Altered Temporal Profile of Heat Shock Factor 1 Phosphorylation and Heat Shock Protein 70 Expression Induced by Heat Shock in Nucleus Tractus Solitarii of Spontaneously Hypertensive Rats

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Background—We demonstrated recently that heat shock (HS)–induced heat shock protein 70 (HSP70) expression in bilateral nucleus tractus solitarii (NTS), the terminal site in the brain stem for primary baroreceptor afferents, confers cardiovascular protection against heatstroke by potentiating baroreceptor reflex (BRR) response. This study evaluated the hypothesis that altered regulation of HSP70 expression may be associated with the heightened susceptibility to heatstroke during hypertension.

Methods and Results—Spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats anesthetized with propofol were used. Compared with WKY rats, significant induction in HSP70 or phosphorylation of heat shock factor 1 (HSF1), but not HSF2, in the NTS and potentiation of BRR response in SHR occurred earlier (4 versus 8 hours), reaching peak magnitude sooner (16 versus 24 hours), and declined more rapidly after a brief hyperthermic HS (42±0.5°C for 15 minutes). The protection conferred by HS against hypotension and bradycardia during the onset of heatstroke (45°C for 60 minutes), although effective, was less effective in SHR. Microinjection bilaterally into the NTS of the selective protein kinase A (PKA) inhibitor H-89 (100 pmol) or the selective PKC inhibitor calphostin C (100 pmol) significantly attenuated all of the above events induced in SHR by HS. However, only H-89 was effective in WKY rats.

Conclusions—An altered temporal profile of HS-induced HSP70 expression or potentiation of BRR response by concurrent activation via both PKA and PKC pathways of phosphorylation of HSF1 in the NTS may be associated with greater susceptibility to heatstroke during hypertension. (Circulation. 2003;107:339-345.)

Key Words: blood pressure ■ heart rate ■ nervous system ■ stroke

Environmental stresses, including heat shock (HS), induce synthesis of a multigene family of proteins known as heat shock proteins (HSPs) that increase the ability of cells to withstand subsequent and otherwise lethal heat challenges. In terms of central cardiovascular regulation, we reported recently that a brief hyperthermic HS increases the expression of HSP70, the major inducible form of HSPs, in the nucleus tractus solitarii (NTS), the terminal site of baroreceptor afferent fibers in the medulla oblongata. This HS-induced HSP70 expression in turn confers cardiovascular protection against heatstroke by potentiating the baroreceptor reflex (BRR) response.

The HS response is primarily regulated at the level of transcription and is mediated by an interaction between heat shock transcription factors (HSFs) and the heat shock element (HSE), a specific regulatory element present in the promoter region of hsp genes. Recent studies showed that HSP70 expression after HS is attributable to phosphorylation of HSF. In addition, cAMP-dependent protein kinase A (PKA) and calcium/phospholipid-dependent protein kinase C (PKC) stimulate phosphorylation of HSF after HS.

Patients with underlying cardiovascular diseases or risk factors such as hypertension are more prone to heatstroke, although the underlying mechanism is not fully understood. That hypertensive animals or patients respond to HS with enhanced phosphorylation of HSF or increased accumulation of hsp70 mRNA in blood vessels or lymphocytes offers a clue. It follows that altered regulation of HSP70 expression and differential activation of HSF by PKA and PKC may be associated with the heightened susceptibility to heatstroke seen during hypertension. The present study validated this hypothesis.
Methods

Adult male spontaneously hypertensive rats (SHR) or Wistar-Kyoto (WKY) normotensive rats (180 to 215 g, n=171) obtained from the Experimental Animal Center of the National Science Council, Taiwan, were used. All experimental procedures were carried out in compliance with the guideline of our institutional animal care committee.

HS Induction

Under pentobarbital anesthesia, SHR or WKY rats were placed on a temperature-controlled electric heating pad set at 45°C. Hyperthermic HS was induced by maintaining the core temperature of 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 stipulated for each experiment. Normothermic (NT) controls were similarly anesthetized but received no HS induction.

Protein Extraction and Western Blot Analysis

Proteins extracted from the dorsomedial medulla that included NTS were subjected to Western blot analysis of HSP70, HSF1, HSF2, or β-tubulin (as loading control). Primary antisera used included mouse monoclonal antiserum against the inducible form of HSP70 (1:500; SPA-810, StressGen) or rat monoclonal antiserum against HSF1 (1:500, MAB88078; Chemicon) or HSF2 (1:500, MAB88079; Chemicon).

Secondary antisera used included horseradish peroxidase–conjugated goat anti-mouse IgG (1:5000, Jackson) for HSP70 or goat anti-rat IgG (1:5000, Jackson) for HSF1 or HSF2. 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

BRR control of heart rate (HR) was evaluated by measuring the slope of regression line that relates reflex changes in HR with increase or decrease in systemic arterial pressure (SAP) evoked by intravenous administration of phenylephrine (2.5, 5, or 10 μg · kg⁻¹ · h⁻¹) or nitroprusside (5, 10, or 15 μg · kg⁻¹ · h⁻¹). Microinjection of Test Agents into the NTS

Microinjection bilaterally into the NTS of test agents was carried out immediately after HS. The coordinates were −0.5 to +0.5 mm from obex, 0.3 to 0.8 mm lateral to midline, and 0.5 to 1.0 mm below dorsal surface of the medulla oblongata. These included the selective PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) or the selective PKC inhibitor calphostin C. A total of 50 nL was delivered to each side over 1 to 2 minutes to allow for complete diffusion of the test agents.

Heatstroke Induction

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. SHR or WKY rats after hyperthermic HS. NT controls received the same heatstroke induction.

The instant at which SAP or HR began to decrease from its peak level was taken as the onset of heatstroke, and observation continued for another 60 minutes or until the animal died.

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 histologic verifications of the location of microinjection sites.

Statistical Analysis

All values are expressed as mean±SEM, n=5 to 6 animals per group. One-way or 2-way ANOVA with repeated measures was used, as appropriate, followed by the Scheffé multiple-range test for post hoc assessment of individual means. P<0.05 was considered statistically significant.

Results

Differential Time-Course Changes in HSP70 Expression in Dorsomedial Medulla of SHR or WKY Rats After HS

Exposure to a brief hyperthermic HS induced in both SHR and WKY rats expression of HSP70 protein in the dorsomedial medulla that contains the bilateral NTS. Whereas the peak magnitude of the induced expression was comparable, the time course was different (Figure 1). HSP70 in the dorsomedial medulla of SHR underwent a significant increase at 4 hours, followed by a dramatic augmentation at 8 hours, peak at 16 hours, and a gradual decline that reached baseline 48 hours after HS. On the other hand, discernible increase in HSP70 was detected at 8 hours in WKY rats, reaching its peak at 24 hours, and returned to baseline by 48 hours after HS. HSP70 was not detected in the dorsomedial medulla of both strains of rats at all time intervals examined in NT controls.
Differential Time-Course Changes in HSF1 Phosphorylation in Dorsomedial Medulla of SHR or WKY Rats After HS

In NT controls, HSF1 or HSF2 from the dorsomedial medulla appeared on SDS-polyacrylamide gels as an ~70-kd protein (Figure 2). A brief hyperthermic HS in both rat strains resulted not only in augmented expression but also appreciable shift to a higher molecular weight form attributable to phosphorylation in HSF1 but not HSF2 (Figure 2).19,20 Intriguingly, whereas the peak of HSF1 phosphorylation was comparable, the slope was significantly steeper in the SHR (0.25 ± 0.01 versus 0.16 ± 0.01, n = 4 animals per group; P < 0.05, multiple linear regression analysis). As such, the time course of discernible phosphorylation of HSF1 paralleled that observed for HS-induced HSP70 expression in the dorsomedial medulla of SHR or WKY rats.

Differential Time-Course Changes in Potentiation of BRR Response in SHR or WKY Rats After HS

Similar to normotensive Sprague-Dawley rats,3,15 a brief hyperthermic HS elicited significant potentiation of BRR control of HR (Figure 3). It is of interest that the temporal profile of this HS-induced BRR potentiation was compatible with that of expression of HSP70 (Figure 1) or phosphorylation of HSF1 (Figure 2) in the dorsomedial medulla of SHR or WKY rats. We noted that along with discernibly reduced baseline BRR slope,21 the amplitude of peak potentiation of BRR response after HS was appreciably lower in SHR (75.7 ± 5.4% versus 96.3 ± 6.3%, n = 6 animals in each group).

We confirmed that bolus injection of phenylephrine or nitroprusside resulted in comparable increases or decreases in mean SAP (MSAP) in NT and HS animals. We also observed that brief hyperthermic HS did not appreciably alter baseline MSAP or HR at all post-HS intervals in both strains of rats compared with their respective NT controls.

Differential HS-Induced Cardiovascular Protection Against Heatstroke in SHR or WKY Rats

Heatstroke, characterized by hyperthermia, severe hypotension, and bradycardia, was observed in SHR or WKY rats that were subjected to heat stress at 45°C for 60 minutes (Figure 4). The slope of cardiovascular depression during heatstroke was discernibly steeper and the survival time appreciably shorter in SHR (33.6 ± 3.7 versus 47.5 ± 4.3 minutes, n = 6 animals per group). Compared with NT controls, these manifestations of heatstroke were significantly alleviated by a brief hyperthermic HS, delivered 16 hours in SHR or 24 hours in WKY rats before the induction of prolonged heat stress. Closer examination revealed that at maximal HSP70 expression in the dorsomedial medulla, this HS-induced cardiovascular protection against heatstroke was appreciably retarded in SHR (Figure 4).

Differential Participation of PKA and PKC in HS-Induced HSP70 Expression or HSF1 Phosphorylation in Dorsomedial Medulla of SHR or WKY Rats

Blockade of PKA activity by microinjection bilaterally into the rostral ventrolateral medulla (RVLM) of H-89 (100 pmol)
significantly reduced the temporal increase in HSP70 expression (Figure 5) or HSF1 phosphorylation (Figure 6) induced by HS in the dorsomedial medulla of both SHR and WKY rats. On the other hand, pretreatment with the PKC inhibitor calphostin C (100 pmol) blunted only the HS-induced HSP70 expression or HSF1 activation in SHR but not WKY rats.

**Differential Participation of PKA and PKC in HS-Induced BRR Potentiation or Cardiovascular Protection Against Heatstroke in SHR or WKY Rats**

Microinjection bilaterally into the RVLM of H-89 (100 pmol) significantly attenuated the potentiation of BRR response in both strains of rats detected 24 hours in WKY rats or 16 hours in SHR after HS (Figure 7). On the other hand, pretreatment with calphostin C (100 pmol) was only effective in SHR. Similarly, protection by prior HS against fatal cardiovascular depression seen during the onset of heatstroke was partially but significantly reversed in both strains of rats (Figure 8) that received microinjection bilaterally into the RVLM of H-89 (100 pmol). However, pretreatment with calphostin C (100 pmol) attenuated only the cardiovascular protective action of HS in SHR.

**Discussion**

A unique feature of hypertension is heightened susceptibility to ambient temperature, and genes were suggested to be attractive candidates for such an altered response to environmental stress. The present study expounded on this suggestion and provided novel evidence to support the notion that an altered temporal profile of HS-induced HSF1 phosphorylation, HSP70 expression or potentiation of BRR response, and engagement of both PKA and PKC pathways in the activation of HSF1 at the NTS may be associated with greater susceptibility to heatstroke during hypertension.

We reported recently that HS-induced expression of HSP70 in the NTS confers cardiovascular protection during heatstroke by potentiating the BRR response via upregulation of glutamatergic neurotransmission. Heightened expression of HSP70 was reported in vascular smooth muscle cells of SHR. The present study, however, revealed that instead of quantity, it is the kinetics of HSP70 induction by HS in the dorsomedial medulla that is crucial to the altered heat stress response in SHR. We found that compared with WKY rats, the induction of HSP70 after HS in the dorsome-
dial medulla of SHR occurred earlier, reached peak magnitude sooner, and declined more rapidly. Equally important were the observations that this altered temporal profile of HS-induced HSP70 expression correlated positively with that of BRR potentiation observed in SHR. Results from preliminary experiments (not shown) additionally revealed the same association between the magnitude of HS-induced HSP70 expression in the dorsomedial medulla and protective efficacy against heatstroke. As a molecular chaperone for the maintenance of intracellular homeostasis and protein folding,\(^1,^2\) it is conceivable that the altered temporal pattern of HSP70 expression in the dorsomedial medulla and potentiation of BRR response after HS contributes to the decrease in thermal tolerance during heatstroke in hypertension. In support of this suggestion, we found that whereas prior HS significantly potentiated BRR response and protected against hypotension and bradycardia during heatstroke, the magnitude of such potentiation and protection was appreciably retarded in SHR.

Transcriptional regulation of HS response is mediated by HSFs that interact with a specific regulatory element, HSE, in the promoter region of\(^{5-8}\) the\(^{hsp}\) gene.\(^{19}\) An abnormal expression of HSPs caused by accelerated transcriptional rate of the\(^{hsp}\) gene was demonstrated during hypertension.\(^{13,24}\) It is therefore of interest that a brief hyperthermic HS induced phosphorylation of HSF1, but not HSF2, in the dorsomedial medulla of both strains of rats. More intriguingly, we observed that SHR showed an accelerated but transient phosphorylation of HSF1 in response to HS. These observations are concordant with the present contention that HSF1 is involved in the regulation of\(^{hsp}\) induction in response to heat stress and HSF2 is involved in\(^{hsp}\) induction during hemin-induced differentiation.\(^{8,19}\) HSF1-null mice do not exhibit classic HS response nor acquire thermotolerance.\(^{25}\) Tissues from hypertensive animals manifest an increase in transcription rate and accumulation of\(^{hsp70}\) mRNA.\(^{24,26}\) This enhanced expression of\(^{hsp70}\) gene seems to be transient, with a rapid decrease of\(^{hsp70}\) mRNA and protein levels in SHR.\(^{26}\)

Our present results additionally suggest that the temporal

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**Figure 5.** HSP70 levels detected from the dorsomedial medulla of WKY rats or SHR at 0, 8, 16, or 24 hours after animals were subjected to a brief hyperthermic HS or in NT controls. These rats received in addition microinjection bilaterally into the NTS of artificial cerebrospinal fluid (aCSF), H-89 (100 pmol), or calphostin C (100 pmol) immediately before induction of HS. Values are mean±SEM, n=5 to 6 animals per group. *P<0.05 vs NT group; #P<0.05 versus HS+aCSF group at corresponding time intervals; and ++P<0.05 versus corresponding WKY group in the Scheffé multiple-range test.

**Figure 6.** Representative Western blots of HSF1 detected 24 hours (WKY rats) or 16 hours (SHR) from the dorsomedial medulla of animals after they were subjected to a brief hyperthermic HS or in NT controls. These rats received in addition microinjection bilaterally into the NTS of artificial cerebrospinal fluid (aCSF), H-89 (100 pmol), or calphostin C (Cal; 100 pmol) immediately before induction of HS. Arrow indicates the position of slower migrating forms of HSF1 protein.

**Figure 7.** BRR slope evaluated at various time intervals in WKY rats or SHR that were subjected to a brief hyperthermic HS or in NT controls. These rats received in addition microinjection bilaterally into the NTS of artificial cerebrospinal fluid (aCSF), H-89 (100 pmol), or calphostin C (100 pmol) immediately before induction of HS. Value are mean±SEM, n=5 to 6 animals per group. *P<0.05 vs NT group; #P<0.05 vs HS+aCSF group at corresponding time intervals; and ++P<0.05 vs corresponding WKY group in the Scheffé multiple-range test.
profile of HSF1 phosphorylation may be related to the altered HS-induced HSP70 expression and BRR potentiation in SHR.

Another major contribution of this study is the identification of differential engagement of intracellular signal transduction pathways in the regulation of HSF1 phosphorylation in the dorsomedial medulla of SHR and WKY rats after HS. Phosphorylation of HSF1 occurs at serine 230 and is mediated by at least PKA and PKC. Both protein kinases phosphorylate HSF1 independently or synergistically after HS. We found that whereas PKA plays a major role in the activation of HSF1 after HS in the dorsomedial medulla of WKY rats, both PKA and PKC are involved in HSF1 phosphorylation in SHR. Coactivation of PKA and PKC may provide a feasible explanation for the accelerated upregulation of HSF1 phosphorylation, leading to early HSP70 expression in the dorsomedial medulla of SHR after HS. Overexpression of HSP70 also inhibits the binding of phosphorylated HSF1 to the HSE in the promoter region of hsp70 gene. Exactly how HS triggers differentially the activation of HSF1 via PKA or PKC in the NTS of SHR and WKY rats, however, remains to be answered. At the same time, we are aware that phosphorylation of HSF1 at serine 230 by calcium/calmodulin-dependent protein kinase II has been reported.

Summary
The present study provides the first demonstration of an altered temporal profile of HS-induced HSP70 expression or phosphorylation of HSF1 in the dorsomedial medulla and potentiation of BRR response in SHR. We additionally show that both PKA and PKC pathways were engaged in these processes. We speculate that these correlated events may be associated with the heightened susceptibility to heatstroke during hypertension. One caveat to this speculation is the applicability of data derived from SHR to the clinical situation. Increased sensitivity to heat stress and abnormal accumulation of HSPs were reported in hypertensive patients. However, because factors contributing to the hypertensive state are many and varied and SHR is an animal model...
model of genetic hypertension, additional clinical evaluations of our identified phenomena are warranted.

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