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Up-Regulation of Glutamate Receptors in Nucleus Tractus Solitarii Underlies Potentiation of Baroreceptor Reflex by Heat Shock Protein 70

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ABSTRACT

Whereas induction of the 70-kDa heat shock protein (HSP70) in the nucleus tractus solitarii (NTS), the terminal site in the brain stem for primary baroreceptor afferents, augments baroreceptor reflex (BRR) response, the underlying cellular and molecular mechanism is essentially unexplored. In Sprague-Dawley rats, we evaluated the hypothesis that HSP70 may potentiate BRR response by up-regulating the molecular synthesis and functional expression of glutamate receptors in the NTS. Animals subjected to brief hyperthermic heat shock (HS; 42°C for 15 min) exhibited augmented expression of NR1 or NR2A subunit of *N*-methyl-D-aspartate (NMDA) receptors, GluR1 or GluR4 subunits of α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors and KA1 subunit of kainate receptors in the NTS. Intriguingly, this up-regulation of glutamate receptors was preceded by an increase in HSP70 expression at the NTS. The HS-induced augmentation in responsiveness of barosensitive NTS neurons to transient hypertension or potentiation of BRR response was discernibly blunted by MK-801 or 6-cyano-7-nitroquinoxaline-2,3-dione. Bilateral microinjection into the NTS of an antisense *hsp*70 oligonucleotide (50 pmol) before HS significantly suppressed the induced expression of HSP70 or the increase in glutamate receptor subunits in the dorsal medulla and discernibly attenuated the potentiation of BRR response. Control microinjection into the NTS of sense or scrambled *hsp*70 oligonucleotide (50 pmol) was ineffective. These findings suggest that HSP70 induced by HS may enhance BRR response by up-regulating the molecular synthesis and functional expression of NR1 or NR2A subunit of NMDA receptors and GluR1, GluR4, or KA1 subunit of non-NMDA receptors in the NTS.

Acute exposure of animals to an elevated ambient temperature induces heatstroke characterized by reduced cerebral blood flow, hypotension, and bradycardia (Lin, 1997; Lin et al., 1997). These hemodynamic dysfunctions seen during the onset of heatstroke can be protected by prior sublethal heat shock (HS) (Yang et al., 1998; Yang and Lin, 1999; Li et al., 2001). We reported recently (Li et al., 2001) that HS induces expression of the 70-kDa heat shock protein (HSP70) in the nucleus tractus solitarii (NTS), the principal recipient of baroreceptor afferent fibers in the medulla oblongata (Ciriello, 1983). More importantly, this up-regulation of HSP70 in the NTS confers cardiovascular protection during heatstroke by potentiating the baroreceptor reflex (BRR) control of peripheral hemodynamic performance. The cellular and molecular mechanism that underlies this HSP70-promoted BRR potentiation in the NTS, however, is currently unknown.

Glutamate is the major neurotransmitter that mediates synaptic transmission at the baroreceptor afferent terminals in the NTS (Talman et al., 1980; Lawrence and Jarrott, 1994). Molecular cloning of cDNA encoding glutamate receptors revealed multiple groups of ionotropic glutamate receptor subunits (Hollmann and Heinemann, 1994; Dingledine et al., 1999). These included six *N*-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A to NR2D, and NR3), four α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits (GluR1–GluR4), and five kainite (KA) receptor subunits (GluR5 to GluR7, KA1, and KA2). Whereas glutamate affects neuronal activity in the NTS via activation of both NMDA and non-NMDA receptors (Ohta and Talman, 1994; Chan et al., 1998; Zhang and Mifflin, 1998; Yen et al., 1999), the contribution of individual glutamate receptor sub-

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S.H.H.C. and J.Y.H.C. contributed equally to this work.

ABBREVIATIONS: HS, heat shock; HSP70, 70-kDa heat shock protein; NTS, nucleus tractus solitarii; BRR, baroreceptor reflex; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NT, normothermic; SAP, systemic arterial pressure; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; HR, heart rate; KA, kainate; aCSF, artificial cerebrospinal fluid.

units in the NTS to cardiovascular regulation is not well documented.

The cytoprotective mechanism of HSP70 is believed to be related to its chaperone functions, particularly in the mediation of protein folding (Morimoto and Santoro, 1998; Fink, 1999). It follows that HSP70 induced by HS may promote potentiation of BRR by enhancing glutamatergic neurotransmission at the NTS via up-regulation of the molecular synthesis and functional expression of glutamate receptors. This hypothesis was validated in the present study, along with identification of the NMDA, AMPA, and KA receptor subunits that are involved.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (weights, 200–230 g, $n = 353$) obtained from the Experimental Animal Center of the National Science Council, Taiwan, were used. All experimental procedures were in compliance with the guidelines of our institutional animal care committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Induction of Heat Shock. Animals were subject to hyperthermic HS according to the procedures described previously (Li et al., 2001). In brief, under pentobarbital anesthesia (50 mg/kg, i.p.), animals 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 min, 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. Animals similarly anesthetized but kept at room temperature served as normothermic (NT) controls.

Protein Extraction and Western Blot Analysis. For biochemical experiments, the brain stem was rapidly removed under pentobarbital anesthesia (50 mg/kg, i.p.) and placed on dry ice. Tissues on both sides of the dorsomedial part of the medulla oblongata, at the level of NTS (1 mm rostral to 1 mm caudal from the obex), were collected by micropunches made with a stainless steel bore (1 mm i.d.) and frozen in liquid nitrogen (Chan et al., 1999). Medullary samples thus obtained from four to six rats under the same experimental condition were stored at −80°C and were pooled to provide sufficient tissue for analysis. Protein extraction and Western blot analysis of HSP70 or NMDA or non-NMDA glutamate receptor subunits at the dorsomedial medulla were performed according to reported procedures (Li et al., 2001). The primary antisera used included: mouse monoclonal antiserum against the inducible form of HSP70 (1:500; SPA-810, StressGen, Victoria, BC, Canada); goat polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) against NR1 (1:500; sc-1467), NR2A (1:500; sc-1468), NR2B (1:500; sc-1469), NR2C (1:500; sc-1470), NR2D (1:500; sc-1471), GluR5 (1:200; sc-7617), GluR6 (1:200; sc-7618), GluR7 (1:200; sc-7620), KA1 (1:200; sc-8917), or KA2 (1:200; sc-8915); or rabbit polyclonal antiserum (Oncogene, Cambridge, MA) against GluR1 (1:1000; PC246), GluR2/3 (1:1000; PC261L), or GluR4 (1:1000; PC262L). The secondary antisera used included: horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for HSP70; rabbit anti-goat IgG (1:10,000; Santa Cruz Biotechnology) for NMDA receptor subunits and GluR5 to GluR7, KA1, and KA2 of KA receptor subunits; or goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology) for GluR1 to GluR4 of AMPA receptor subunits. Specific antibody-antigen complex was detected by an enhanced chemiluminescence Western blot detection system (PerkinElmer Life Sciences, Boston, MA). A parallel run with additional application of the respective antigen (data not shown) confirmed the position of each receptor subunit on the Western blot.

Animal Preparation. Some rats were prepared for electrophysiological experiments or evaluation of BRR response after HS or NT treatment. They were anesthetized initially with pentobarbital sodium (50 mg/kg, i.p.) to carry out preparatory surgery. These included intubation of the trachea to facilitate ventilation and cannulation of the left femoral artery to measure systemic arterial pressure (SAP).

Both femoral veins were also cannulated for the administration of drugs and maintenance of anesthesia by intravenous infusion of pentobarbital sodium at 20 mg/kg/h. This management scheme (Yang et al., 1996) provided satisfactory anesthetic maintenance and preserved the capacity of central cardiovascular regulation, including BRR response.

Extracellular Single-Neuron Recording and Microiontophoresis. Extracellular single-neuron recordings from, and microiontophoretic application of test agents to, NTS neurons, at the level of the obex, of NT or HS animals were carried out as described previously (White et al., 1988; Chan and Chan, 1994). In brief, seven-barrel microdot micropipettes (tip diameter, 7–9 μm; impedance, 3–8 MΩ for the recording barrel and 45–86 MΩ for the drug barrels) were used. The center barrel and one side barrel contained NaCl (4 M) and were used, respectively, for recording and automatic current balancing. The other barrels contained either the NMDA antagonist dizocipline (MK-801; 1 mM, pH 7.2; Sigma/RBI, Natick, MA), the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1 mM, pH 7.0; Sigma/RBI), Pontamine sky blue (Sigma, St. Louis, MO) or 0.9% saline.

Bioelectric signals recorded from NTS neurons were amplified and filtered with a high-impedance preamplifier (DAM-80; WPI, New Haven, CT; AC recording, band-passes: 300-3000 Hz). Action potentials from single neurons were displayed on an oscilloscope, selected with a window discriminator (WPI 121-G), counted with a period generator (NL304; Digitimer, Welwyn Garden City, Hertfordshire, England) coupled with a pulse integrator (NL601, Digitimer), and recorded with a polygraph (2400S; Gould, Cleveland, OH).

NTS neurons that altered their spontaneous discharge rate by a minimum of 30% in response to baroreceptor activation induced by transient hypertension evoked by i.v. injection of phenylephrine (5 μg/kg; Sigma) were taken to be barosensitive neurons. Their responsiveness to comparable degrees of baroreceptor activation in NT or HS animals was evaluated, in conjunction with microiontophoretic application of MK-801 or CNQX. The glutamate receptor antagonists were ejected with a negative current, at an intensity ≦ 40 nA. The retaining current for MK-801 or CNQX was +5 to +10 nA. An ejected test agent was considered to be effective when the evoked neuronal discharge rate was altered by at least 20% and the elicited change was not mimicked by an equivalent current applied to the current control (0.9% saline) barrel. Pontamine sky blue was ejected at the end of each experiment to mark the position of the recorded NTS neuron.

Evaluation of BRR Response. Two methods were used to evaluate BRR response (Li et al., 2001). First, BRR control of heart rate (HR) was assessed by the slope of the regression line that relates changes in HR with increase or decrease in SAP induced by intravenous bolus administrations of phenylephrine (2.5, 5, or 10 μg/kg) or nitroprusside (5, 10, or 15 μg/kg; Sigma). Second, reflex alterations in BRR-mediated neurogenic sympathetic vasomotor tone were evaluated by on-line spectral analysis of SAP signals. Changes in the integrated power density of the low-frequency component (0.25–0.8 Hz) in the SAP spectrum (Li et al., 2001) to a decrease in SAP induced by 10 min of intravenous infusion of nitroprusside (5 μg/kg/h) were determined. BRR response was evaluated immediately after HS or at 8, 16, 24, or 48 post-treatment.

Microinjection of Oligonucleotide or Test Agent into the NTS. An antisense (5′-CACCTTGCCGTGCTGGAA-3′) oligonucleotide (50 pmol; Genemed Biotechnologies, San Francisco, CA) that targets against the coding region (nt 61–78) of the mouse heat-inducible *hsp*70 gene (Hunt and Calderwood, 1990) was employed to block the molecular synthesis of HSP70 (Robertson et al., 1999). A sense (5′-TTCCAGCACGGCAAGGTG-3′) and a scrambled (5′-TG-

GATCCGACATGTCAGT-3′) *hsp*70 oligonucleotides (Robertson et al., 1999) were used as our control to confirm the specificity of the elicited blockade of HSP70 expression. MK-801 (500 pmol) or CNQX (10 pmol) was used to block glutamate neurotransmission. Antisense, sense, or scrambled *hsp*70 oligonucleotide, MK-801, or CNQX was microinjected bilaterally and sequentially at a volume of 50 nl into the NTS (Chan et al., 1998; Li et al., 2001). The coordinates for NTS 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. The dose and treatment regimen were adopted from previous reports that used those oligonucleotides (Sato et al., 1996; Robertson et al., 1999) or glutamate receptor antagonists (Chan et al., 1998) for the same purpose as in this study.

Histology. The brain stem was removed at the end of each electrophysiological or pharmacological experiment and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥72 h. Frozen 25-μm sections of the medulla oblongata was stained with 1% neural red for histological verification of the recording pipette or location of microinjection sites. Evans blue (1%) was added to the microinjection solution to facilitate this process.

Statistical Analysis. All values are expressed as mean ± S.E. One-way or two-way analysis of variance with repeated measures was used, as appropriate, to assess group means. This was followed by Scheffé's multiple range test for post hoc assessment of individual means. A value of $p < 0.05$ was considered to be statistically significant.

Results

Temporal Changes in Expression of Glutamate Receptor Subunits or HSP70 in Dorsomedial Medulla after Hyperthermic Heat Shock. Various subunits of glutamate receptors were identified under NT condition by Western blot analysis in the dorsomedial medulla that contains the NTS. These included NR1, NR2A, or NR2B subunit of NMDA receptors (Fig. 1); GluR1, GluR2/3, or GluR4 subunit of AMPA receptors (Fig. 2); and GluR5 or KA1 subunit of KA receptors (Fig. 3). Protein expression corresponding to NR2C or NR2D subunit of NMDA receptors or GluR6, GluR7, or KA2 subunit of KA receptors, on the other hand, was below our detection limit.

Exposure of animals to brief hyperthermic HS (42°C for 15 min) induced differential up-regulation of NMDA, AMPA, and KA receptor subunits at the dorsomedial medulla. Western blot analysis revealed that significant increase in expression of NR1 or NR2A subunit of NMDA receptors (Fig. 1), GluR1 or GluR4 subunit of AMPA receptors (Fig. 2), and KA1 subunit of KA receptors (Fig. 3) was detected at 16 h, optimized at 24 h, and returned to baseline 48 h after HS. In contrast, no discernible change in the expression of NR2B subunit of NMDA receptors, GluR2/3 subunit of AMPA receptors, or GluR5 subunit of KA receptors was observed. The expression of NR2C or NR2D subunit of NMDA receptors and GluR6, GluR7, or KA2 subunit of KA receptors was again below our detection limit under hyperthermic HS condition.

As we demonstrated recently (Li et al., 2001), HS also induced a temporal increase in the expression of HSP70 at the dorsomedial medulla. It is particularly noteworthy that this up-regulation of HPS70 took place before the increased expression of NMDA, AMPA, and KA receptor subunits. As such, an appreciable augmentation of HSP70 expression (Fig. 4) was first detected at 8 h, optimized at 24 h, and returned to baseline 48 h after HS. In NT controls, in which animals were similarly anesthetized but without subsequent hyperthermic treatment, HSP70 was undetectable in the dorsomedial medulla (Fig. 4).

Effects of NMDA or Non-NMDA Receptor Antagonist on Responsiveness of Barosensitive NTS Neurons after Hyperthermic Heat Shock. Single-neuron recording in the NTS 24 h after animals received HS treatment revealed an appreciable augmentation (+48.2 ± 6.6% over NT control, $n = 10$ neurons in each group) in the responsiveness of barosensitive NTS neurons (Fig. 5) to similar degrees of transient elevation in SAP (HS, +48.4 ± 2.3 mm Hg; NT, +46.7 ± 3.5 mm Hg; $n = 10$ trials in each group). Although maintaining the differential response pattern, the responsiveness of those barosensitive NTS neurons in both HS and NT control animals was discernibly blunted (Fig. 5) by microiontophoretically applied MK-801 (40 nA) or CNQX (40 nA). These glutamate receptor antagonists, however, did not appreciably affect baseline spontaneous NTS neuronal activities in NT and HS animals.

Effects of NMDA or Non-NMDA Receptor Antagonist on the Potentiated BRR Response Induced by Hyperthermic Heat Shock. Similar to our previous observations (Li et al., 2001), both BRR control of HR (Fig. 6A) and reflex

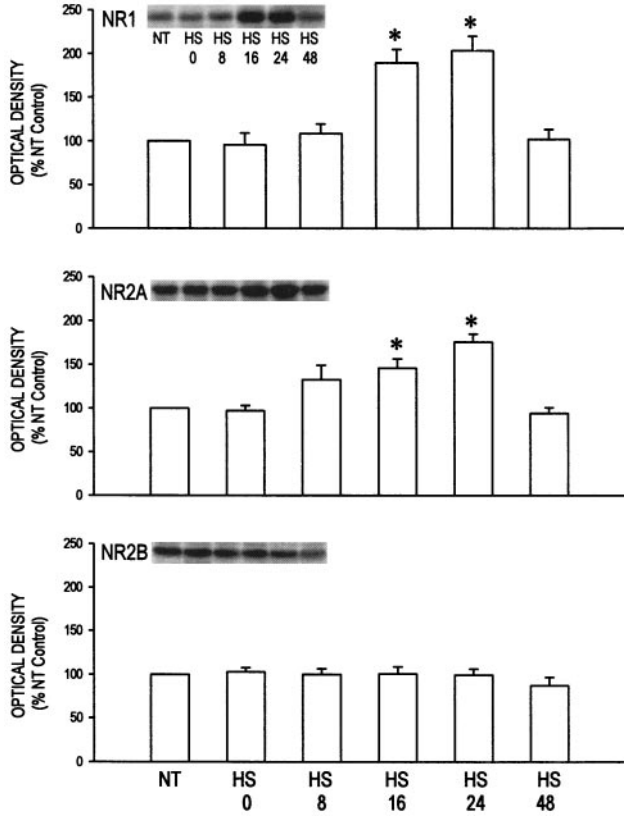

Fig. 1. Representative Western blots of NR1, NR2A, or NR2B subunit (inset) or levels of these NMDA receptor subunits detected from the dorsomedial medulla of rats that were subject to brief hyperthermic heat shock (HS; 42°C for 15 min). Lane 1 represents expression of individual NMDA receptor subunits under normothermic (NT) condition, and lanes 2 through 6 represent their expression at 0, 8, 16, 24, or 48 h after HS. Western blot of β-tubulin was carried out to verify that all lanes contain equal amounts of protein (100 μg/lane, data not shown). Protein expression of NR2C or NR2D receptor subunit is not shown as it was below our detection limit. Values are expressed in percentage against corresponding levels determined in NT controls and are mean ± S.E. of quadruplicate analysis; $n = 6$ to 7 animals per group. *, $p < 0.05$ versus NT group in the Scheffé multiple range test.

sympathoexcitatory response to unloading of baroreceptors (Fig. 6B) were appreciably potentiated 16 or 24 h after brief hyperthermic HS. This elicited augmentation by HS of vagally and sympathetically mediated BRR response (Chan et al., 1998; Li et al., 2001) was significantly reversed in animals that received microinjection bilaterally into the NTS of MK-801 (500 pmol) or CNQX (10 pmol) 10 min before BRR evaluations (Fig. 6). Local application of MK-801 or CNQX into the NTS of NT control animals also resulted in a significant suppression in vagally and sympathetically mediated BRR response (Fig. 6). Microinjection of either glutamate receptor antagonist into the NTS, on the other hand, did not affect baseline SAP or HR.

Effects of Antisense hsp70 Oligonucleotide on Temporal Expression of HSP70 or Glutamate Receptor Subunits in the Dorsomedial Medulla Induced by Hyperthermic Heat Shock. Microinjection bilaterally into the NTS of an antisense *hsp*70 oligonucleotide immediately after HS significantly reduced the expression of HSP70 in the dorsomedial medulla, measured 8, 16, or 24 h after hyperthermic treatment (Fig. 4). The same antisense treatment also reversed to control level the up-regulation of NR1, NR2A, GluR1, GluR4, or KA1 receptor subunit manifested 16 or 24 h after HS (Fig. 7). Microinjection of antisense *hsp*70 oligonucleotide into the NTS, on the other hand, exerted no discernible effect on the expression of NR2B, GluR2/3, or GluR5 subunit under NT or hyperthermic HS condition (data not shown).

Effects of Antisense hsp70 Oligonucleotide on the Potentiated BRR Response Induced by Hyperthermic Heat Shock. Both BRR control of HR (Fig. 8A) and reflex sympathoexcitatory response to unloading of baroreceptors (Fig. 8B) appreciably potentiated 16 or 24 h after brief hyperthermic HS were significantly attenuated in animals that were pretreated with microinjection bilaterally into the NTS

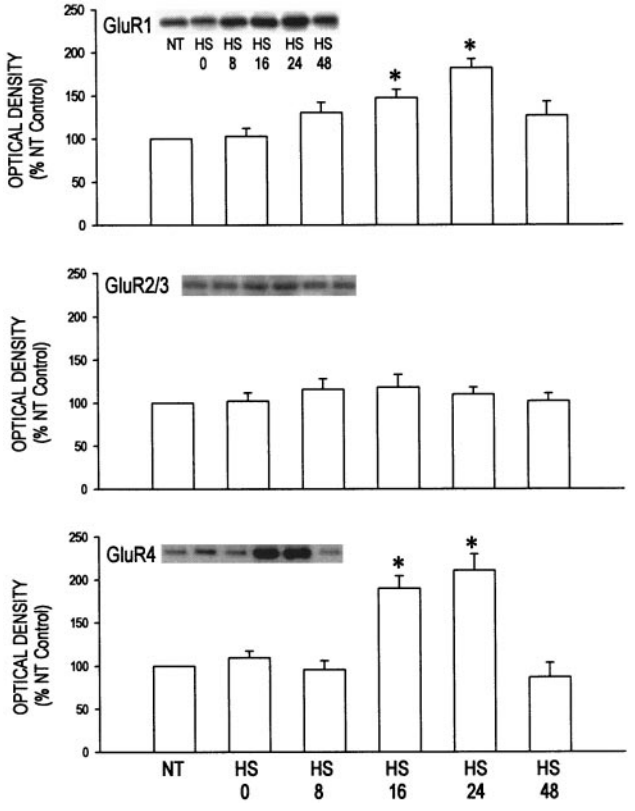

Fig. 2. Representative Western blots of GluR1, GluR2/3, or GluR4 subunit (inset) or levels of these AMPA receptor subunits detected from the dorsomedial medulla of rats that were subject to brief hyperthermic HS (42°C for 15 min). Lane 1 represents expression of individual AMPA receptor subunits under NT condition, and lanes 2 through 6 represent their expression at 0, 8, 16, 24, or 48 h after HS. Western blot of β-tubulin was carried out to verify that all lanes contain equal amounts of protein (100 μg/lane, data not shown). Values are expressed in percentage against corresponding levels determined in NT controls and are mean ± S.E. of quadruplicate analysis; n = 6 to 7 animals per group. *, $p < 0.05$ versus NT group in the Scheffé multiple range test.

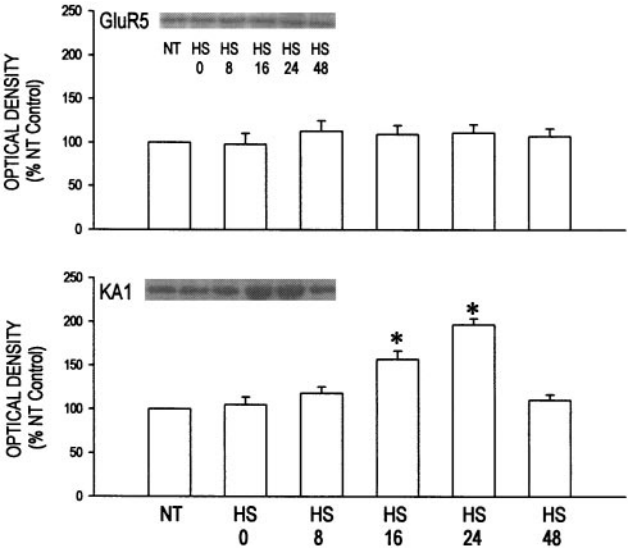

Fig. 3. Representative Western blots of GluR5 or KA1 subunit (inset) or levels of these KA receptor subunits detected from the dorsomedial medulla of rats that were subject to brief hyperthermic HS (42°C for 15 min). Lane 1 represents expression of individual KA receptor subunits under NT condition, and lanes 2 through 6 represent their expression at 0, 8, 16, 24, or 48 h after HS. Western blot of β-tubulin was carried out to verify that all lanes contain equal amounts of protein (100 μg/lane, data not shown). Protein expression of GluR6, GluR7, or KA2 receptor subunit is not shown as it was below our detection limit. Values are expressed in percentage against corresponding levels determined in NT controls and are mean ± S.E. of quadruplicate analysis; n = 6 to 7 animals per group. *, $p < 0.05$ versus NT group in the Scheffé multiple range test.

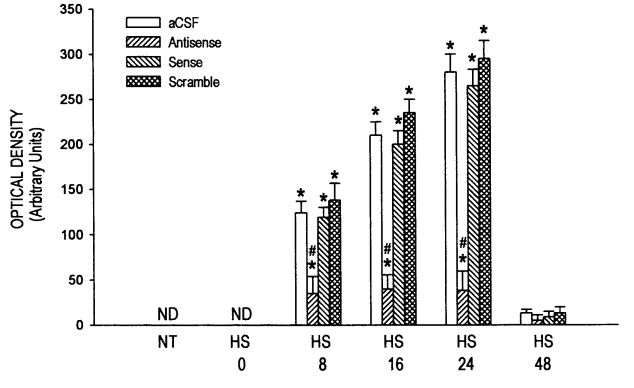

Fig. 4. HSP70 levels detected by Western blot analysis from the dorsomedial medulla of rats 0, 8, 16, or 24 h after hyperthermic HS or in NT controls. Animals in the HS group also received microinjection bilaterally into the nucleus tractus solitarii (NTS) of antisense, sense, or scrambled *hsp*70 oligonucleotide (50 pmol) or artificial cerebrospinal fluid (aCSF), delivered immediately after hyperthermic treatment. ND denotes undetectable level of HSP70. Values are mean ± S.E. of quadruplicate analysis; n = 6 to 7 animals per group. *, $p < 0.05$ versus NT group; #, $p < 0.05$ versus aCSF group in the Scheffé multiple range test.

of an antisense *hsp*70 oligonucleotide. On the other hand, both baseline SAP and HR were essentially unaltered at all time points evaluated after HS, delivered alone or together with antisense pretreatment.

Lack of Effects of Control hsp70 Oligonucleotides. We ascertained the specificity of the observed biological activity of our antisense *hsp*70 oligonucleotide by evaluating the effect of two control oligonucleotides. Pretreatment with microinjection bilaterally into the NTS of the sense or scrambled *hsp*70 oligonucleotide, similar to aCSF group or baseline control, resulted in no discernible alteration in the enhanced expression of NMDA, AMPA, or KA receptor subunits in the dorsomedial medulla 16 or 24 h after HS (Fig. 7). The same treatment also did not significantly affect the potentiation of BRR response (Fig. 8) in animals that received prior hyperthermic HS.

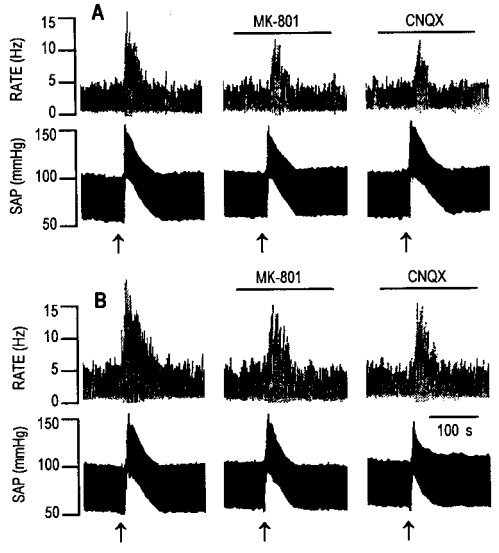

Fig. 5. Representative examples of single-neuron responses from spontaneously active NTS neurons to transient hypertension induced by phenylephrine (5 μg/kg, i.v.; at arrow) in NT controls (A) or rats 24 h after hyperthermic HS (B). The responsiveness of these barosensitive NTS neurons to comparable increase in SAP was also evaluated in the presence of microiontophoretically applied NK-801 or CNQX (40 nA; at bar). Note the lack of effect on baseline neuronal activity by both glutamate receptor antagonists.

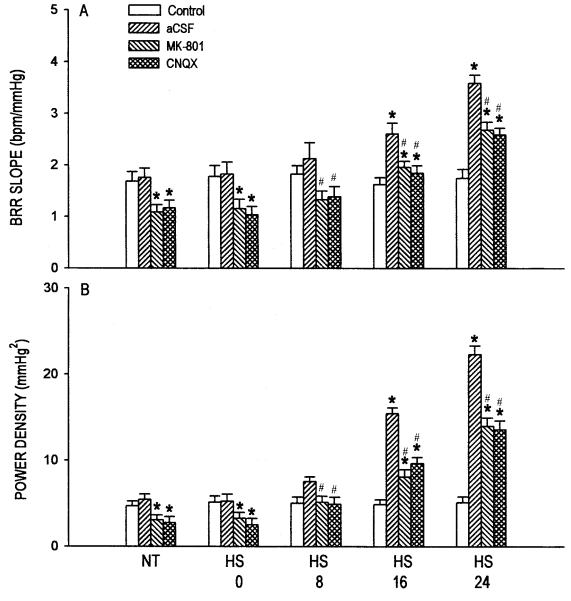

Fig. 6. Slope of baroreceptor reflex (BRR)-mediated change in heart rate in response to either hypotension or hypertension (A) or total power density of the low-frequency component of systemic arterial pressure spectrum, our experimental index for BRR-mediated sympathetic neurogenic vasomotor tone, over 10 min of sustained hypotension (B), evaluated in rats 0, 8, 16, or 24 h after hyperthermic HS or in NT controls. Animals in the HS group also received microinjection bilaterally into the NTS of aCSF, MK-801 (500 pmol), or CNQX (10 pmol) delivered 10 min before BRR evaluation. Values are mean ± S.E., $n = 5$ to 6 animals per group. *, $p < 0.05$ versus baseline control group; #, $p < 0.05$ versus aCSF group in the Scheffé multiple range test.

Discussion

The present study provided the first demonstration of a temporal association between the increase in HSP70 expression, up-regulation of NMDA, AMPA, and KA receptor subunits in the dorsomedial medulla that includes the NTS, augmentation in responsiveness of barosensitive NTS to transient hypertension, and potentiation of BRR response in animals that were subject to hyperthermic HS. We further established a causative relationship between these biochem-

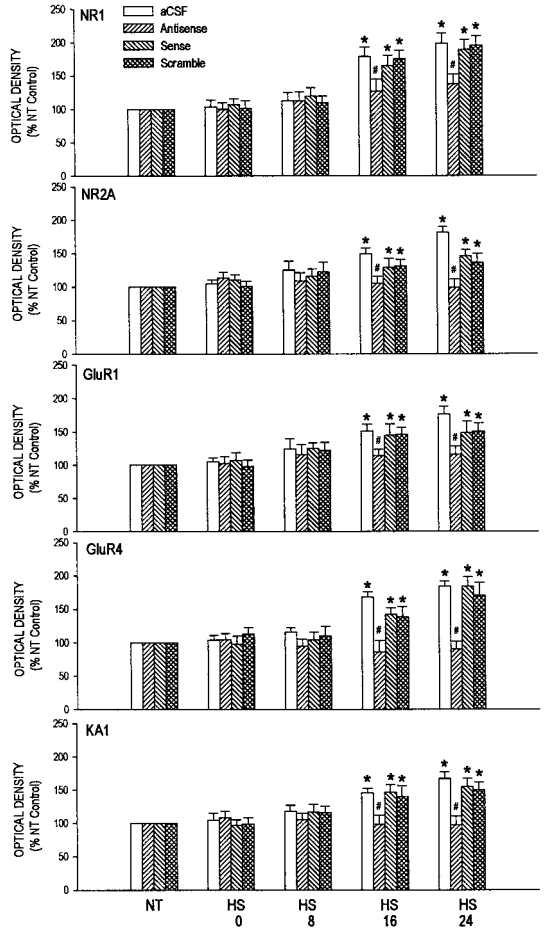

Fig. 7. Levels of glutamate receptor subunits detected by Western blot analysis from the dorsomedial medulla of rats 0, 8, 16, or 24 h after hyperthermic HS or in NT controls. Animals in the HS group also received microinjection bilaterally into the NTS of aCSF, antisense, sense or scrambled *hsp*70 oligonucleotide (50 pmol), delivered immediately before hyperthermic treatment. Values are expressed in percentage against corresponding levels determined in NT controls, and are mean ± S.E. of quadruplicate analysis; $n = 6$ to 7 animals per group. *, $p < 0.05$ versus NT group; #, $p < 0.05$ versus aCSF group in the Scheffé multiple range test.

ical and physiological events by showing that both NMDA and non-NMDA antagonists reversed the HS-induced augmentation in responsiveness of barosensitive NTS neurons or potentiation of BRR response. In addition, blockade of HSP70 expression in the NTS with an antisense *hsp*70 oligonucleotide not only attenuated the HS-induced up-regulation of those NMDA, AMPA, and KA receptor subunits, but also reversed the potentiation of BRR response. These findings together suggest that an enhanced molecular synthesis and functional expression of NR1 and NR2A subunit of the NMDA receptors, GluR1 and GluR4 subunit of the AMPA receptors, or KA1 subunit of KA receptors in the dorsomedial medulla underlies the promotion of BRR potentiation by HSP70 induced in the NTS by HS.

Heat stress with a sublethal increase in temperature of a few degrees above the physiological level induces heat shock response, resulting in the synthesis of a multigene family of proteins known as HSPs. The functional expression of these HSPs increases the ability of cells or tissues to withstand an otherwise lethal subsequent heat challenge (Lindquist and Craig, 1988). Several studies (Yang et al., 1998; Yang and Lin, 1999) reported that HS-induced expression of HSP70, the major inducible form of HSPs, confers cardiovascular protection during the onset of heatstroke promoted by severe hyperthermic heat stress (45°C for 60 min). Of particular relevance is our recent demonstration (Li et al., 2001) that HSP70 synthesized in the NTS participates in cardiovascular protection during heatstroke by potentiating the BRR response. The present study extended these observations to suggest that an enhancement of glutamatergic neurotransmission in the NTS may underlie the BRR potentiation seen after hyperthermic HS. We further provided novel evidence to support the notion that an up-regulation of both molecular synthesis and functional expression of NR1, NR2A, GluR1, GluR4, or KA1 subunit of glutamate receptors in the NTS underlies such an enhancement.

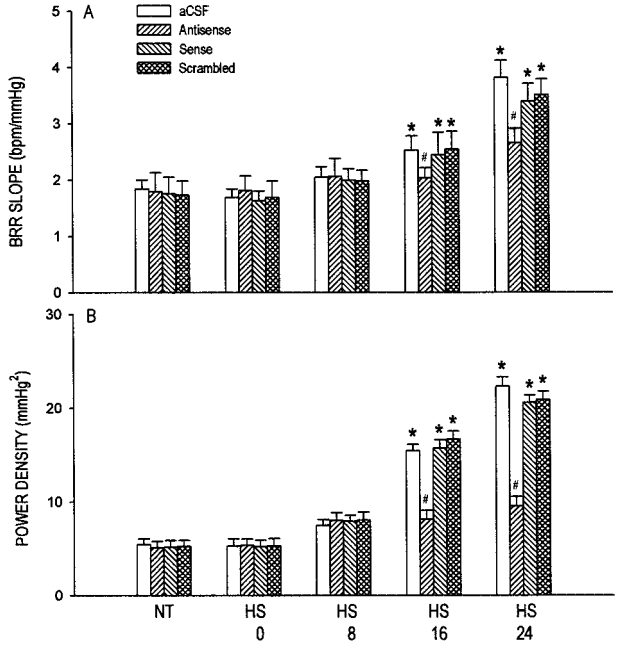

Fig. 8. Slope of baroreceptor reflex (BRR)-mediated change in heart rate in response to either hypotension or hypertension (A) or total power density of the low-frequency component of systemic arterial pressure spectrum, our experimental index for BRR-mediated sympathetic neurogenic vasomotor tone, over 10 min of sustained hypotension (B), evaluated in rats 0, 8, 16, or 24 h after hyperthermic HS or in NT controls. Animals in the HS group also received microinjection bilaterally into the NTS of aCSF, antisense, sense, or scrambled *hsp*70 oligonucleotide (50 pmol), delivered immediately after hyperthermic treatment. Values are mean ± S.E.; $n = 6$ to 7 animals per group. *, $p < 0.05$ versus NT group; #, $p < 0.05$ versus aCSF group in the Scheffé multiple range test.

Despite previous reports on the expression of glutamate receptor subunits in the NTS (Ambalavanar et al., 1998; Lacassagne and Kessler, 2000; Ohtake et al., 2000), a complete evaluation on all subunits in this nucleus is still lacking. Earlier studies (Aicher et al., 1999; Huang et al., 2000; Ohtake et al., 2000) described the expression of NR1 subunit of NMDA receptors in the NTS. The present study provided, in addition, the first demonstrated expression of NR2A and NR2B receptor subunits in the dorsomedial medulla. The lack of expression of NR2C or NR2D subunits confirmed previous observations that these two subunits are almost exclusively expressed in cerebellum, thalamus, and olfactory bulb (Wenzel et al., 1995). In line with previous reports (Ambalavanar et al., 1998; Kessler and Baude, 1999), we also detected the expression of all four subunits of AMPA receptors in the dorsomedial medulla. The expression of GluR5 or KA1, but not GluR6, GluR7, or KA2 subunit, of KA receptors further fills a void on the distribution of KA receptor subunits in the NTS. Together, our results indicate that ionotropic glutamate receptor subunits exhibited differential presence in the dorsomedial medulla.

Relatively little is known of the contribution of NMDA, AMPA, and KA receptor subunits to the cardiovascular regulatory functions of the NTS (Sato et al., 1993; Ambalavanar et al., 1998). NMDA, AMPA, and KA receptors play a differential role in synaptic response of NTS neurons to activation of afferent fibers (Kubo and Kihara, 1991; Dingledine et al., 1999; Yen et al., 1999). Blockade of NR1 mRNA in the NTS with an antisense oligonucleotide attenuates BRR sensitivity (Dean et al., 1998). Superimposed on this information, the present study demonstrated that NR1, NR2A, GluR1, GluR4, or KA1 subunit undergoes an augmentation in molecular synthesis after brief hyperthermic HS and contributes functionally to the potentiation of glutamatergic neurotransmission in the NTS by HSP70 seen after HS. Our Western blot analysis also identified the presence of NR2B, GluR2/3, or GluR5 subunit in the dorsomedial medulla. Because these glutamate receptor subunits did not exhibit significant expressional changes after HS, they may subserve functions of the NTS other than HSP70-induced BRR potentiation.

We are aware that our Western blot analysis of NMDA, AMPA, or KA receptor subunits was carried out on tissues collected from the dorsomedial medulla. In addition to the NTS, this sampled region also contains area postrema, dorsal motor nucleus of the vagus nerve, and hypoglossal nucleus, where glutamate receptors are known to be present (Willis et al., 1996; Aylwin et al., 1998; Kessler and Baude, 1999; Lacassagne and Kessler, 2000). In this regard, we found that microinjection of antisense *hsp*70 oligonucleotide into these medullary sites was ineffective (data not shown) in attenuating the up-regulation of NMDA, AMPA, or KA receptor subunits and reversing the potentiation of BRR response induced by prior HS. It is therefore highly likely that the temporal changes in expression of HSP70 and glutamate receptor subunits detected after HS in this study originate mainly from the NTS.

Our experimental design did not allow us to decipher the mechanisms that underlie the up-regulation of NMDA, AMPA, or KA receptor subunits by HSP70 in the NTS after HS. The cellular protective mechanism of HSP70 is believed to be related to its chaperone functions, which lead to the prevention of protein denaturation and promotion of refolding of damaged proteins after stress (Morimoto and Santoro, 1998; Fink, 1999). In addition, HSP70 chaperone may sustain proteins in the productive folding pathway or maintain newly synthesized proteins in an unfolded conformation suitable for translocation across membranes (Beckmann et al., 1990; Nelson et al., 1992). We may speculate, therefore, that HSP70 induced by HS up-regulates the molecular synthesis of NR1, NR2A, GluR1, GluR4, or KA1 subunit in the NTS by acting as a protein chaperone.

We recognize that the establishment of a causative relationship between the increase in HSP70 expression after hyperthermic HS and up-regulation of NR1, NR2A, GluR1, GluR4, or KA1 subunit in the NTS or potentiation of BRR response depends on the specificity of the antisense *hsp*70 oligonucleotide used in the present study. In this regard, the same antisense oligonucleotide has been demonstrated to inhibit *hsp*70 transcription (Robertson et al., 1999) and to reverse the neuroprotective effect of HS on hippocampal neurons (Sato et al., 1996). Nonetheless, two control oligonucleotides were employed to further ascertain the specificity of the biological activity of our antisense *hsp*70 oligonucleotide. We demonstrated that a sense oligonucleotide complimentary to the antisense *hsp*70 sequence or an oligonucleotide with scrambled sequences elicited indiscernible alterations in all of the biochemical and cardiovascular events that we evaluated after hyperthermic HS. Thus, we are confident that the blunting effects of antisense *hsp*70 oligonucleotide we observed on these same events were related to its complementarity with the *hsp*70 gene. That antisense pretreatment did not result in discernible changes in baseline SAP or HR further indicated that the elicited reversal of the potentiation of BRR response was not secondary to cardiovascular perturbations.

Among the cascade of events subsequent to activation of both NMDA and non-NMDA receptors in NTS on stimulation of the baroreceptors is the elicitation of BRR response (Ohta and Talman, 1994; Chan et al., 1998). It is intriguing to note, therefore, that the HS-induced BRR potentiation and up-regulation of glutamate receptor subunits in the NTS exhibited parallel time courses. That glutamate receptor antagonists or antisense *hsp*70 oligonucleotide did not exert effects on baseline NTS neuronal activity, SAP, or HR further indicate the close association between the augmented HSP70 expression, enhanced synthesis of functional glutamate receptors, increased responsiveness of barosensitive NTS neurons, and potentiated BRR response in animals that received HS treatment. Whether the implied up-regulation of glutamate receptors elicited by HS also involves an enhanced glutamate release is subject to further elucidation. It is also likely that protein molecules other than glutamate receptors may participate in HS-induced BRR potentiation. A possible candidate is glucocorticoid receptor, which is present in the NTS (Harfstrand et al., 1986), and is enhanced by hyperthermic HS through transcriptional activation (Sanchez et al., 1994).

In conclusion, the present study provided novel findings to associate HS-induced HSP 70 with augmented glutamatergic neurotransmission in the NTS and potentiation of BRR response. We demonstrated that HSP70 induced by HS in the NTS up-regulates the molecular synthesis and functional expression of NR1 or NR2A subunit of NMDA receptors, GluR1 or GluR4 subunit of AMPA receptor, or KA1 subunit of KA receptors in dorsomedial medulla, leading to augmentation in responsiveness of barosensitive NTS neurons to transient hypertension and potentiation of BRR response. BRR is a fundamental mechanism through which the central nervous system regulates peripheral hemodynamic performance. By rendering the cardiovascular system less vulnerable through HSP70-induced up-regulation of glutamatergic neurotransmission at the NTS, the enhanced BRR response, in turn, confers crucial protection against hemodynamic dysfunctions during the onset of heatstroke.

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