEL-positive neurons in hippocampus, compared the number of hippocampal apoptotic neurons between mice subjected to chronic psychological stress only and mice subjected to heat shock stress plus chronic psychological stress.

MATERIALS AND METHODS

Subjects

Animals and Housing. The subjects were 2 months old mice, wild type of heat shock factor 1 (HSF1) gene. The mice were reared 3–5 mice per cage, in a room at the Animal Center of 2nd Xiangya Hospital, Central South University, at $22 \pm 2^{\circ}$C in a 12-h light–dark cycle (lights on at 07:00 h and off at 19:00 h). Food and water were available ad libitum.

Grouping. Ninety-four mice were divided into four groups: unstressed group (NS), heat shock stress group (HS), psychological stress group (PS), and heat shock stress plus psychological stress (HS+PS).

Establishment of Animal Models

The NS group were placed in a quiet room, without psychological stress or heat shock stress administered to them ($n = 23$). The HS group were submitted to heat shock stress once a week ($n = 23$). The PS group were submitted to psychological stress, including restraint stress and water-drinking conflict stress ($n = 24$). HS+PS group were submitted to heat shock stress and psychological stress ($n = 24$). Mice were decapitated after being submitted to one month, two months, or three months of a particular kind of stress. The experimental paradigms are shown in Figure 1.

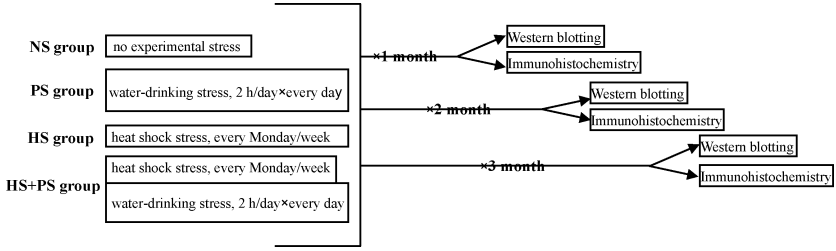

Figure 1. Experimental paradigms.

Psychological Stress Animal Model

Psychological stress includes restraint stress and water-drinking conflict stress. In the restraint stress (Pare & Glavin, 1986; Glavin et al., 1994; Wu et al., 2004; Tahera et al., 2006), a mouse was restrained in a narrow box (4 cm in diameter, 8 cm in length, with a hole at the top of the box to supply enough oxygen), to restrain its movements therefore making it helpless and depressed. Mice were restrained 3 times per week, 1 h every time. The water-drinking conflict stress was designed according to the Vogel model and Geller-Seifter model (Geller & Seifter, 1960; Hjorth et al., 1987; Millan and Brocco, 2003; Yamashita et al., 1996). Drinking was punished by a mild but aversive shock delivered via the spout of the bottle. This stress was given every day, 2 h per day. The time of stress was randomly controlled by computer procedure.

Heat Shock Stress Animal Model

Mice in the HS group and HS+PS group were submitted to heat shock stress. Mice were heated in a incubator, with a anorectal thermometer in their anus, keeping the average rectal temperature maintained at $42 \pm 1^{\circ}$C for 15 min (Saganek et al., 1997; Kelty et al., 2002).

Prepation of Hippocampus Tissue

When decapitated, two hippocampi in each group at each time point were rapidly removed and stored in liquid nitrogen for western blotting; other brains were paraffin-embedded and cut in 5-μm-thick serial coronal sections starting at a random position at the beginning of the hippocampus for immunohistochemistry and TUNEL detection.

HSP72 Expression

Protein Extraction and Western Blots. Hippocampi were removed from liquid nitrogen and stored at –70°C until time of tissue processing. Homogenization procedure was carried out on ice or at 4°C. Mice hippocampi were homogenized in approximately 5 volumes of 100 mM Tris/HCl (pH7.4) containing 150 Mm NaCl, 1.0% (w/v) Triton X-100, 0.1% (w/v) SDS, 5 mM EDTA, 1.0 mM PMSF, and 10 Ag/ml aprotinin by using a sonifier (4–6 bursts for 1 s each, Branson Cell Disrupter, Dunbury, CT, USA). Then, protein concentration was determined according to Bradford assay (Bio-Rad) with BSA as standard. Equal amounts of proteins (10–20 μg) were loaded in each lane and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresisthe; resolved proteins were transferred to nitrocellulose membrane. After blocking with 2% albumin in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C, the blot was probed with anti-HSP72 (Stressgen) antibodies at the appropriate concentration for 2 h at room temperature. The membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The membrane was washed 3 times in TBST and developed by DAB assay kit using the manufacturer's protocol (Boster Biological Technology). Because multiple gels were analyzed for quantification of marker proteins, immunopositive bands of the control protein GAPDH were used for normalization of optical densities of marker protein bands of each probe.

Immunohistochemistry Staining for HSP72 Expression in Hippocampal Neurons. Immunohistochemical analysis for HSP72 was performed on formalin-fixed and paraffin-embedded sections. Immunohistochemical staining was performed by the biotin–streptavidin–peroxidase method. A mouse monoclonal antibody against HSP72 (Stressgen) was used as the primary antibody. In brief, tissue sections were dewaxed, deparaffinized in xylene, rehydrated through a series of graded alcohols and washed in water. The slides were rinsed in running water, bathed in TBS buffer for 5 min and covered with 0.03% hydrogen peroxide for a further 5 min to reduce nonspecific staining. The slides were then incubated with a 1:500 dilution of a mouse monoclonal primary antibody for HSP72 (Stressgen) at room temperature in a humid chamber for 30 min. After further washing in buffer for 5 min, the secondary-peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins was applied for 30 min. The sections were washed in buffer

and HSP72 antigen visualized using DAB and hematoxylin counterstain. Slides were dehydrated through graded alcohols and coverslips applied.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) for Apoptotic Hippocampal Neurons

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on formalin-fixed and paraffin-embedded sections to detect apoptotic neurons. Section processing began with dewaxed, deparaffinized in xylene, rehydrated through a series of graded alcohols, and washed in water. Sections subsequently were rinsed in PBS (5 min) and treated with 20 μg/ml proteinase K (Promega) in PBS (15 min, RT). Then sections were washed in 4 $\times$ PBS (2 min per wash) and rinsed in 3% hydrogen peroxide in PBS (5 min) to inactivate endogenous peroxidase. Subsequently, sections were incubated at RT in TdT-mediated dUTP-biotin nick-end labeling (TUNEL) reaction solution for 60 min. After washing, Streptavidin HRP was applied to the sections for 30 min at 37°C. Finally, sections were stained with DAB and then counterstained lightly with hematoxylin.

Detection of Positive Cells

Observe and count the positive cells stained by immunohistochemistry and TUNEL respectively in the hippocampal CA3 region at $\times$200 magnification under a Olympus light microscope. Take micrographs in the selected visual fields. The HSP72 could be expressed in neurons and neuronal glia, but the authors could distinguish them according to the standard for HSP72 positive cells by Bodega (Bodega et al., 2002): the neuron was brown, the volume of the cell was larger, and the thick axon was obvious (Figure 4). The apoptotic neurons was recognized by its condensed chromatin, smaller and dark brown nucleus.

Statistical Analysis

All data were analyzed with SPSS for windows 12.0 software. The results were presented as mean $\pm$ S.E.M. Data was analyzed using one-way ANOVA and three-way ANOVA. Post hoc multiple comparisons were carried out using LSD tests. Statistical significance was accepted at $p < .05$.

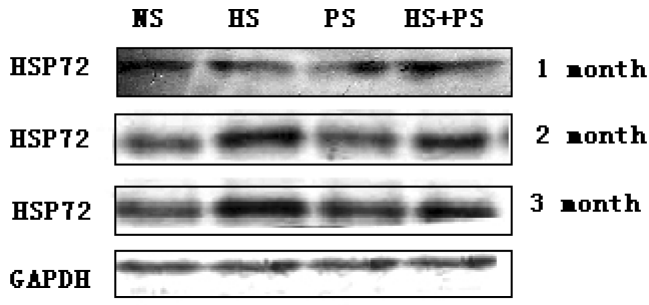

Figure 2. The expression of the HSP72 in hippocampi. Western blots showing effect of HS on HSP72 induction in mice. Mice in HS group (lane 2) and HS+PS group (lane 4) were submitted to heat shock (42°C ± 1°C)for 15 min, the HSP72 expression was significantly higher than NS group (lane 1) and PS group (lane 3) after 1 month, 2 month, and 3 month. With GAPDH lane as loading controls for HSP72.

RESULTS

HSR-Induced HSP72 Expression

Western blots showed that HSR-induced HSP72 overexpressed in mice of HS group and HS+PS group, compared with NS group and PS group after 1 month, 2 months, or 3 months of treatment (Figure 2).

To localize the expression of HSP72 in hippocampal neurons, immunohistochemistry was used for brain section. Immunohistochemistry of mice brains showed that 12 h after HSR, HSP72 was induced in hippocampal neurons (Figure 3). One-way ANOVA was used to compare the means of HSP72 positive neurons, and the result showed that, after one month, two months, and three months of treatment, the number of HSP72 positive neurons was significantly higher in HS group and HS+PS group than in NS group and PS group ($p < .05$) (Table 1, Figure 3). To explore the effect of heat shock stress, psychological stress, and time on HSP72 expression, a three-way ANOVA was used to analysis the number of HSP72 positive CA3 neurons, and revealed a significant heat shock stress effect ($F1,70 = 48.12$, $p = .000 < .05$), time effect ($F1,70 = 4.38$, $p = 0.017 < .05$), but no significant psychological stress effect ($F1,70 = 3.55$, $p = 0.065 > .05$).

HSR Decreased TUNEL-Positive Cells

TUNEL staining showed that TUNEL-positive cells were located primarily in CA3 region. Many of these cells showed chromatin margination or apoptotic

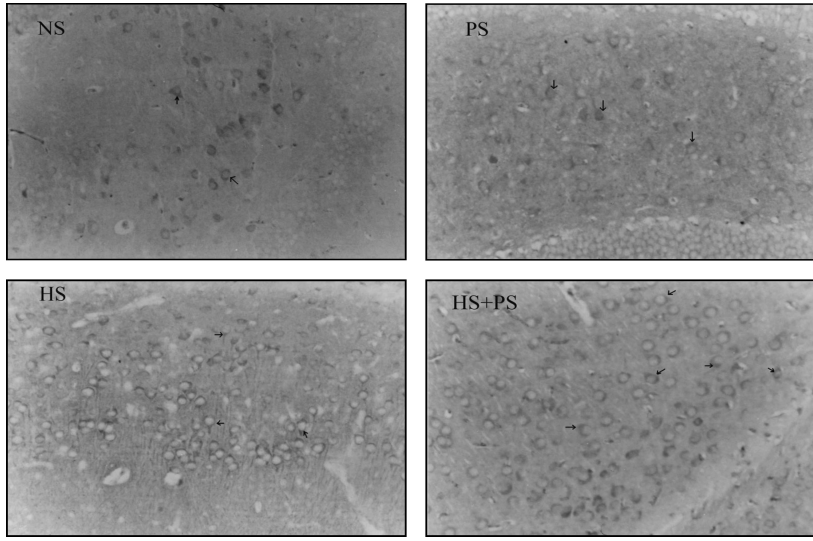

Figure 3. HSP72 expression in CA3 neurons in different groups after 3 months of treatment (DAB×200).

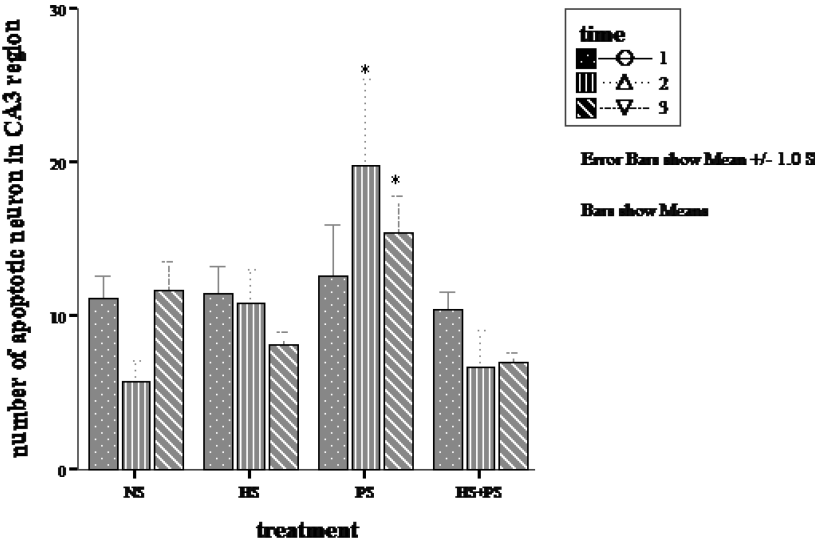

Figure 4. The number of the apoptotic neurons in the hippocampal CA3 region among different groups at three time points (mean ± S.E.M.) $^{*}p < .05$, PS vs. NS, HS+PS.

Table 1. The comparision of the HSP72 positive neurons in the hippocampal CA3 region among different groups at three time points (mean ± S.E.M.)

Time	NS	HS	PS	HS+PS
1 month	21.60 ± 7.41 ($n = 5$)	109.67 ± 18.26* ($n = 6$)	48.33 ± 14.70 ($n = 6$)	107.83 ± 15.55* ($n = 6$)
2 months	23.80 ± 3.29 ($n = 5$)	78.33 ± 23.86* ($n = 6$)	34.50 ± 9.05 ($n = 6$)	117.50 ± 21.05* ($n = 6$)
3 months	57.00 ± 13.25 ($n = 6$)	120.00 ±27.45* ($n = 6$)	71.67 ± 11.67 ($n = 6$)	145.00 ± 21.41* ($n = 6$)

bodies. TUNEL-positive cells were counted in all groups and the results are shown in Figures 4 and 5. Data was analyzed using one-way ANOVA and three-way ANOVA. One-way ANOVA showed that, after 1 month of treatment, no significant difference was observed in the number of TUNEL-positive cells; after 2 months of treatment, the number of TUNEL-positive hippocampal CA3 neurons was 6.67 ± 2.47 in HS+PS group, lower than that of PS group (19.83 ± 5.59); after 3 months, it was 7.00 ± 0.68 in HS+PS group, lower than that of PS group too (15.50 ± 2.41) (Figure 5). Three-way ANOVA revealed a significant heat shock stress effect ($F1,70 = 6.38$, $p = .014 < .05$) and a

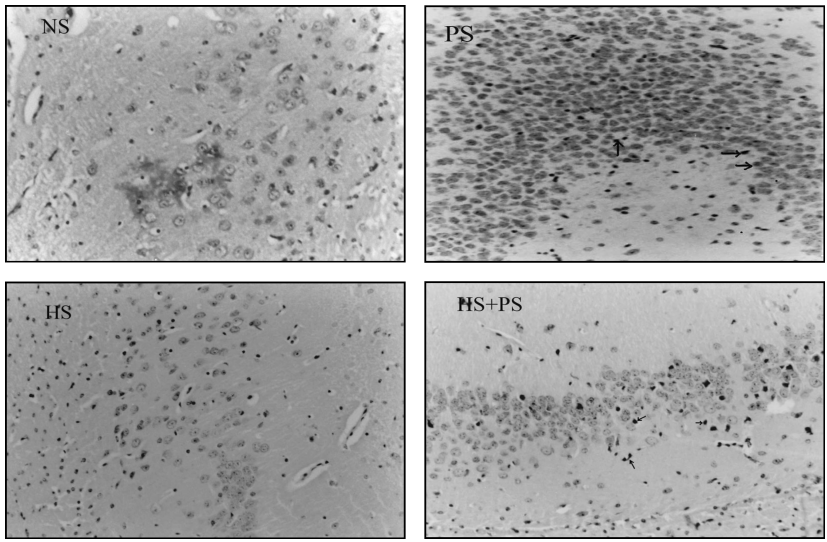

Figure 5. Apoptosis in CA3 neurons in different groups after 3 months of treatment (DAB×200).

significant heat shock stress × psychological stress interaction ($F1,70 = 8.68$, $p = .005 < .05$), but no significant psychological stress effect and time effect ($p > .05$). When analyzed to explore the simple effect of heat shock stress, it showed that without heat shock stress, psychological stress increased apoptotic neurons in CA3 area ($p = .004 < .05$), with the presence of heat shock stress, psychological stress can't increase apoptotic neurons in CA3 area ($p = .300 > .05$). Heat shock stress significantly decreased the number of apoptotic neuron in CA3 area in mice subjected to psychological stress ($p = .000 < .05$), but no significant simple effect in mice without psychological stress ($p = .819 > .05$).

DISCUSSION

In this study, HSR-induced HSP72 expression in hippocampal neurons, and decreased TUNEL-positive hippocampal CA3 neurons caused by chronic psychological stress. The protective role was not significant after one month of treatment, when one month of psychological stress did not increase hippocampal neuron apoptosis, but was significant after 2 months and 3 months of treatment, when 2 months and 3 months of psychological stress caused the increase of hippocampal CA3 neuron apoptosis. This result was consistent with previous researches. Previous studies demonstrated that overexpression of HSP72 decreased TUNEL-positive hippocampal cells caused by ischemic insult (Hoehn et al., 2001; Alves et al., 2002; Nagai et al., 2001) and glutamate excitotoxicity (Sato and Matsuki, 2002; Kwong et al., 2003). The current study showed that HSP72 not only protected hippocampual neurons against ischemic *in vivo*, but also protected hippocampal CA3 neurons against chronic psychological stress *in vivo*.

The protective role of HSP72 may be due to its anti-apoptosis effect. Apoptosis is a kind of cellular self-destroy controlled by genes. Many genes expressed and proteins synthesized during apoptosis. HSP72 act together with these genes and proteins involved in apoptosis to depress apoptosis, including: HSP72 control molecules crucial for cell cycle, such as P53, Cdk4, Wee-1, C-myc, and so on (Kontogeorgos et al., 1999); HSP72 cooperate with Bcl-2, which depress apoptosis to resist apoptosis (Beere & Green, 2001); HSP72 depress the Fas-mediated apoptosis signal pathway (Schett et al., 2000). HSP72 alleviate stress-induced inhibition of JNK dephosphorylation and prevent its activation (Gabai et al., 2000). HSP72 prevent the association of procaspase-9 with Apaf-1 and inhibited caspase-9 and caspase-3 activation (Oleana et al., 1998; Beere et al., 2000; Beere & Green, 2001). HSP72 prevent the release of cytochrome c from mitochondria (Mosser et al., 2000).

In summary, these findings suggested that HSR-induced HSP72 overexpression and protected hippocampal neurons against chronic psychological stress. The pre- induction of heat shock proteins might provide whole-body protection against chronic psychological stress, including disfunctioning of hippocampus, that is, impairment of long-term memory or spatial memory; further researches are needed to answer these questions.

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