Thermal preconditioning protected cerebellar granule neurons of rats by modulating HSP70 expression[1]

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KEY WORDS thermal preconditioning; heat; heat shock proteins; cerebellum; neurons; apoptosis; antisense oligodeoxynucleotides; Western blotting

ABSTRACT

AIM: To explore the possibility that expressions of different heat shock proteins (HSPs) are specifically involved in the protection of thermal preconditioning against apoptosis of cerebellar granule neurons (CGNs) induced by repolarization. **METHODS:** Western blotting was used to detect expressions of HSP27, HSP70, and HSP90 induced by thermal preconditioning (TP) in CGNs; reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the expression level of HSP70 mRNA; apoptosis of CGNs was induced by switching culture medium containing KCl 25 mmol/L to one containing KCl 5 mmol/L. **RESULTS:** No expression of HSP27 in cerebellar granule neurons was observed with TP at 44 ºC. Expression of HSP90 was obvious in CGNs both without and with TP at 44 ºC for different periods. Expression of HSP70 protein in CGNs was lower with TP at 44 ºC for 5 min, but it increased gradually after the period was prolonged to 30, 60, or 90 min. HSP70 mRNA was detected after TP 44 ºC for 30, 60, and 90 min and increased gradually with time. HSP70 antisense oligodeoxynucleotides 10 μmol/L for 72 h inhibited the protective effects of TP at 44 ºC on apoptosis of CGNs induced by repolarization. **CONCLUSION:** HSP70 is involved in protective effects of thermal preconditioning on apoptosis in cerebellar granule neurons induced by repolarization.

INTRODUCTION

Heat shock proteins (HSPs) were first discovered in 1962[1] as a set of highly conserved proteins whose expression was induced by different kinds of stress such as heat shock, ischemia damage, infection, oxidants, heavy metals and *etc*. HSPs function as molecular chaperones that transport, fold, and assemble nascent polypeptide chains to well-defined comformation of functional proteins[2], as thermaltolerance, that is, HSPs can protect cultured cells or organisms from stress-damages[3]. In addition to its above roles, there is increasing evidence that HSPs can protect cultured cells or organisms from apoptosis. In the previous studies, we have found thermal preconditioning (TP) can protect cerebellar granule neurons (CGNs) from apoptosis induced by repolarization[4]. The present experiments were designed to investigate the possibility that expressions of different heat shock proteins induced by heat were specifically involved in the protection of TP against apoptosis of CGNs induced by repolarization.

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MATERIALS AND METHODS

Animals Seven or 8-d-old Sprague-Dawley rats (15±2 g, Grade II) of either sex were purchased from Laboratory Animal Center, Sun Yat-sen University of Medical Sciences.

Antibodies Antibodies HSP70 (W27), HSP27 (C-20), and HSP90 (H-114) were from Santa Cruz Biotechnology, Inc, Santa Cruz, CA. Anti-biotin-IgG-HRP, anti-goat-IgG-HRP, anti-mouse-IgG-HRP, and anti-rabbit-IgG-HRP were obtained from New England Biolabs, Inc, Beverly, MA.

Cell culture and cell viability assay CGNs were prepared from 8-d-old Sprague-Dawley rats pups as described by Yan *et al*[5]. Neurons were cultured in basal Eagle's medium containing KCl 25 mmol/L and 10 % fetal bovine serum (FBS). To prevent replication of non-neural cells, after 24 h, the cultures were supplemented with cytosine arabinoside (10 μmol/L), these cultures contained >95 % granule neurons. *D*-Glucose (5 mmol/L) was added to the cultures on d 7. Cell viability assays were the same as before[4].

HSPs induction in cerebellar granule neurons Neurons were seeded on poly-*L*-lysine coated 35 mm culture dishes (CORNING, USA). After 8 d of culture, cell dishes were subjected to TP at 44 ºC for 90 min, 60 min, 30 min, 15 min and 5 min, respectively, then recovered at 37 ºC for 60 min, to induce expression of HSPs in CGNs.

Western blot analysis Cultured cells were harvested and lysed in SDS buffer containing Tris-HCl 62.5 mmol/L, 2 % (w/v) SDS, 10 % glycerol, DTT 50 mmol/L, and 0.1 % (w/v) bromphenol blue. The resulting lysates were boiled for 5 min at 100 ºC, and then were clarified by microcentrifugation at 10 000×*g* for 5 min at 4 ºC. Total concentration of protein was measured in DU 640 Nuclear Acid and Protein Analyzer. The resultant soluble fraction was subjected to SDS-PAGE. After gel electrophoresis, the separated proteins were transferred by electroblotting onto nitrocellulose (NC, Amersham). The NC membrane was then blocked with Tris-buffered saline solution pH 7.4, containing 0.1 % Tween 20 and 5 % non-fat milk. The blotted proteins were probed with primary antibody. The secondary antibody was conjugated to horseradish peroxidase. The blots were developed with an enhanced chemiluminescence system.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was extracted with TRIzol Reagent (GIBCO BRL) and the concentration was measured in DU 640 Nucleic Acid and Protein Analyzer. The mRNA expression of HSP70 was evaluated by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified parallelly as an internal control. Total RNA of 1 μg from each dish was reverse-transcribed into cDNA by using the SUPERSCRIPT ONE-STEP RT-PCR kit (GIBCO BRL). The primers for HSP70 were synthesized by Shanghai Sangon Biotechnology Inc. Forward primer for HSP70: 5'-CAAGATCAGCGAGGCTGACAAG-3'; reverse primer for HSP70: 5'-AACTGTACACAGGGTGGCAGTG-3'. The cDNA were amplified by 35 cycles (each cycle consisting of DNA denaturation at 94 ºC for 30 s, primer annealing at 56 ºC for 30 s and primer extension at 72 ºC for 60 s). The amplified cDNA (303 bp and 500 bp for GAPDH and HSP70, respectively) were separated on 1.5 % agarose gel with 0.5 mL/L ethdium bromide (EB).

Antisense oligodeoxynucleotides (ODN) of HSP70 on HSP70 expression and cell survival Antisense ODN (5'-TGTTTTCTTGGCCAT-3') and sense ODN (5'-ATGGCCAAGAAAACA-3') of HSP70 (phosphorothionate conjugated oligo) were synthesized by Shanghai Sangon Biotechnology Inc. Rat granule cells were cultured for 3 d with the antisense 10 μmol/L added fresh every 24 h (three additions in total). Then cell dishes were subjected to TP at 44 ºC for 90 min, and reversed at 37 ºC for 60 min. Apoptosis of cerebellar granule neurons was induced by switching culture medium containing KCl 25 mmol/L to one containing KCl 5 mmol/L. HSP70 protein expression were measured by Western blotting and cell survival was counted in a hemacytometer[6].

Image analysis Germany KONTRON IBAS 2.0 automatically image analysis system and JVC ky-F30B 3-CCD coloring camera import were used.

Statistics Data were expressed as mean±SD. A one-way analysis of variance (ANOVA), followed by Dunnett test was performed.

RESULTS

Expression of HSP70, HSP27, and HSP90 protein in CGNs induced by TP Expression of HSP70 was very lower in control (CGNs without TP) and CGNs with TP at 44 ºC for 5 min. It increased a little after TP at 44 ºC for 15 min and increased gradually for 30, 60, or 90 min ($P<0.01$ *vs* Control, Fig 1A, 1B).

There was no expression of HSP27 protein in both CGNs without TP at 44 ºC and CGNs with TP at 44 ºC

for different periods (5, 15, 30, 60, or 90 min, Fig 2).

Expression of HSP90 protein was observed in both CGNs without TP at 44 ºC and CGNs with TP at 44 ºC for different periods (5, 15, 30, 60, or 90 min, Fig 3A). The difference was not significant between each group (P>0.05, Fig 3B).

Expression of HSP70 mRNA in CGNs induced by TP Expression of HSP70 mRNA was detected a little in CGNs with TP at 44 ºC for 30 min. It increased gradually while duration of TP prolonged to 60 or 90 min (Fig 4).

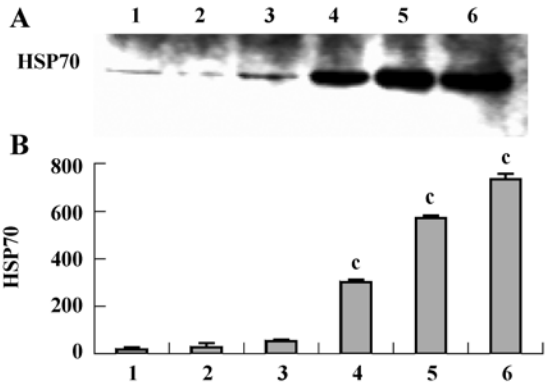

Fig 1. Expression of HSP70 protein in cerebellar granule neurons induced by thermal preconditioning. (1) Control; (2) 44 ºC, 5 min; (3) 44 ºC, 15 min; (4) 44 ºC, 30 min; (5) 44 ºC, 60 min; (6) 44 ºC, 90 min. n=3. Mean±SD. ^cP<0.01 compared with control. HSP70 content was expressed as absolute value OPTDI (AREA×OPTDM).

Fig 2. Expression of HSP27 protein in cerebellar granule neurons and Hela cells induced by thermal preconditioning. (1) Control; (2) 44 ºC 5 min; (3) 44 ºC 15 min; (4) 44 ºC 30 min; (5) 44 ºC 60 min; (6) 44 ºC 90 min (1-6: in cerebellar granule neurons); (7) Hela cells (44 ºC 90 min).

Effect of HSP70 antisense ODN on expression of HSP70 protein and neuron survival Expression of HSP70 in CGNs with TP at 44 ºC for 90 min was obviously higher than that of control (without TP at 44 ºC, P<0.01). Treatment of CGNs with HSP70 antisense ODN 10 μmol/L for 72 h reduced expression of HSP70 induced by TP at 44 ºC for 90 min (P<0.01, Fig 5).

Repolarization by KCl 5 mmol/L reduced neuron decreased cell survival rate to 45.0 %±2.6 %. Cell viability was increased to 96.3 %±5.4 % by TP at 44 ºC for 90 min before repolarization (P<0.01). But it decreased to 47.8 %±5.0 % after treatment with HSP70 antisense ODN 10 μmol/L for 72 h before TP at 44 ºC and repolarization (P<0.01). Sense ODN had no such effect.

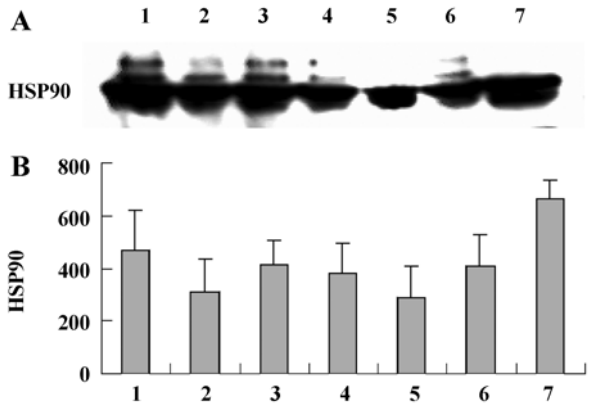

Fig 3. Expression of HSP90 protein in cerebellar granule neurons and Hela cells induced by thermal preconditioning. (1) Control; (2) 44 ºC, 5 min; (3) 44 ºC, 15 min; (4) 44 ºC, 30 min; (5) 44 ºC, 60 min; (6) 44 ºC, 90 min (1-6: in cerebellar granule neurons); (7) Hela cells (44 ºC, 90 min). n=3. Mean±SD. HSP90 content was expressed as absolute value OPTDI (AREA×OPTDM).

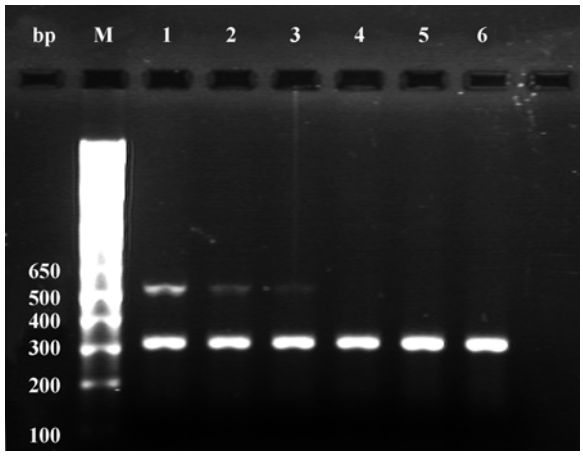

Fig 4. Expression of HSP70 mRNA in cerebellar granule neurons inducedby thermal preconditioning. (1) 44 ºC 90 min; (2) 44 ºC 60 min; (3) 44 ºC 30 min; (4) 44 ºC 15 min; (5) 44 ºC 5 min; (6) Control. Upper bands (500 bp): HSP70; lower bands (300 bp): GAPDH.

DISCUSSION

Induction of heat shock proteins (HSPs) is thought to play a protective role in apoptosis.

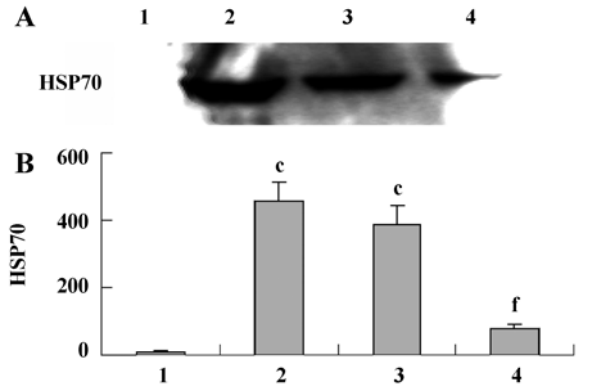

Fig 5. Effect of HSP70-antisense on expression of HSP70 protein in cerebellar granule neurons induced by thermal preconditioning. (1) Control; (2) 44 ºC, 90 min; (3) Sense ODN 10 μmol/L plus TP at 44 ºC, 90 min; (4) Antisense ODN 10 μmol/L plus TP at 44 ºC, 90 min. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* TP at 44 ºC 90 min. (ANOVA following by Dunnett test) . HSP70 content was expressed as absolute value OPTDI (AREA×OPTDM) .

There are several HSPs in mammalian. Three of them are known only by their subunit molecular weights: HSP27, HSP70, and HSP90. Some scientists believe that HSP27 and HSP70 are antiapoptotic proteins[7-9]; while other thinks it is HSP90 that protect cells from apoptosis[10].

Our study found that there was no expression of HSP27 in CGNs induced by TP at 44 ºC. Expression of HSP90 in CGNs was obvious both with TP at 44 ºC and without TP at 44 ºC. Only HSP70 mRNA and protein in CGNs increased gradually while duration of TP was prolonged from 5 min to 90 min. Furthermore, HSP70 antisense ODN inhibited expression of HSP70 protein in CGNs induced by TP and abolished the protective effects of TP on CGNs apoptosis induced by repolarization. It suggested that TP induced an up-regulation of HSP70 in a time-dependent manner but not HSP27 or HSP90 in CGNs. Hyperthermic pre-conditioning protected against repolarization-induced apoptosis in CGNs by modulating HSP70 expression.

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