Potentiation of Baroreceptor Reflex Response by Heat Shock Protein 70 in Nucleus Tractus Solitarii Confers Cardiovascular Protection During Heatstroke

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Background—Whereas hypotension and bradycardia seen during the onset of heatstroke may be protected by prior induction of heat shock protein 70 (HSP70) in the brain, the underlying mechanism is not fully understood. We evaluated the hypothesis that HSP70 may confer cardiovascular protection during heatstroke by potentiating the baroreceptor reflex (BRR) control of peripheral hemodynamic performance.

Methods and Results—Adult male Sprague-Dawley rats subjected to a brief hyperthermic heat shock (HS; 42°C for 15 minutes) induced discernible expression of HSP70 in the bilateral nucleus tractus solitarii (NTS), the terminal site in the brain stem for primary baroreceptor afferents. This HSP70 expression was detected at 8 hours, peaked at 24 hours, and returned to baseline by 48 hours after HS. Brief hyperthermia also significantly potentiated the BRR response in a temporal profile that correlated positively with changes in HSP70 expression at the NTS. Prior HS also appreciably alleviated hyperthermia, severe hypotension, and bradycardia manifested during the onset of heatstroke (45°C for 60 minutes) elicited 24 hours later. Microinjection bilaterally of anti-HSP70 antiserum (1:20) into the NTS or denervation of the sinoaortic baroreceptor afferents significantly reversed the enhancement of BRR response and cardiovascular protection during heatstroke induced by prior HS.

Conclusions—These results suggest that HS-induced expression of HSP70 in the NTS may alleviate severe hypotension and bradycardia exhibited during the onset of heatstroke by potentiating both the sensitivity and capacity of BRR response. (Circulation. 2001;103:2114-2119.)

Key Words baroreceptors ■ blood pressure ■ heart rate ■ nervous system, autonomic ■ stroke

Animals exposed to heat, toxins, seizure, trauma, or ischemia express a set of highly conserved proteins known as heat shock proteins (HSPs) in brain and other tissues. These HSPs are thought to be involved in cellular repairs, resulting in protection of the central nervous system (CNS) and other tissues from subsequent lethal damage. For example, expression of HSP70 or HSP72 increases tolerance of hippocampal neurons to ischemic injury, and overexpression of HSP70 alone protects cultured hippocampal neurons against severe heat shock (47°C). HSPs also provide protection against cerebral ischemia, hemodynamic dysfunctions, and monoamine overload during heatstroke. Transfer of viral vectors that overexpress HSP72 into the brain is neuroprotective in a rat model of stroke.

Exposure of animals to an elevated ambient temperature induces heatstroke that is characterized by reduced cerebral blood flow, hypotension, and bradycardia. Such hemodynamic dysfunctions seen during the onset of heatstroke can be protected by prior heat shock (HS). More importantly, the time course of this protection correlated with the temporal profile of HSP70 or HSP72 expression. The mechanism that underlies the protective actions of HS-elicited HSP70 in the CNS against heatstroke-induced cerebral ischemia, hypotension, and bradycardia, however, is not fully understood.

Baroreceptor reflex (BRR) is one of the fundamental mechanisms through which the CNS controls peripheral hemodynamic performance. It follows that HSP70 induced by HS may protect animals against heatstroke-induced hemodynamic dysfunctions by potentiating the BRR response through an action on the nucleus tractus solitarii (NTS), the principal recipient of baroreceptor afferent fibers in the medulla oblongata. This hypothesis was validated in the present study. We demonstrated that HSP70 expressed in the NTS after HS may alleviate the severe hypotension and bradycardia during the onset of heatstroke by potentiating both the sensitivity and capacity of BRR response.

Methods

Adult male Sprague-Dawley rats (weight, 200 to 250 g; n=265) were purchased from the National Animal Experimental Center of the National Science Council, Taiwan. All experimental procedures...
were carried out in compliance with the guidelines of our institutional animal care committee.

**HS Induction**

Animals subjected to hyperthermic shock were anesthetized with sodium pentobarbital (40 mg/kg IP) and were placed on a temperature-controlled electric heating pad set at 45°C. HS was induced by maintaining the core temperature of heated animals at 42±0.5°C for 15 minutes, as monitored by a thermistor probe placed in the colon. Animals were thereafter allowed to recover at room temperature for the time interval as determined for each experiment.

**Protein Extraction and Western Blot Analysis**

Western blot analysis of HSP70 at the dorsomedial medulla was performed with the use of mouse monoclonal antiserum against the inducible form of HSP70 (HSPAb, 1:500; SPA-810, StressGen) as the primary antiserum and horseradish peroxidase–conjugated goat anti-mouse IgG (1:2500; Jackson) as the secondary antiserum. Specific antibody-antigen complex was detected by an enhanced chemiluminescence Western blot detection system (NEN™, Life Science Products).

**Evaluation of BRR Control of Heart Rate**

As in our previous studies, an increase in systemic arterial pressure (SAP) was induced by intravenous infusion of phenylephrine (10 µg·kg⁻¹·h⁻¹) for 30 minutes. The slope of the regression line that relates the reduction in heart rate (HR) with elevation in SAP during the ramp phase of pressor response was taken as the sensitivity of BRR control of HR. The maximal reflex bradycardia during the plateau stage of pressor response was taken as the capacity of BRR control of HR.

**Evaluation of BRR Control of Sympathetic Vasomotor Tone**

On-line spectral analysis of SAP signals was carried out as reported previously. Changes in the integrated power density of the low-frequency (LF) component (0.25 to 0.8 Hz) of SAP signals, which represents the BRR-mediated neurogenic sympathetic vasomotor tone, to a decrease in SAP induced by 10 minutes of intravenous infusion of nitroprusside (5 µg · kg⁻¹ · h⁻¹) were determined.

**Sinuovasic Denervation**

Sinuovasic denervation (SAD) or sham control was performed according to the procedures of Osborn. The effectiveness of SAD was confirmed 4 weeks after surgery, with the slope of the regression line that relates changes in HR to SAP (BRR sensitivity) reduced to 0.16 against 1.17 bpm/mm Hg in the sham controls.

**Microinjection of Antiserum Against HSP70 Into the NTS**

The same anti-HSPAb (1:50 or 1:20) used in Western blot analysis was microinjected sequentially, at a volume of 50 nL, into the bilateral NTS. The coordinates were 0.5 to +0.5 mm from the obex, 0.3 to 0.8 mm lateral to the midline, and 0.5 to 1.0 mm below the dorsal surface of the medulla oblongata. We added 0.02% Triton X-100 to the microinjection solution to facilitate transport of the antiserum across the cell membrane. Microinjection of normal mouse serum (1:20) or Triton X-100 served as our controls.

**Induction of Heatstroke**

A modification of the procedures for inducing heatstroke was used. Animals maintained under pentobarbital anesthesia (20 mg · kg⁻¹ · h⁻¹ IV) were placed for 60 minutes on a heating pad kept at 45°C. The instant at which SAP or HR began to decrease from their peak levels was taken as the onset of heatstroke.

**Histology**

The brain was removed and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥72 hours. Frozen 25-µm sections of the medulla oblongata were stained with 1% neutral red for histological verifications of the location of microinjection sites. One percent Evans blue dye was added to the microinjection solution to facilitate this process.

**Statistical Analysis**

All values are expressed as mean±SEM. One-way or 2-way ANOVA with repeated measures was used, as appropriate, followed by Scheffé’s multiple range test for post hoc assessment of individual means. Mortality rate was assessed by the Fisher’s exact test. A value of P<0.05 was considered statistically significant.

**Results**

**Temporal Changes in HSP70 Expression in Dorsomedial Medulla After HS**

Exposure of animals to a brief hyperthermic shock (42°C for 15 minutes) induced temporal changes in HSP70 expression in the dorsomedial medulla, including the bilateral NTS (Figure 1). Western blot analysis indicated that HSP70 underwent a significant increase at 8 hours, followed by a progressive augmentation that peaked at 24 hours, and a return to baseline by 48 hours after HS. In normothermic controls, in which animals were similarly anesthetized but without subsequent hyperthermic treatment, HSP70 was not detected in the dorsomedial medulla at all time intervals examined.

**Effect of HS on BRR Response**

On the basis of analysis of reflex sensitivity (Figure 2A) and maximal reflex bradycardiac capacity (Figure 2B) in response to a sustained increase in SAP, we found a significant potentiation of BRR control of HR 16 hours and 24 hours after animals were subjected to a brief hyperthermic shock. We confirmed that infusion of phenylephrine for 30 minutes resulted in comparable elevations in SAP in normothermic controls (plateau: +45.3±3.6 mm Hg, n=12) and hyperthermic animals (plateau: +44.5±2.8 mm Hg, n=14). We also observed that brief hyperthermic shock (n=14) did not elicit discernable effect on baseline mean SAP or HR when compared with normothermic controls (n=12) 16 hours after treatment (109.4±4.3 versus 104.6±3.4 mm Hg; 380±11 versus 376±13 bpm) or 24 hours after treatment (106.3±5.1 versus 108.4±2.9 mm Hg; 374±9 versus 382±17 bpm).

A brief hyperthermic shock also augmented the sympathoexcitatory response to unloading of baroreceptors (Figure 3A). We found in untreated and normothermic controls an increase in the power density of the LF component in SAP signals in response to hypotension induced by intravenous infusion of nitroprusside. Such a BRR-mediated increase in sympathetic vasomotor tone was significantly potentiated (+77.2±9.7%, n=7) 24 hours after animals were subjected to HS. We again confirmed that nitroprusside induced comparable hypotension in normothermic (−43.6±2.4 mm Hg, n=7) and hyperthermic (−45.6±3.5 mm Hg, n=7) animals.

**Effect of Microinjection Bilaterally of Anti-HSPAb Into the NTS on Potentiation of BRR Response by HS**

Microinjection bilaterally of anti-HSPAb into the NTS, delivered 2 hours before BRR evaluations, dose-dependently
attenuated the potentiation of BRR slope (Figure 4A) or maximal reflex bradycardia (Figure 4B) in response to hypertension or the reflex increase in power density of the LF component in SAP signals (Figure 3B) in response to hypotension (1:50, 1:42.1±6.4%, n=7; 1:20, 1:17.7±6.4%, n=7), determined 24 hours after brief hyperthermic preconditioning. In contrast, local application of normal mouse serum (1:20) or 0.02% Triton X-100 into the NTS minimally affected the HS-induced enhancement of BRR control of HR (Figure 4). When evaluated in normothermic controls at corresponding time intervals, microinjection of normal mouse serum (1:20, 0.95±0.12, n=6), 0.02% Triton X-100 (1.05±0.17, n=7), or anti-HSPAb (1:20, 1.02±0.18, n=6) resulted in minimal alterations in BRR control of HR.

**Effect of HS and/or Microinjection Bilaterally of Anti-HSPAb Into the NTS on Hyperthermia, Hypotension, and Bradycardia During Heatstroke**

Heatstroke, characterized by hyperthermia, severe hypotension, and bradycardia, was observed in normothermic control animals that were subjected to prolonged heat stimulation at 45°C for 60 minutes (Figure 5). These manifestations of heatstroke were significantly alleviated (Figure 5) by a brief HS, delivered 24 hours before the incidence of heatstroke. The causative engagement of HSP70 expressed at the NTS in cardiovascular protection against heatstroke was revealed (Figure 5) when microinjection bilaterally into the NTS of anti-HSPAb (1:20), 2 hours before the induction of heatstroke, discernibly albeit partially attenuated the alleviated hypotension and bradycardia elicited by brief HS. We also noted that 2 of 6 animals that received microinjection into the NTS of anti-HSPAb died within 120 minutes after heatstroke induction, our cutoff time of evaluation. In contrast, none of the control animals died during the same observation period after heatstroke. In addition, blockade of endogenous HSP70 by local administration of its specific antiserum (1:20) into the NTS of normothermic controls was ineffective in attenuating the manifestations of hemodynamic dysfunctions during the onset of heatstroke. Local application of anti-HSPAb...
into the NTS also minimally affected the HS-induced thermotolerance during heatstroke (Figure 5).

Effect of Sinoaortic Denervation on HS-Induced Cardiovascular Protection Against Heatstroke

Compared with the sham control, which essentially duplicated the HS group, the alleviation of hypotension and bradycardia by a brief HS delivered 24 hours before heatstroke was profoundly reversed in the SAD animals (Figure 5). Baroreceptor afferent denervation also significantly increased the mortality rate; 4 of 6 SAD animals died within 120 minutes after the onset of heatstroke. Nonetheless, SAD elicited no discernible effect on the expression of HSP70 in the dorsomedial medulla, measured 24 hours after HS (255±40 versus 248±26, n=6).

Histological Verifications of Microinjection Sites

Histological verifications indicated that the tip of the micropipettes used to deliver anti-HSPAb was located within the dorsomedial or medial part of caudal NTS. Microinjection of anti-HSPAb into areas outside the confines of NTS (ie, nucleus gracilis, areas dorsolateral to NTS) elicited minimal effect on HS-induced cardiovascular protection against heatstroke.

Discussion

This study provides novel insights into the cardiovascular regulatory role of brain HSPs in the protection against heatstroke. Our results substantiate the notion that the inducible form of HSP70, expressed in the NTS of animals after a brief HS, contributes to central circulatory regulation by potentiating the BRR response. By rendering the cardiovascular system less vulnerable, such an enhancement of BRR response in turn confers crucial protection against hemodynamic dysfunctions during the onset of heatstroke.

In response to hyperthermia and other stresses, cells exhibit an upregulation of HSP expression, which contributes to cellular repair and protective mechanisms. It is well established that this classic HS response protects the nervous system from subsequent stress. As such, a major contribution of this study is the demonstration that HS-induced HSP70 expression in the NTS may contribute to central cardiovascular regulation by potentiating both parasympathetic and sympathetic arms of the BRR loop. Reduced cerebral blood flow and cerebral ischemia have been proposed to be the main causes of neuronal damage during heatstroke. It is therefore conceivable that by enhancing both the sensitivity and capacity of BRR control of the heart and peripheral vasculature, brain HSP70 may confer neuronal protection by sustaining a stable SAP in the face of hemodynamic perturbations during heatstroke, resulting in maintained cerebral perfusion pressure to brain tissues.

Prior HS induces CNS HSP70 synthesis and protects against heatstroke-induced hyperthermia, arterial hypotension, and bradycardia in a temporally correlated manner.
Superimposed on these crucial correlative evaluations, this study provides direct evidence for a causative role of CNS HSP70 in cardiovascular protection during HS-induced thermodurability. We found that the expression of HSP70 in the dorsomedial medulla was upregulated in a temporal and graded fashion that correlated positively to the potentiation of BRR response after HS (see Figures 1 through 3). More intriguingly, neutralization of the induced HSP70 by administration into the NTS of anti-HSPAb, at a post-HS interval when maximal expression of HSP70 was induced, significantly reversed the potentiation of BRR response (see Figures 3 and 4). The same anti-HSPAb treatment also precluded the acquisition of thermotolerance by discernibly attenuating the HS-induced cardiovascular protection during subsequent heatstroke (see Figure 5). Application of an antiserum against HSP70 also disables the survival of rat fibroblasts to a brief incubation at 45°C after mild HS (37°C) treatment.20

Another novel finding of our study is that BRR potentiation and cardiovascular protection against heatstroke by HS are causatively related. We found that the alleviation of hypotension and bradycardia during heatstroke by prior HS was profoundly reversed in the SAD animals (see Figure 5). Elimination of baroreceptor afferent inputs also significantly increased mortality rate during heatstroke. We also established that such a reversal of HS-induced cardiovascular protection against heatstroke was not due to lack of expression of brain HSP70.

We noted that whereas a graded increase in HSP70 expression in the dorsomedial medulla was detected 8, 16, and 24 hours after HS (see Figure 1), significant potentiation of BRR response was detected only at 16 and 24 hours (see Figure 2). These observations suggest that a threshold level of HSP70 expression in the NTS is required for the enhancement of BRR response to take place. Because our samples also included tissues from area postrema and dorsal motor nucleus of the vagus nerve, two medullary structures that are involved in BRR control of blood pressure,21,22 it is possible that HS-induced HSP70 expression in these two nuclei may also participate in the modulation of BRR response. This possibility, however, is deemed unlikely because HS-induced BRR potentiation was almost completely reversed by microinjection of anti-HSPAb into the bilateral NTS (see Figures 3 and 4).

It is also noteworthy that despite a significant reversal of HS-induced BRR potentiation, microinjection bilaterally of anti-HSPAb into the NTS did not completely attenuate the HS-promoted cardiovascular protection during heatstroke (see Figure 5). This observation suggests that HSP70 induced in other regions of the CNS are also involved in cardiovascular protection during heatstroke. A recent study23 reported that whereas the brainstem contains the essential neural circuitry required for mediating heating-induced changes in the frequency components of sympathetic nerve activity, changes in sympathetic nerve activity induced by acute heating depend only on sympathetic spinal structures. Hyperthermia induces expression of HSP70 mRNA in forebrain regions that are involved in neuroendocrine control of blood pressure.24 HSP70 expression induced in the peripheral vasculature25 and cardiac myocytes26 by HS also confer cardiovascular protection during heatstroke. Induction of HSP70 by prior HS also reduces the augmented production of interleukin-1 and other cytokines in the plasma, resulting in protection against heatstroke-induced hypotension and bradycardia.8

One of the major actions of HS-induced CNS HSP70 is to protect animals against hyperthermia during lethal HS.2,7,8 Our results indicate that such an acquired thermotolerance may not be attributable to upregulation of HSP70 in the NTS elicited by HS. We found that neutralization of the induced HSP70 by microinjection bilaterally of anti-HSPAb into the NTS did not discernibly affect the acquired thermotolerance against severe HS (see Figure 5). In this regard, expression of HSP70 induced by HS in the hypothalamic areas24 that are engaged in neural regulation of body temperature may play a more crucial role.

Figure 5. Time-course changes in colonic temperature (T col), mean systemic arterial pressure (MSAP), or HR, evaluated in normothermic controls (NT) or 24 hours after animals were subjected to hyperthermic HS. Some HS rats received in addition SAD or microinjection bilaterally into NTS of anti-HSPAb (1:20) 2 hours before induction of heatstroke (at bar). For clarity, data on sham animals are not shown because they essentially duplicated HS group. Values are mean ± SEM, n = 5 to 6 animals per group. *P < 0.05 vs NT group, #P < 0.05 vs HS group in Scheffe’s multiple range test.
During the heatstroke experiments, animals were maintained under intravenous infusion of sodium pentobarbital at 20 mg · kg\(^{-1}\) · h\(^{-1}\). This management scheme was found\(^{14}\) to provide stable anesthesia while preserving the capability of cardiovascular regulation, including the BRR response. To facilitate the transport of anti-HSPAb across the cell membrane to the cytoplasm,\(^{27,28}\) 0.02% Triton X-100 was added to our microinjection solution. We found that local application of Triton X-100 into the NTS did not affect the NTS neurons to execute BRR response.

**Summary**

This study provides the first demonstration of a causative role for HSP70 expression induced by HS in the NTS in the potentiation of BRR response in the rat. By rendering the circulatory system less vulnerable, such an enhancement of BRR response in turn confers crucial protection against hemodynamic dysfunctions during heatstroke.

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