EXPRESSION OF HSP72 IN LYMPHOCYTES IN PATIENTS WITH FEBRILE CONVULSION

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The pathophysiology of febrile convulsion, the most common childhood neurologic disease, remains unclear. In this study, we investigated what role a heat shock protein plays in this disease. We enrolled eight boys and two girls with febrile convulsion and 10 age-matched healthy controls. We did a biosynthetic evaluation of both groups by separating lymphocytes and measuring the expression of heat shock protein 72 before and after heat shock treatment. Before the treatment, both groups were found to have small amounts of constitutive heat shock protein 72. Afterwards, its expression increased in both groups, and no statistical difference was found between the increases in the two groups. In addition, there was no obvious difference in the susceptibility to produce heat shock proteins. However, the febrile convulsion group was found to have a significant decrease in phosphorylation of heat shock protein 72. These results suggest the possible involvement of post-translational modification of heat shock proteins, most likely phosphorylation, in the pathogenesis of febrile convulsion.

Key Words: febrile convulsion, heat shock protein, phosphorylation
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Febrile convulsion (FC), the most common childhood neurologic disease, has an incidence of 1–10% in children [1]. The National Institutes of Health defines FC as "a convulsion that happens to children between 3 months and 5 years of age associated with a febrile illness excluding central nervous system infection" [2]. Clinically, FC generally occurs during acute rises in body temperature and manifests as general tonic-clonic convulsion that lasts for less than 10 minutes. Two attacks during one episode of febrile illness are rare. Cerebrospinal fluid (CSF) is generally normal. The causes of fever vary although upper respiratory tract infection (URTI) is the most common [3]. Although FC has been investigated extensively, its exact pathogenesis has not yet been discovered. One study has reported that high temperatures may decrease neuronal threshold for excitement [4]. Another study has found inflammatory mediators, such as lipopolysaccharide-induced interleukin-1 of monocytes, to be more prominent in children with FC than in controls [5]. Still another study suggested that the cause may be related to synthesis of gamma amino butyric acid (GABA), which was found to decrease with elevated temperatures and increase with age [6]. These phenomena, however, are not consistently found in all patients.

FC is harmless, but when it occurs, parents become anxious about their child's health. Three to six percent of the children presenting with FC may later be diagnosed as having real epilepsy [7]. However, although several risk factors have been statistically associated with epilepsy, there is still no way of positively differentiating between the benign form of FC and that caused by epilepsy.

Fever is a warning sign of inflammation in all mammals. Extremely high fevers cause cellular damage by disrupting the Golgi apparatus, swelling the mitochondria [8], changing cellular permeability [9], or disrupting the nucleus and aggregation of chromatin [10]. In response to fever or heat,

all living cells respond to transient, sublethal higher temperatures by synthesizing heat shock proteins (Hsps), a highly conserved protein family that contains several types of molecules. The Hsp72 subgroup is the most inducible Hsp in mammalian cells. It is believed that sublethal stress leads to the synthesis of Hsps, which helps cells to protect themselves under subsequent lethal conditions [11,12]. Mice without Hsp70-producing genes have been found to have neuronal defects and to be vulnerable to beta-amyloid peptide-related neurotoxicity [13].

This study measured the expression of Hsp72 before and after heat treatment to investigate this Hsp's relationship to FC.

MATERIALS AND METHODS

Subjects and samples collection

We enrolled 10 children (mean age, 24.4 ± 12.8 months; range, 9–44 months) diagnosed by a pediatric neurologist as having simple FC at Kaohsiung Medical University Hospital. All patients were diagnosed according to the criteria stated by Fukuyama et al [14]. We also enrolled 10 age-matched children with no past history or family history of FC as healthy controls. All parents were informed and signed consent obtained. Using a heparin-rinsed syringe, 5 mL of whole peripheral blood was drawn from the peripheral vein of each subject, once he or she had been free from febrile illness and FC for at least 2 weeks. Ficoll-paque solution (Bio-Rad Laboratories, Hercules, CA, USA) and centrifugation were used to separate the lymphocytes. Each lymphocyte sample was divided for direct Hsp72 detection and biosynthesis study.

Preparation of cytosolic protein extraction

Lymphocytes were lysed by distilled water containing 0.1 mM phenylmethylsulfonylfluoride (PMSF, Sigma, St. Louis, MO, USA). The suspension was sonicated at 4°C with an ultrasonic processor sonicator (VibraCell-VC50, Sonics & Materials Inc, Daubury, CT, USA). The supernatant was separated after centrifugation at $12,000g$ for 5 minutes and added with the same volume of lysis buffer, containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 10% glycerol.

SDS-PAGE and immunochemical detection of Hsp72

Thirty micrograms of the sample were applied to a 10% SDS-PAGE at 30 mA/gel. After electrophoresis, the gel was transblotted to a 0.22 nm nitrocellulose membrane (11307, Sartorius AG, Gottingen, Germany) with a semidry transblot apparatus (Horizblot, AE6675; Atto, Tokyo, Japan) and blocked with milk saline. The membranes were reacted with monoclonal anti-Hsp72 antibody (Amersham International, Amersham, UK) as the primary antibody and then anti-mouse IgG from goat as the secondary antibody. Target protein bands were visualized by the horseradish peroxidase immunostaining system (Biogenex Laboratories, San Ramon, CA, USA).

Biosynthesis (chase and pulse) study of Hsp72 in cultured lymphocytes

The cell suspension was divided into six tubes and exchanged the medium by 0.5 mL methionin-free RPMI 1640 medium. To exhaust the intracellular methionin, all tubes were incubated at 37°C in an incubator supplied with 95% O_2 and 5% CO_2 for 30 minutes. Five of the six tubes were then heated in a thermo-controlled water bath at 41°C for 20 minutes and ^{35}S-methionin was added at a concentration of 1 mCi/mL of medium. The tubes were then incubated at 37°C in an incubator supplied with 95% O_2 and 5% CO_2. One or 2 hours later (pulse), cells were harvested and washed with phosphate-buffered saline (PBS). Cells of one tube were collected and stored at –70°C. The other cells were further cultured in medium replaced by full RPMI 1640. The cells were harvested by centrifugation and washed with PBS twice 16, 24, and 48 hours later (chase). Cytosolic proteins were extracted and SDS-PAGE was prepared as described above. The gel was dried and autoradiographed.

Phosphorylation of Hsp72 in vitro

The cell suspension was divided into two tubes and the medium was exchanged by full RPMI 1640 medium. All the cells were incubated in a phosphate-free medium and heated in a thermo-controlled water bath at 39°C for 20 minutes. One hundred and twenty mCi/mL ^{32}P-H_3PO_4 was added to each tube and then incubated in a 37°C incubator supplied with 95% O_2 and 5% CO_2. The cells were harvested by centrifugation and washed with PBS twice, 4 and 24 hours later. Cytosolic proteins were extracted and SDS-PAGE was prepared as described above. Gels were dried and autoradiographed.

RESULTS

Ten patients (8 boys and 2 girls; mean age, 24.4 ± 12.8 months) met the criteria of simple FC and were enrolled in this study. Mean age at onset was 20.5 ± 11.5 months

(Table). All patients were full term and had relatively normal birth histories. Two had family histories of FC. In total, the 10 patients had 14 episodes of FC. In all patients, the seizures were of the generalized tonic-clonic type and lasted no more than 15 minutes, most less than 3 minutes. Body temperatures were between 38°C and 40.2°C (peak, 38.5–39.5°C) on arrival. The most common cause of fever was upper respiratory infection followed by acute gastroenteritis. We admitted four patients, two of whom received CSF examinations that were normal. All patients received EEG examinations, and, except for two patients who had central sharp waves, all were found to be normal. None of the patients received anticonvulsant therapy. At 15 months' follow-up, eight more episodes occurred (a recurrence rate of 60%).

Both patients and controls were found by immuno-chemical studies to have expression of Hsp72 before heat shock treatment (Figure 1). Two hours after heat shock treatment, both groups had increased levels of Hsp72, but there was no significant difference between the two groups. In doing the biodynamic assay, we evaluated Hsp synthesis susceptibility to elevated temperatures by heating the lymphocytes at 38°C, 39°C and 40°C for 20 minutes. ^{35}S-methionine-contained Hsp72 was observed 15, 30, 45 and 60 minutes later. As shown in Figure 2A, there was little detectable Hsp72 at 38°C. However, Hsp72 could be observed as early as 15 minutes after heating to 39°C or 40°C (Figures 2B & 2C). It is clear that Hsp72 could be induced in human lymphocytes 20 minutes after hyperthermic treatment at 39°C. FC patients and controls were not significantly different from each other with regard to susceptibility (Figure 3).

For a better understanding of the post-translational modification of Hsp72 in lymphocytes of FC, two further bioassays were performed: pulse-chase assay and phosphorylation detection. In the pulse-chase assay, a band with molecular weight of 72 kd, which could not be identified in non-heated cells using the current method, was over-expressed 2 hours after pulse. Twenty-four hours after chase, the target band was still visible, while most of the newly synthesized proteins were fading. Similarly, there was no significant difference between the FC and control group (Figure 4). Phosphorylation was compared by adding ^{32}P-H$_3$PO$_4$ in culture media. In the controls, the phosphorylation phenomenon was prominent after hyperthermic treatment, creating a band of 72 kd. In FC patients, the intensity of phosphorylation decreased, including the band of 72 kd (Figure 5).

DISCUSSION

The clinical symptoms and signs of FC in this study were similar to those reported in previous studies [14]. The majority of these patients had their first febrile seizures between the ages of 8 and 18 months. Body temperatures registered between 38.0°C and 40.2°C. The most common cause of fever was URTI. All seizures occurred within 24 hours of fever onset. As others have reported, males outnumbered females in our study. Two (20%) of our patients had family histories of FC. Based on these findings, fever, age and genetic predisposition seem to be major factors affecting the pathophysiology of FC.

A study by Ritossa found that short-term hyperthermia can induce a molecular response in cells [11]. In stressful circumstances, cells produce large amounts of proteins, Hsps in cases of heat stress, which can protect cells from subsequent injury. These Hsps can be induced in the central

Table. Personal data of patients with febrile convulsions

Patient	Sex	Age	Family history	Age of first attack	Episodes	EEG
1	F	11 mo	+ve	11 mo	1	Normal
2	F	2 yr 7 mo	–ve	1 yr 5 mo	4	Abnormal*
3	M	1 yr 6 mo	–ve	1 yr 6 mo	1	Abnormal†
4	M	3 yr 8 mo	–ve	3 yr 8 mo	1	Normal
5	M	9 mo	–ve	9 mo	1	Normal
6	M	1 yr 10 mo	–ve	1 yr 8 mo	2	Normal
7	M	3 yr 11 mo	+ve	2 yr 11 mo	2	Normal
8	M	2 yr 1 mo	–ve	2 yr 1 mo	4	Normal
9	M	1 yr 7 mo	–ve	8 mo	2	Normal
10	M	1 yr 6 mo	–ve	1 yr 6 mo	4	Normal

*Asymmetric central sharps; †bilateral central sharps. EEG = electroencephalogram; +ve = positive; –ve = negative.
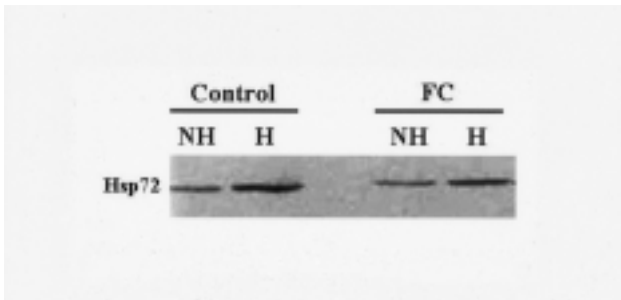
Figure 1. *Inducible Hsp72 present in non-heated (NH) and heated (H) lymphocytes from control and febrile convulsion patients. Hsp72 was detected by separation on 1-D SDS-PAGE and immunochemical study with anti-Hsp72 monoclonal antibody from mouse and antimouse IgG antibody. Visualization was developed by 4-CN system.*
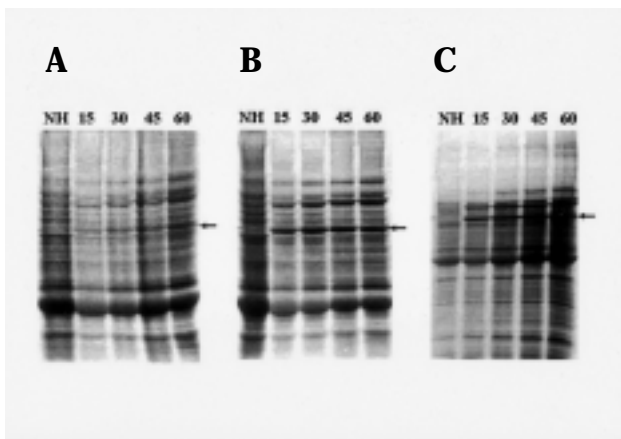

Figure 2. *Effects of various heat shock temperatures and incubation times on the newly synthesized ^{35}S-methionine labeled polypeptide from control lymphocytes. The duration of heat shock treatment is 20 minutes for all conditions. Arrows indicate the inducible Hsp72. (A) 38°C; (B) 39°C; (C) 40°C. NH = nonheated lymphocytes. 15, 30, 45, 60 = 15, 30, 45, and 60 minutes after heat shock, respectively.*

Figure 3. *Comparison of newly synthesized ^{35}S-methionine labeled polypeptide of lymphocytes between control and febrile convulsion (FC) patients. All cells were labeled with ^{35}S-methionine and heat shocked at 39°C for 20 minutes. Lymphocytes were harvested 15 or 30 minutes after heat shock treatment. Lanes NH, 15, and 30 indicate nonheated, 15, and 30 minutes after heat shock treatment of control and FC patients, respectively.*

nervous system as well as other tissues. Besides high fever, sublethal stereotaxic injection of kainic acid, flurothyl-induced status epilepticus, tissue injury, spinal cord injury, or ischemia can also induce Hsp synthesis [15–17]. Even though tissue specificity was present in our previous study, Hsp72 induced by heat shock in the brain was similar to the lymphocytes [18].

Recently, Hsps have been investigated in greater detail. On the cellular level, they act as molecular chaperones and are able to enhance the folding, refolding, assembly, transport of protein or newly synthesized polypeptide, achieving the goal of cell repair or protection [19,20]. Microinjecting anti-Hsp72 antibodies, we found cellular survival rates decreased rapidly at high temperatures.
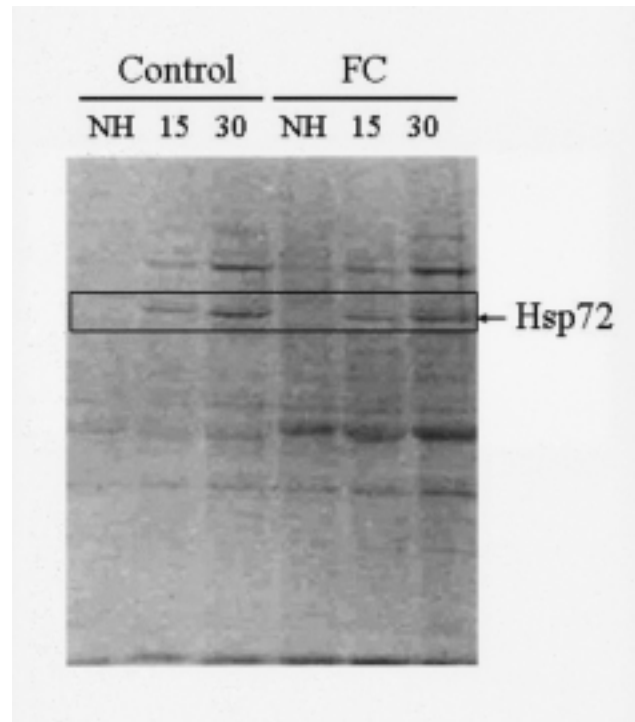
Previous *in vivo* heat shock treatment has been found to decrease retina damage against UV light [21], decrease the cortical damage from ischemia, and increase the recovery ability from hypoxia-induced cortical function [22]. In one of our previous studies, we also showed that hyperthermic pretreatment attenuated bicuculline-induced (a GABA antagonist) convulsions in rats [18]. This evidence suggests that using heat shock to induce Hsp synthesis protects or stabilizes neuronal cells in future adverse environments.

In this study, Hsps could be detected by immunostaining before hyperthermic treatment in controls and FC patients. Hsps in humans may be caused by daily stress from work or the environment. Pulse-and-chase studies show that, once induced in humans, Hsps can be sustained for more than 24 hours. Therefore, it is reasonable to assume that we could detect Hsps before heat shock treatment in human lymphocytes. However, although Hsp72 could be detected before heat shock treatment, synthesis of Hsps was not a continuous process. Without heat shock, lymphocytes use ^{35}S-methionine in the medium to synthesize proteins other
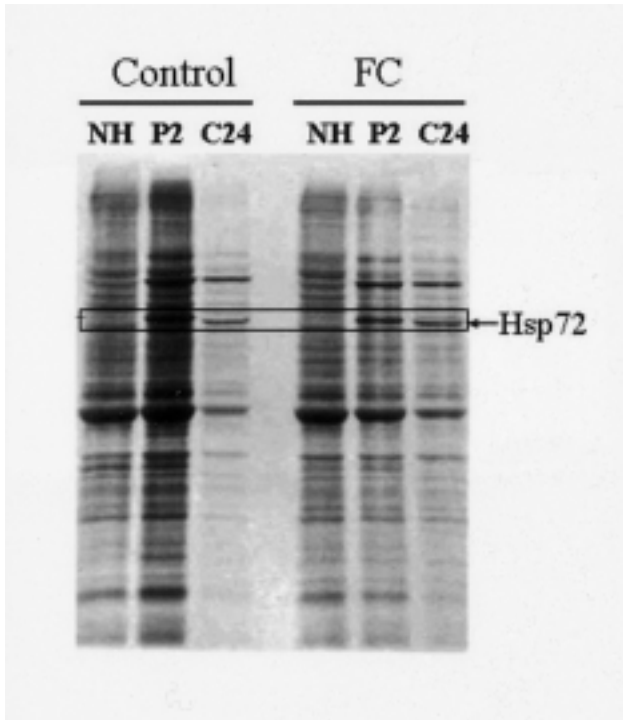
Figure 4. *Comparison of newly synthesized ^{35}S-methionine labeled polypeptides during pulse and chase phases between normal control and febrile convulsion (FC) patients. Cells were heat shocked and labeled with ^{35}S-methionine for 2 hours (pulse) and the medium changed into full medium without isotope amino acid for 24 hours (chase). Lanes NH, P2 and C24 indicate samples of nonheated, pulse 2 hours and chase 24 hours of control and FC patients, respectively.*
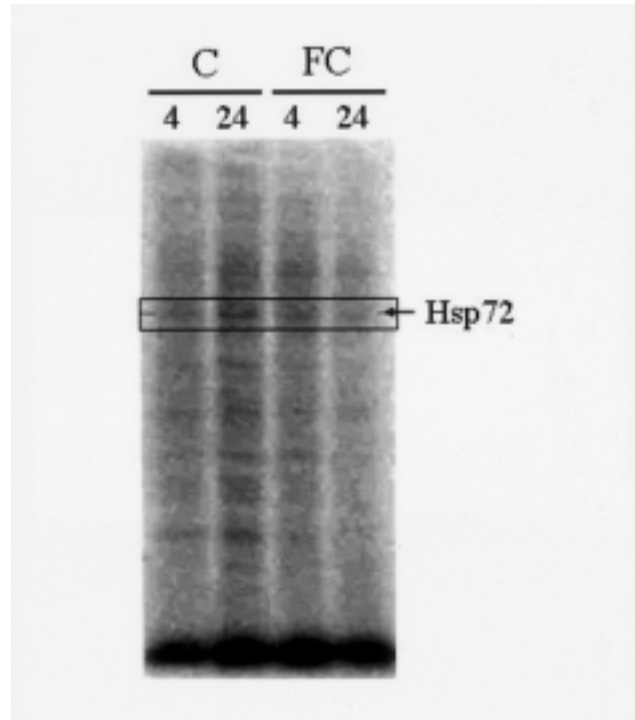

Figure 5. *Phosphorylation of Hsp72 in lymphocytes of normal control and febrile convulsion (FC) patients after hyperthermia. Lymphocytes were heated in vitro at 39°C for 20 minutes and labeled with ^{32}P-H$_3$PO$_4$. Lanes 4 and 24 indicate 4 and 24 hours after heat shock of control (C) and FC patients, respectively.*

than Hsp72. Hsp72 increased in both groups 2 hours after heat shock treatment, indicating that heat shock (41°C for 20 minutes) can induce Hsp72 in lymphocytes obtained from FC patients.

The synthesis of Hsp72 was induced by stress, which stimulated the heat shock factor and then activated the promoter of the *Hsp72* gene. In one previous study, the temperature of activation was reported to be 3–5°C above body temperature [23], though different tissues or different cell types in the same tissue may respond differently to these temperatures. In this study, we used various sub-heat shock temperatures, 38°C, 39°C, and 40°C, to treat the lymphocytes in both groups and found 39°C for 20 minutes able to induce the synthesis of Hsp72 in both groups, indicating, for the first time, that human lymphocytes are more susceptible to mild stresses than other mammals. However, because no significant difference could be found between the two groups, our findings do not provide us with a clue to the pathogenesis of FC.

"Turnover" is found in various proteins, especially in

those that are functional. Proteins are metabolized and then degraded into small particles. Finally, these proteins degrade into amino acids and are used to form new proteins. In this study, we found no significant difference between the FC biosynthesis of Hsps and that of the controls. Therefore, we used the pulse-and-chase method to study the difference of the rate of metabolism in newly formed Hsp72 of both groups and found neither group to have significant differences in biosynthesis of Hsp72, 15 minutes after 20 minutes of 39°C heat shock treatment. Although we could not find a difference in the period of time it took for the FC patients and controls to synthesize Hsp72, we found something interesting about the kinetics of Hsp72 synthesis. While many authors have stated that Hsps can be induced 2 hours after 15 minutes of 43°C heat shock treatment [23], we found large amounts of Hsp72, 5 minutes after 20 minutes of 39°C heat shock treatment. The Hsp72 increased gradually, peaking 16–24 hours later, before ultimately degrading. Furthermore, no newly formed protein was found until 48 hours later in the chase study. Therefore,

Hsp72 may be metabolized in its original form, but how this is done needs further investigation.

During protein synthesis, a polypeptide is modified into the final product by hydroxylation, carboxylation, glycosylation, phosphorylation, or the packaging, folding or breaking of amino acids. Few reports have mentioned the phosphorylation of Hsp72, though there has been some mention of the phosphorylation of the small particles, such as Hsp28, Hsp25 or Hsp27. Lavoie et al found that thermo-tolerance was not linearly correlated with the amount of Hsp27 in Chinese hamster cells, but correlated with phosphorylation [24]. Schafer et al also found that phos-phorylation of Hsp27 in Chinese hamsters is required to stabilize the actin cytoskeleton and to protect the cells from the effects of high concentrations of cholecystokinin [25]. Our study is the first, as far as we know, to find either a post-translational modification to Hsp72 in human lymphocytes or phosphorylation to be significantly different between non-FC and FC subjects. Although the biologic function needs further investigation, decreased phosphorylation might attenuate the cytoprotective effect of heat shock proteins.

In conclusion, we did not find the synthesis, susceptibility to heat, or degradation rate of Hsp72 in lymphocytes of FC patients to be significantly different from that of non-FC subjects. However, we did find evidence that phosphoryla-tion of Hsp72 might play a role in the pathogenesis of FC.

REFERENCES

1. Kaplan RE. Febrile seizures. When is treatment justified? *Postgrad Med* 1987;82:63–6, 69–71.
2. Nelson KB, Ellenberg JH (eds). *Febrile Seizure.* New York, NY: Raven Press, 1981:301–6.
3. Lee P, Jones KV. Urinary tract infection in febrile convulsions. *Arch Dis Child* 1991;66:1291–5.
4. Lewis HM, Parry JV, Parry RP, et al. Role of viruses in febrile convulsions. *Arch Dis Child* 1979;54:869–76.
5. Helminen M, Vesikari T. Increased interleukin-1 (IL-1) production from LPS-stimulated peripheral blood monocytes in children with febrile convulsions. *Acta Pediatr Scand* 1990; 79:810–6.
6. Schmiegelow K, Johnsen AH, Ebbesen F, Sorensen O. Gamma-aminobutyric acid concentration in lumbar cerebrospinal fluid from patients with febrile convulsions and controls. *Acta Pediatr Scand* 1990;79:1092–8.
7. Koyama A, Matsui T, Sugisawa T. Febrile convulsions in northern Japan: A quantitative and qualitative analysis of EEG and clinical findings. *Acta Neurol Scand* 1991;83:411–7.
8. Welch WJ, Suhan JP. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli and the modulation of plasma membrane sodium-potassium ATPase activity. *J Cell Biol* 1985;101:1198–211.
9. Burdon RH, Cutmore DMM. Human heat shock gene expression and the modulation of plasma membrane sodium-potassium ATPase activity. *FBES Lett* 1982;140:45–8.
10. Lewis MJ, Pelham HRB. Involvement of ATP in the nuclear and nucleolar function of the 70 kd shock protein. *EMBO J* 1985;4:3137–43.
11. Ritossa F. A new puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia* 1962;18:571–3.
12. Ashburner M, Bonner JJ. The induction of gene activity in drosophila by heat shock. *Cell* 1979;17:241–54.
13. Wang G, Zhang J, Moskophidis D, Mivechi NF. Targeted disruption of the heat shock transcription factor (hsf)-2 gene results in increased embryonic lethality, neuronal defects, and reduced spermatogenesis. *Genesis* 2003;36:48–61.
14. Fukuyama Y, Kagawa K, Tanaka K. A genetic study of febrile convulsions. *Eur Neurol* 1979;18:166–82.
15. Krapfenbauer K, Berger M, Lubec G, Fountoulakis M. Changes in the brain protein levels following administration of kainic acid. *Electrophoresis* 2001;22:2086–91.
16. White DJ, Carlson D, Ordway GA, Horton JW. Protective role of heat stress in burn trauma. *Crit Care Med* 2004;32:1338–45.
17. Lai Y, Kochanek PM, Adelson PD, et al. Induction of the stress response after inflicted and non-inflicted traumatic brain injury in infants and children. *J Neurotrauma* 2004;21:229–37.
18. Yang SL, Jing SH, Yang RC, et al. The effect of hyperthermic treatment on the recovery of EEG after interruption of respiration in rats. *Exp Brain Res* 1994;99:431–4.
19. Nover L. *Heat Shock Response.* Florida, CRC Press, 1991.
20. Parnham MJ, Feige U. Structural and functional properties of heat shock proteins in inflammation and immunity. *Agents Actions* 1992;35:34–6.
21. Barbe MF, Tyeli M, Gower DJ, Welch WJ. Hyperthermia protects against light damage in the rat retina. *Science* 1988; 241:1817–20.
22. Chopp M, Chen H, Ho KL, et al. Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. *Neurology* 1989;39:1396–8.
23. Krueger-Naug AM, Hopkins DA, Armstrong JN, et al. Hyperthermic induction of the 27 kDa heat shock protein (Hsp27) in neuroglia and neurons of the rat central nervous system. *J Comp Neurol* 2000;428:495–510.
24. Lavoie JN, Lambert H, Landry J, et al. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol and Cell Biol* 1995;15:505–16.
25. Schafer C, Clapp P, Williams JA, et al. Hsp 27 expression regulates CCK-induced changes of the actin cytoskeleton in CHO-CCK-A cells. *Am J Physiol* 1999;277:C1032–C1043.

熱休克蛋白 72 在熱痙攣病人淋巴球的表現

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雖然熱痙攣在小兒神經科的領域中是一種相當常見的疾病，但目前為止對它的致病機轉還不是很清楚。在這個研究中，我們主要探討熱休克蛋白在熱痙攣所扮演的角色。十個年齡從九個月到三歲八個月的熱痙攣病人，八男二女，當做實驗組；另外找十個年齡相當的正常兒童作為對照組，分別抽取他們的淋巴球做熱休克蛋白 72 合成動力學的分析，結果顯示兩組在熱休克處理前都可以測到微量的熱休克蛋白 72，在熱休克處理後，兩組的熱休克蛋白 72 都有明顯的增加，但兩組增加的量並沒有顯著的差異。但是在蛋白質磷酸化的探討上，熱痙攣病人這一組熱休克蛋白 72 的磷酸化卻明顯比對照組少。我們認為，熱休克蛋白的磷酸化也許是熱痙攣的致病機轉之一。

關鍵詞：熱痙攣，熱休克蛋白，磷酸化

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