Heat shock treatment protects osmotic stress–induced dysfunction of the blood-brain barrier through preservation of tight junction proteins

Tzong-Shi Lu,¹ Hsiang-Wen Chen,² Maw-Hsiung Huang,³ Shu-Jung Wang,¹ and Rei-Cheng Yang^{1,4}

¹Department of Physiology, Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung City 807, Taiwan, Republic of China

²Department of Microbiology, Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung City 807, Taiwan, Republic of China

³Department of Rehabilitation Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung City 807, Taiwan, Republic of China

⁴Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung City 807, Taiwan, Republic of China

Abstract The blood-brain barrier (BBB) is a specialized structure in the central nervous system (CNS), which participates in maintenance of a state of cerebrospinal fluid homeostasis. The endothelial cells of the cerebral capillaries and the tight junctions between them form the basis of the BBB. Research has shown that destruction of the BBB is associated with diseases of the CNS. However, there is little research on how the BBB might be protected. In this study, we used a high osmotic solution (1.6 M D-mannitol) to open the BBB of rats and Evans blue dye as a macromolecular marker. The effect of heat shock treatment was evaluated. The results show that increased synthesis of heat shock protein 72 (Hsp72) was induced in the heated group only. BBB permeability was significantly less in the heat shock–treated group after hyperosmotic shock. The major tight junction proteins, occludin and zonula occludens (ZO)-1, were significantly decreased after D-mannitol treatment in the nonheated group, whereas they were preserved in the heated group. The coimmunoprecipitation studies demonstrated that Hsp72 could be detected in the precipitates of brain extract interacting with anti-ZO-1 antibodies as well as those interacting with anti-occludin antibodies in the heated group. We conclude that the integrity of tight junctions could be maintained by previous heat shock treatment, which might be associated with the increased production of Hsp72.

INTRODUCTION

The blood-brain barrier (BBB), a unique structure between the central nervous system (CNS) and the peripheral circulation system, acts as a metabolic and physical barrier to maintain brain homeostasis. The BBB is characterized by 2 particular biological phenomena that make it different from other systemic vascular barriers. The first is the low transcytotic activity of the BBB endothelial cells; the second is that the vascular endothelial cells of the brain contain highly resistant tight junctions fusing

the plasma membranes of neighboring vascular endothelial cells.

Tight junctions of the BBB are composed of an intricate combination of transmembrane and cytoplasmic proteins linked to an actin-based cytoskeleton, which allows the tight junctions to form a seal (Petty and Lo 2002). The tight junctions form a continuous permeability barrier between adjacent cells and influence the flux of molecules through the paracellular pathway of the brain vessels. At the molecular level, tight junctions are made up of several accessory proteins that are necessary for structural support. This support is primarily composed of the 2 kinds of proteins. One is occludin, a transmembrane protein that forms dimers and binds homotypically to adjacent endothelial cells to form the primary seal of the tight

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Correspondence to: Rei-Cheng Yang, Tel: 886-7-3121101; Fax: 886-7-3234687; E-mail: rechya@kmu.edu.tw.

junction. The other kind is the zonula occludens proteins (ZO-1, ZO-2, and ZO-3), which serve as recognition proteins for tight junction placement as well as a support structure for signal transduction proteins.

Research indicates that many diseases are caused by the opening of the BBB. Its destruction can lead to brain edema and subsequent pathological changes. Furthermore, recent studies indicate that dysfunction of the tight junction proteins is a hallmark of many CNS pathologies. Destruction of the BBB components induces permeability changes and lethal complications in several diseases such as bacterial meningitis (Kieseier et al 1999), acute inflammation (Kim et al 1997), septic encephalopathy, human immunodeficiency virus infection (Dallasta et al 1999), multiple sclerosis (Kondo and Suzuki 1993), Alzheimer's disease (Stadelmann et al 1998), and toxic chemical invasion (Richmon et al 1995). To date, there has been little mention of whether the BBB could be modulated to help prevent disease, and currently there is no effective therapy to stop or prevent the destruction of the BBB in the disease cascade. Searching for protective interventions to prevent destruction of the BBB might eventually help treat the life-threatening conditions that featured BBB breakdown.

Heat shock proteins (Hsps) have been the subjects of research for many years. They are characterized as a family of highly conserved and universally inducible proteins found in almost all organisms and cultured cells in response to stress (Chopp et al 1989; Chen et al 2000). Since first mentioned by Ritossa (1962), it is now well accepted that living cells, from plants to humans, react to heat and other physiological or pathological stresses by synthesizing Hsps. Hsps act against subsequent damage by increasing the tolerance of the organisms, known as thermotolerance or cross-tolerance phenomena. In previous studies, we have shown that overexpression of Hsps induced by hyperthermia treatment could protect the brain cortex from hypoxic damage (Yang et al 1994) and decrease the bicuculline-triggered convulsive effects in rats (Yang et al 1996). In addition, heat shock pretreatment attenuated the vascular permeability change and subsequently preserved the blood pressure in an anaphylactic shock model of rats (Chen et al 2001). Similar effects have also been seen in the barrier function of colonic and intestinal epithelial cells (Musch et al 1996; Urayama et al 1998). These studies suggest that further investigation of BBB permeability and regulation could be valuable for developing efficient therapeutic interventions to protect or arrest the damage of the BBB as well as of the CNS.

In this study, we investigated the effects of heat shock treatment on BBB permeability by evaluating the extravasation of Evans blue (EB) dye in rats after hypertonic solution infusion. We also investigated possible mechanisms for the observed effects.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley rats (300–350 g) were purchased from the National Laboratory Animal Breeding and Research Center (Nan-Kang, Taipei, Taiwan). The experiments conducted in this study were approved by the animal committee of Kaohsiung Medical University, and the authors adhered to the guidelines of the National Institutes of Health for the use of experimental animals. The animals were housed in an animal center of the Kaohsiung Medical University. Rats were divided into the following groups: (1) control group (no D-mannitol treatment or heat shock treatment) ($n = 6$), (2) heat-control group (no D-mannitol treatment but heat shock treatment) ($n = 3$), (3) nonheated group (D-mannitol treatment without heat shock treatment) ($n = 9$), and (4) heated group (D-mannitol treatment with heat shock treatment) ($n = 9$).

Heat shock treatment

The rats in the heat shock-treated group were chosen randomly and treated with whole-body hyperthermia to induce the overexpression of Hsps (Yang et al 2000) 24 hours before D-mannitol (Sigma Chemical Co, St Louis, MO, USA) infusion. They were anesthetized with sodium pentobarbital (50 mg/kg body weight; intraperitoneal [ip]) and placed on automatic heating pads. When the rectal temperature reached 41°C, it was maintained between 41°C and 42°C for 15 minutes under anesthesia. After the heating pad was removed, the rectal temperature was maintained at 37°C until the rat completely regained consciousness. During the procedure, we ensure that the rat's airway was unobstructed. The heated rats were then returned to their cages to recover for 24 hours. The rats of the nonheated group were also anaesthetized, then returned to their cages.

Induction of BBB opening

BBB opening was induced by 1.6 M D-mannitol infusion. The rats were anesthetized with sodium pentobarbital (50 mg/kg body weight; ip), and the femoral vein was catheterized with a PE50 tube linked with a 3-way stopcock to which 2 other tubes were connected. The first was then connected to a syringe containing normal saline mixed with heparin (1 unit/mL) and the second was connected to the EB dye (20 mg/mL) (Sigma). The left common carotid artery, internal carotid artery, and external carotid artery were exposed, and the distal portion of the external carotid artery was ligated. The common carotid artery was then catheterized with a 24G catheter linked with a peristaltic pump (E500, ATTO Co, Tokyo, Japan). After catheterization, 5 mL of 1.6 M D-mannitol (37°C, filtered,

in normal saline) was injected into the left common carotid artery at a rate of 1 mL/min. After D-mannitol infusion, we waited 15 minutes, then injected EB as a tracer of intravascular fluid extravasation. After EB administration, the rats were observed for an additional 60 minutes to confirm the complete extravasation of intravascular fluid. They were then sacrificed and exsanguinated with 0.9% NaCl solution, followed by 10% buffered formalin (Sigma) fixation through the left ventricle.

Immunohistochemistry study

Frozen 10- μ m sections were used to observe the brain morphology. The brain tissue was fixed in 10% buffered formalin for 24–48 hours at 4°C and then transferred to 30% sucrose in phosphate-buffered saline (PBS) for 48 hours at 4°C. The brain tissue was then embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc, Torrance, CA, USA), frozen in liquid nitrogen, and stored at –70°C until sectioning. The frozen sections were further sectioned by a frozen section machine (Leica, Solms, Germany) at –25°C and placed on a coated glass slide (Dako Cytomation, Glostrup, Denmark). The sections were washed with t-PBS (0.2% Triton X-100 in PBS) for 10 minutes, 3 times, and then soaked in 10% nonfat milk–t-PBS blocking solution at room temperature (20°C) for 1 hour to block the reaction. They were incubated with a t-PBS solution overnight at 4°C, containing 1 of the following antibodies: monoclonal anti-Hsp72 antibody (StressGen Biotechnologies, Victoria, British Columbia, Canada), anti-ZO-1 antibody (Chemicon, Temecula, CA, USA), or anti-occludin antibody (Zymed, South San Francisco, CA, USA). The sections were then washed with t-PBS and incubated with a t-PBS solution containing either fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgG (StressGen Biotechnologies) or FITC-conjugated anti-rat IgG (Chemicon) as the secondary antibody for 2 hours at room temperature. Finally, the sections were washed with t-PBS for 15 minutes 3 times and then cover sealed with fluorescence mounting medium (Dako). Target proteins were observed using fluorescence microscopy (Leica) and photographed. Alkaline phosphatase, a specific marker, was also stained in the brain tissue to identify the location of brain endothelial cell (Orte et al 1999).

Tissue preparation for electrophoresis and Western blot analysis

After exsanguination with 0.9% NaCl solution, the rat brains were removed for electrophoretic assays. Each brain was separated into 2 parts, the control side and the lesion side. They were then ground up in liquid nitrogen and sonicated with lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid, 0.42 M NaCl,

1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid [EDTA], 0.5 mM dithiothreitol, 0.5 mM phenylmethane sulfonyl fluoride [PMSF], 2% NP-40, 25% glycerol) by an ultrasonic processor sonicator (model XL2020, Misonix Inc, Farmingdale, NY, USA) at 4°C. The mixture was centrifuged at 12 000 rpm at 4°C for 30 minutes. The supernatant was harvested and frozen at –20°C for further experiments. Equal amounts (10 μ g) of protein extract were loaded and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins on the gel were transblotted to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA, USA) for 4 hours. The PVDF membrane was soaked in 5% nonfat milk–t-TBS (0.1% tween-20 in Tris-buffered saline) blocking solution at 4°C overnight. After blocking, the membranes were incubated with a t-TBS solution containing monoclonal anti-Hsp72 antibody (StressGen Biotechnologies) as the primary antibody. β -Tubulin (Chemicon) was also tested and used for comparison for protein content in different groups. The membranes were then washed with t-TBS for 5 minutes, 4 times, and incubated with a t-TBS solution containing goat anti-mouse IgG (Amersham Pharmacia, Uppsala, Sweden) as the secondary antibody for 2 hours. Target proteins were stained by enhanced chemiluminescence (ECL kit, NEN Life Science Products) and autographed with X-ray film (Fujifilm, Tokyo, Japan). The results were quantitated and analyzed with a densitometer and assay software (Bio-1D V.97, Vilber Lourmat, Marne-la-Vallée France).

Permeability assay

To assay permeability, vascular protein leakage in the brain was measured using the EB technique (Udaka et al 1970; Baluk et al 1997). Fifteen minutes after 1.6 M D-mannitol infusion, EB dye was injected intravenously (20 mg/kg body weight) using the PE50 tubing cannulated into the femoral vein and observed for 1 hour. The brain was removed and separated into the control and lesion sides and soaked in formamide (4 mL/g of brain tissue) at 60°C for 72 hours. Eluted EB dye was measured by a spectrophotometer (620 nm) and expressed as grams per gram of brain tissue.

Immunoprecipitation study

To immunoprecipitate tight junction protein from brain tissue, the specific antibody–protein A Sepharose mixtures were prepared first. Five micrograms of ZO-1 or occludin antibody was incubated individually with 70 μ L of immunoprecipitation binding buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 mM PMSF) and 20 μ L protein A

Sepharose (Amersham Pharmacia) and gently shaken at room temperature for 60 minutes. Then, the mixture was centrifuged at 6000 rpm at room temperature for 20 seconds to remove the suspension and washed 2 times with immunoprecipitation binding buffer. Next, the specific antibody-protein A Sepharose mixtures were incubated with total brain protein extracts (1.5 mg) and gently rotated at 4°C overnight. The following day, the mixtures were centrifuged at 6000 rpm at room temperature for 20 seconds. The precipitates were washed 3 times with immunoprecipitation binding buffer. The specific antibody-protein A Sepharose-protein mixtures were resuspended in 50 μ L 0.1 M citric acid (pH 2) and vortexed for 10 minutes. The suspension was centrifuged, and the supernatant was collected. The products were mixed with 10 μ L 1 M Tris buffer (pH 9) and separated by SDS-PAGE. After electrophoresis, the target proteins were observed by the methods described above.

Statistical analysis

Values of permeability assay are presented as mean $\pm$ SD. The 1-way analysis of variance (ANOVA) test was used to compare the means of 3 groups of data. If the 1-way ANOVA test indicated an overall significant difference ($P < 0.05$), the Tukey's multiple comparison test was used to determine the significance of a difference in the mean between any 2 groups. The independent *t*-test was used to analyze the data of Western blot assay. Differences were considered significant when *P* value is less than 0.05.

RESULTS

Expression of Hsp72 after heat shock treatment

As shown in Figure 1, there was significantly more Hsp72 content in the total protein extraction from the heated group than that found in the nonheated group, the normal saline-infused rats, or the D-mannitol-treated group ($P < 0.05$). The immunohistochemical study (Fig 2) confirms the location of inducible Hsp72, and the results were identical to the Western blot assay. The inducible Hsp72 was found in brain endothelial cells of heated group. These findings indicate that Hsp72 could be induced successfully by heat shock treatment.

Extravasation of EB in the brain after D-mannitol treatment

BBB permeability was evaluated with the EB technique. EB dye binds macromolecules of the plasma and acts as an indicator of plasma leakage. As shown in Figure 3, extravasation of EB dye could be easily seen without mag-

Fig 1. The Western blot and quantitative analysis study of heat shock protein 72 (Hsp72) expression in the brains of different groups. The data indicated mean $\pm$ SD of the samples in each group. Hsp72 content clearly increased in the heat treatment group only. D-Mannitol treatment did not alter the overexpression of Hsp72 ($n = 3$; *, $P < 0.05$).

nification. The lesion side of the brain in the nonheated rats was deeply stained, especially in the territory of middle and anterior cerebral artery. These areas were only faintly stained in the heated group, similar with those on the control side in all 3 groups and on the saline-infused side. The cross sections show the distribution of EB dye extravasation, from the frontal lobe to the occipital lobe, maximally in the parietotemporal area. Furthermore, the brain cortex appeared to be more significantly stained than the white matter. The corpus callosum appears to be the most sheltered. Only faint staining was found in the heated groups. The extravasation of EB dye in the brain was then eluted and measured by a spectrophotometer at 620 nm. The results showed that EB eluted from the lesion side of the brain was 13.81 ± 3.85 μ g of EB/g of tissue in the nonheated group, whereas it was 5.23 ± 1.42 in the heated group ($P < 0.05$). No difference was noticed in the normal side of the brains in the 3 groups and the saline-infused side (1.39 ± 0.64 μ g of EB/g of tissue; 2.75 ± 0.63 μ g of EB/g of tissue; 2.81 ± 0.88 μ g of EB/g of tissue) (Fig 4).

Immunohistochemical study of tight junction proteins after D-mannitol treatment

To evaluate the alteration of tight junction proteins, occludin and ZO-1, an immunohistochemical study was performed. As shown in Figure 5 A,B (panel A: occludin; panel B: ZO-1), both these 2 major tight junction proteins were observed around the vessels in the normal, saline-infused as well as in the heated, D-mannitol-infused hemispheres. In contrast, occludin and ZO-1 were undetectable in the nonheated, D-mannitol-treated hemisphere. It is clear that both ZO-1 and occludin were de-

Fig 2. (A) The immunohistochemical study of heat shock protein 72 (Hsp72) expression in the different groups of brain tissue using fluorescence microscopy. Hsp72 was strongly expressed in the heated group only. The arrows indicate the Hsp72-abundant endothelial cells. C: no D-mannitol treatment or heat shock treatment; M: D-mannitol treatment without heat shock treatment; H + M: D-mannitol treatment with heat shock treatment; original magnification, 100 $\times$, 400 $\times$. (B) The double staining of Hsp72 and specific brain vascular endothelial cell marker, alkaline phosphatase, of D-mannitol treatment with heat shock treatment specimen. The arrows indicate that the abundant inducible Hsp72 was indeed observed at the brain vascular endothelial cells (original magnification, 400 $\times$).

natured or down-regulated after osmotic stress that could be attenuated by previous heat shock treatment.

The tight junction proteins interact with Hsps

To demonstrate the correlation between Hsps and tight junction proteins, a co-immunoprecipitation study was performed. In samples of the heated group, Hsp72 could be detected in the immunoprecipitate reacting with both anti-ZO-1 and anti-occludin antibodies (Fig 6). The evidence suggests that Hsp72, induced by heat shock treat-

ment, could interact with the tight junction proteins, ZO-1 and occludin.

DISCUSSION

Homeostasis of the microenvironment of the brain is important to its proper functioning and subsequently the functioning of the many physiologic systems it controls. The BBB is 1 way the brain is able to maintain this specialized microenvironment. Normally, the BBB is highly permeable to small molecules such as water, carbon di-

Fig 3. The distribution of Evans blue dye extravasation by the gross view and cross-section view of brain tissue in the different groups. The upper panel shows the dorsal view and ventral view of the whole brain. The lower panel shows the sequential cross sections of the brain. C: no D-mannitol treatment or heat shock treatment; M: D-mannitol treatment without heat shock treatment; H + M: D-mannitol treatment with heat shock treatment; F: frontal lobe; P: parietal lobe; T: temporal lobe; Cb: cerebellum; Po: pons; *: corpus callosum.

oxide, or oxygen, but impermeable to plasma proteins and large, non-lipid soluble, organic molecules. Permeability changes result in leakage of vascular proteins or water into the brain tissue leading to perivascular or vasogenic edema and increased intracranial pressure. In this study, we used the hyperosmotic stress model, which has shown that the BBB opening may persist at least 30 minutes after hyperosmotic stress (Rapoport 2000). Our result confirmed that the BBB could be opened by hyperosmotic stress by demonstrating the extravasation of the macromolecular marker, EB, 15 minutes after hyperosmotic solution challenge. Our results show that the extravasation could be significantly attenuated by previous heat shock treatment. This result is coincident with our previous findings in systemic vascular barriers (Chen et al 2001), indicating that heat shock pretreatment may modulate the endothelium barrier both in the systemic and CNS. In addition, House and coworkers (2001) reported that heat shock pretreatment put rat aortic endothelium in an anti-inflammatory state.

Tight junctions are specialized membrane domains at

the most apical region of the endothelial cells that not only create a primary barrier to prevent paracellular transport of solutes but also restrict the lateral diffusion of membrane lipids and proteins to maintain cellular polarity (Morita et al 1999). Several accessory proteins are required to form the structural support of this barrier. Occludin is an integral protein localized at junction site with 4 transmembrane domains and a long COOH terminal cytoplasmic domain. Cytoplasmic proteins ZO-1, ZO-2, and ZO-3 have been identified and shown to form the submembranous plaque of tight junctions. ZO-1, ZO-2, and ZO-3 bind directly to the carboxy terminal tail of occludin through their guanylyl kinase domains. Then, the primary cytoskeletal protein, F-actin, binds to the carboxy terminal portions of ZO-1 and ZO-2 (Furuse et al 1994; Fanning et al 1998; Haskins et al 1998; Itoh et al 1999). The interactions between occludin, ZO protein, and F-actin are essential in the regulation and assembly of tight junctions. Coincident with the changes in permeability, our results show that ZO-1 as well as occludin could not be detected on the lesion side of the brain after

Fig 4. The quantification of Evans blue (EB) dye extravasation in the control and lesion sides of brains in different groups. Data were expressed as grams of EB per gram of tissue and mean $\pm$ SD in each group and were analyzed statistically by 1-way analysis of variance and the Tukey's multiple comparison test as the posttest. The results show that EB eluted from the lesion side of the brain in the nonheated group was significantly higher than those in the heated group after D-mannitol treatment. No significant differences were noted on the control side of the brain in the 3 groups on the saline-infused side (*, $P < 0.05$).

1.6 M D-mannitol infusion, whereas nearly no change was found on the control side of the same rat. These results suggest that hyperosmotic stress induced the disassembling or reorganization of tight junctions, leading to increased vascular permeability. Moreover, our results show this change could be attenuated by heat shock treatment.

Hsps belong to multigene families and are universally induced in all living cells, organisms, and cultured cells by heat shock treatment as well as by many other chemical or physical stresses (Tissieres et al 1974; Lindquist 1986; Lindquist and Craig 1988). Sublethal heat pretreatment, inducing the Hsps, is known to play a protective role in all living cells against subsequent stress (Lindquist 1986; Yellon and Latchman 1992; Yang et al 1998; Chen et al 2001). Their physiological roles have been extensively studied in mammals. It has been reported that the cortical neuronal damage caused by hypoxic ischemia is significantly decreased when preceded by previous hyperthermia treatment. To our knowledge, this study is the first to document the protective role of Hsps in maintaining BBB integrity.

In cells, Hsps may act as molecular chaperones by participating in the refolding or folding of misfolded polypeptides or posttranslational protein assembly and translocation (Pelham 1989; Wynn et al 1994; Benjamin and McMillan 1998). There is growing evidence that the cytoskeleton plays an important role in regulating the contraction of endothelial cells, and Hsps have significant effects on the cytoskeleton (Loktionova and Kabakov 1998; Wittchen et al 1999). It is known that Hsp70 may

Fig 5. The immunohistochemical study of tight junction protein expression in the different groups of brain tissue using fluorescence microscopy. Panel A: occludin; panel B: ZO-1. C: no D-mannitol treatment or heat shock treatment; M: D-mannitol treatment without heat shock treatment; H + M: D-mannitol treatment with heat shock treatment; original magnification, 400 $\times$.

stabilize actin filaments in cultured cells (Macejak and Luftig 1991) and act as an Hsp70-related actin capping protein (Weeds and Maciver 1993). Small Hsps also act as modulators of the cytoskeleton components such as microfilaments and intermediate filaments (Hino et al 2000). In stressed cells, some Hsps may function as specialized chaperones for the actin, participating in its protection and recovery (Mounier and Arrigo 2002). It has been reported that Hsps may support neuronal survival in the brain after transient ischemia through refolding of the denatured protein (Yagita et al 2001). Effect of Hsps on the BBB is still poorly documented. The present study demonstrated that Hsp72 could be co-immunoprecipitated with tight junction proteins, ZO-1 and occludin, in

Fig 6. The immunochemical study of heat shock protein 72 from the coimmunoprecipitation products reacted with anti-ZO-1 or occludin antibody. C: no D-mannitol treatment or heat shock treatment; M: D-mannitol treatment without heat shock treatment; H + M: D-mannitol treatment with heat shock treatment. IP ZO-1: immunoprecipitated with anti-ZO-1 antibody; IP occludin: immunoprecipitated with anti-occludin antibody.

preheated rats where the extravasation of EB was significantly decreased. These data suggested that Hsp72 acts as a chaperone for the tight junction proteins to preserve the conformation and function of the BBB.

In conclusion, Hsp72, induced by heat shock treatment, plays an important role in stabilizing the tight junction proteins of the endothelial cells and subsequently maintaining BBB integrity during hyperosmotic stress. These results highlight a possible novel therapeutic intervention in the prevention of pathological states resulting from breakdown or opening of the BBB.

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