Heat Shock Protein 60 or 70 Activates Nitric-oxide Synthase (NOS) I- and Inhibits NOS II-associated Signaling and Depresses the Mitochondrial Apoptotic Cascade during Brain Stem Death*§

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The cellular and molecular basis of brain stem death remains an enigma. As the origin of a “life-and-death” signal that reflects the progression toward brain stem death, the rostral ventrolateral medulla (RVLM) is a suitable neural substrate for mechanistic delineation of this phenomenon. Here, we evaluated the hypothesis that heat shock proteins (HSPs) play a neuroprotective role in the RVLM during brain stem death and delineated the underlying mechanisms, using a clinically relevant animal model that employed the organophosphate pesticide mevinphos (Mev) as the experimental insult. In Sprague-Dawley rats, proteomic, Western blot, and real-time PCR analyses demonstrated that Mev induced de novo synthesis of HSP60 or HSP70 in the RVLM without affecting HSP90 level. Loss-of-function manipulations of HSP60 or HSP70 in the RVLM using antisense or antisense oligonucleotide potentiated Mev-elicited cardiovascular depression alongside reduced nitric-oxide synthase (NOS) I/protein kinase G signaling, enhanced NOS II/peroxynitrite cascade, intensified nucleosomal DNA fragmentation, elevated cytoplasmic histone-associated DNA fragments or activated caspase-3, and augmented the cytochrome c/caspase-3 cascade of apoptotic signaling in the RVLM. Co-immunoprecipitation experiments further revealed a progressive increase in the complex formed between HSP60 and mitochondrial or cytosolic Bax or mitochondrial Bcl-2 during Mev intoxication, alongside a dissociation of the cytosolic HSP60-Bcl-2 complex. We conclude that HSP60 and HSP70 confer neuroprotection against Mev intoxication by ameliorating cardiovascular depression via an anti-apoptotic action in the RVLM. The possible underlying intracellular processes include enhancing NOS I/protein kinase G signaling and inhibiting the NOS II/peroxynitrite cascade. In addition, HSP60 exerts its effects against apoptosis by blunting Mev-induced activation of the Bax/cytochrome c/caspase-3 cascade.

Whereas brain stem death is currently the clinical definition of death in many countries (1, 2), the cellular and molecular underpinnings of this phenomenon of paramount medical importance are wanting. The invariable prognosis, that asystole takes place within hours or days after the diagnosis of brain stem death (3), strongly suggests that permanent impairment of the brain stem cardiovascular regulatory machinery should precede death. It is therefore intriguing that our laboratory demonstrated previously that a common denominator exists among patients who succumbed to systemic inflammatory response syndrome (4), severe brain injury (5), or organophosphate poisoning (6). We found that a dramatic reduction or loss of the power density of the low frequency (LF) component (0.04–0.15 Hz in human) in the systemic arterial pressure (SAP) spectrum, which reflects failure of brain stem cardiovascular regulatory functions, invariably precedes death. We further established that this “life-and-death” signal takes origin from the rostral ventrolateral medulla (RVLM) (7), the brain stem site responsible for tonic sympathetic vasomotor outflow to the blood vessels and maintenance of SAP (8). It follows that evaluation of biochemical changes in the RVLM, whose neuronal activity is reflected in the waxing and waning of the life-and-death signal, should shed light on the cellular and molecular mechanisms of brain stem death.

One suitable experimental animal model for mechanistic evaluation of brain stem death uses the organophosphate poison, mevinphos (3-(dimethoxyphosphinoyl)-2-butenolic acid

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3 The abbreviations used are: LF, low frequency; aCSF, artificial cerebrospinal fluid; CREB, cAMP-response element-binding protein; HR, heart rate; HSF1, heat shock transcription factor 1; HSPs, heat shock proteins; HSP60, 60-kDa heat shock protein; HSP70, 70-kDa heat shock protein; HSP90, 90-kDa heat shock protein; ike, inhibitory κB; Mev, mevinphos (3-(dimethoxyphosphinyloxy)-2-butenolic acid methyl ester); MSAP, mean systemic arterial pressure; NO, nitric oxide; NOS, nitric oxide synthase; PKG, protein kinase G; RVLM, rostral ventrolateral medulla; SAP, systemic arterial pressure; nt, nucleotide(s); MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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methyl ester (Mev)) as the experimental insult (9). The RVLM is a brain stem site via which Mev, a U.S. Environmental Protection Agency Toxicity Category I pesticide, acts to elicit cardiovascular toxicity (10–13). More importantly, the distinct phases of augmentation followed by reduction of the LF component in the SAP spectrum exhibited during Mev intoxication can be designated as “pro-life” and “pro-death” phases in this model of brain stem death (9). Our laboratory demonstrated previously (11, 12) that nitric oxide generated by nitric-oxide synthase (NOS) I in the RVLM, followed by activation of the soluble guanylyl cyclase/cGMP/protein kinase G (PKG) cascade, is responsible for the pro-life phase of Mev intoxication. On the other hand, peroxynitrite formed by a reaction between NOS II-derived NO and superoxide anion in the RVLM underlies the pro-death phase.

Because death represents the end of existence for an individual, we proposed recently (9, 14) that multiple pro-life and pro-death programs must be activated in the RVLM during the progression toward brain stem death. In this regard, the heat shock proteins (HSPs) present themselves as another reasonable candidate for the pro-life program. The HSPs represent a group of intracellular proteins that protect cells from otherwise lethal consequences of exposure to heat, toxins, infection, seizure, trauma, ischemia, or other cellular stresses (15–17). Based on the molecular size, HSPs are grouped into families that include at least HSP60, HSP70, and HSP90. The cellular protective mechanisms of HSPs are related to their chaperone functions (18), which lead to the prevention of protein denaturation and promotion of refolding of damaged proteins after stress (17, 19). Recent reports (14, 20) also suggest that HSPs may interact with NOS at the level of gene regulation or NO-associated signaling cascades. For example, HSP60 (21, 22) or HSP70 (20, 23–25) inhibits NOS II gene expression, and HSP70 protects macrophages against peroxynitrite cytotoxicity (26, 27). There is also ample evidence that links HSP70 or HSP90 to protection against apoptosis (28–30). On the other hand, HSP60 is reportedly pro-apoptotic (31) or anti-apoptotic (32, 33).

Based on an animal model that uses Mev as the experimental insult (9), the present study was undertaken to test the hypothesis that HSP60, HSP70, or HSP90 in the RVLM plays a neuroprotective role during brain stem death and delineate the underlying cellular and molecular mechanisms. This hypothesis was partially validated. We demonstrated that, whereas an underlying cellular and molecular mechanisms. This hypothesis was partially validated. We demonstrated that, whereas an apoptotic role during brain stem death (9). Our laboratory demonstrated previously (11, 12) that nitric oxide generated by nitric-oxide synthase (NOS) I in the RVLM, followed by activation of the soluble guanylyl cyclase/cGMP/protein kinase G (PKG) cascade, is responsible for the pro-life phase of Mev intoxication. On the other hand, peroxynitrite formed by a reaction between NOS II-derived NO and superoxide anion in the RVLM underlies the pro-death phase.

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EXPERIMENTAL PROCEDURES

Animals—Adult male Sprague-Dawley rats (278–362 g, n = 523) purchased from the Experimental Animal Center of the National Science Council, Taiwan, Republic of China, were used. All experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee.

General Preparation—Preparatory surgery for our physiological experiments was performed under an induction dose of pentobarbital sodium (50 mg/kg, intraperitoneal). This included intubation of the trachea and cannulation of the left femoral artery and both femoral veins. During the recording session, which routinely commenced 60 min after the administration of pentobarbital sodium, anesthesia was maintained by an intravenous infusion of propofol (Zeneca, Macclesfield, UK) at 20–25 mg/kg/h. This scheme (34) provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation. The head of the animal was thereafter fixed to a stereotaxic head holder (Kopf, Tujunga, CA), and body temperature was maintained at 37 °C by a heating pad. During the experiment, animals were allowed to breathe spontaneously with room air.

Recording and Power Spectral Analysis of SAP Signals—SAP signals recorded from the femoral artery were subject simultaneously to on-line power spectral analysis (7, 10–12, 14, 35–37). We were particularly interested in the LF (0.25–0.8 Hz in rat) component of SAP signals, which takes origin from the RVLM (7) and mirrors the prevalence of sympathetic neurogenic vasomotor discharges that emanate from this brain stem site (11, 12, 14, 35–37). More importantly, our laboratory demonstrated previously (11, 12) that the power density of this spectral signal exhibited biphasic changes that reflect the pro-life and pro-death phases of Mev intoxication (9). Heart rate (HR) was derived instantaneously from SAP signals. The SAP spectra and power density of LF component were displayed during the experiment, alongside pulsatile SAP, mean SAP (MSAP), and HR, in an on-line and real-time manner.

Microjection of Test Agents—Microinjection bilaterally of test agent into the RVLM, each at a volume of 50 nL, was carried out stereotaxically and sequentially with a 27-gauge needle that was connected to a 0.5-μl Hamilton microsyringe (Reno, NV). The coordinates were: 4.5–5 mm posterior to lambda, 1.8–2.1 mm lateral to midline, and 8.1–8.4 mm below the dorsal surface of cerebellum (10–14, 20, 35–37). Injecting the same amount of artificial cerebrospinal fluid (aCSF) controlled for possible volume effect of microinjection. Test agents used included Mev (kindly provided by Huikwang Corp., Taian, Hsien, Taiwan) and a transcription inhibitor (13, 38), actinomycin D (Tocris Cookson, Bristol, UK), or a translation inhibitor (14, 38), cycloheximide (Tocris Cookson). A goat polyclonal antisera against HSP60 (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal antisera (Stressgen, Victoria, Canada) against HSP70 or HSP90 was used to effect immunoneutralization. 0.02% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was added to facilitate transport of the antisera across the cell membrane of RVLM neurons (supplemental Fig. S1B). Microinjection of normal goat serum (Sigma-Aldrich) or normal mouse serum (Sigma-Aldrich) plus 0.02% Triton X-100 served as our vehicle control. To avoid verbose presentation, however, the phrase “0.02% Triton X-100” is omitted from subsequent narration. Gene knockdown was introduced using an
antisense oligonucleotide (Quality Synthesis, Taipei, Taiwan) against the coding region nt 109–123 of hsp60 (32); 5′-ATAAG- CTCGAACATC-3′; the coding region nt 61–78 of hsp70 (14, 20, 39); 5′-CACCCTGGCAGTGCTTGAA-3′; or the coding region nt 97–116 of hsp90 (14, 40): 5′-CTCACCCTTCTCTCT- CCTCAT-3′ gene. The corresponding sense oligonucleotide: 5′- GATGCTGAGCTTAA-3′ (hsp60), 5′-TTCCAGACGCGCA- AGGTG-3′ (hsp70) or 5′-ATGGAGAGAAAGGTGGAG-3′ (hsp90); or scrambled oligonucleotide 5′-GCTCGTGGTCAT- AC-3′ (hsp60), 5′-TGATCCGACGTGCACT-3′ (hsp70) or 5′-TTCCTACCCTCTCTCATT-3′ (hsp90) was used as the control. The oligonucleotides were phosphorylated in all positions and were diluted in aCSF at pH 7.4. To avoid the confounding effects of drug interactions, each animal received only one treatment regimen.

Protein Extraction—Because the power density of LF component in the SAP spectrum reflects the prevalence of the life-and-death signal detected from patients intoxicated with organophosphate poisons (6), we routinely collected tissue samples (11–13) during the peak of the pro-life and pro-death phases (Mev group), or 30 or 180 min after microinjection of aCSF, or other solvents into the RVLM (vehicle control). Medullary tissues collected from anesthetized animals but without treatment served as the sham-controls. In brief, rats were killed with an overdose of pentobarbital sodium and perfused intracardially with 150 ml of warm (37 °C) saline containing heparin (100 units/ml). The brain was rapidly removed and placed on dry ice. Tissues from both sides of the ventrolateral medulla, at the level of the RVLM (0.5–1.5 mm rostral to the obex), were collected by micropunches made with a 1-mm (inner diameter) stainless steel bore. The tissues thus obtained covered the anatomical boundaries of the RVLM and were co-extensive with the penetration of, for example, the microinjected IgG (supplemental Fig. S1A). Tissues were homogenized on ice in a protein extract buffer and centrifuged. The supernatant was processed for all the cDNA and for the GAPDH control using TRIzol reagent (Invitrogen) (20, 42). All RNA isolated was quantified spectrophotometrically by determining the ratio of optical density at 260/280 nm. As in our recent study (42), reverse transcriptase reaction was performed for first-strand cDNA synthesis. Real-time PCR analysis was performed in duplicate for all the cDNA and for the GAPDH control using protocols reported previously (43). Primers for hsp60, hsp70, hsp90 or GAPDH were designed by the Roche LightCycler® probe design software 2.0 (Mannheim, Germany) using the sequence information of the NCBI data base, and oligonucleotides were synthesized by Genemed Biotechnologies (Taipei, Taiwan). The primer pairs for amplification of hsp60 cDNA (GenBank™ accession NM022229) were 5′-AGGCATGAAGTTGATAGAGG-3′ for the forward primer and 5′-TTFFCAATTTCAAGAGAGG-3′ for the reverse, those of hsp70 cDNA (GenBank™ accession L16764) were 5′-GGCAAGATCGCGGAA- ACG-3′ for the forward primer and 5′-CCGATAGGTTGGAAG-3′ for the reverse primer, and those of hsp90 cDNA (GenBank™ accession S45392) were 5′-TTCCTCCTGTGTT- GTGTT-3′ for the forward primer and 5′-AGTTCTCTTT- GTCTTCCAGC-3′ for the reverse primer, and those of GAPDH cDNA (GenBank™ accession NM017008) were 5′-GCCAAAAGGTTATCATCATC-3′ for the forward primer and 5′-GGGCTATCCACAGTCTCTT-3′ for the reverse primer. Fluorescence
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**A**

- **HSP60**
  - **Phase I**
  - **Phase II**
  - MSAP (mmHg)
  - HR (bpm)
  - LF (mmHg²)

- **HSP70**
  - **Phase I**
  - **Phase II**
  - MSAP (mmHg)
  - HR (bpm)
  - LF (mmHg²)

- **HSP90**
  - **Phase I**
  - **Phase II**
  - MSAP (mmHg)
  - HR (bpm)
  - LF (mmHg²)

**POSTINJECTION TIME (min)**

**B**

- **HSP60/hsp60**
  - **Phase I**
  - **Phase II**
  - DURATION (min)

- **HSP70/hsp70**
  - **Phase I**
  - **Phase II**
  - DURATION (min)

- **HSP90/hsp90**
  - **Phase I**
  - **Phase II**
  - DURATION (min)
signals from the amplified products were quantitatively assessed using the LightCycler® software program (version 3.5). The second derivative maximum mode was chosen with baseline adjustment set in the arithmetic mode. The relative change in hsp60, hsp70, or hsp90 mRNA expression was determined by the -fold change analysis (42, 44) in which \(-\text{fold change} = 2^{-\Delta\Delta Ct} \), where \(\Delta\Delta Ct = (Ct_{\text{hsp}} - C_{\text{GAPDH}})_{\text{Mev treatment}} - (Ct_{\text{hsp}} - C_{\text{GAPDH}})_{\text{sham control}}\). Note that the Ct value is the cycle number at which the fluorescence signal crosses the threshold.

**Qualitative and Quantitative Analysis of DNA Fragmentation**—Total DNA was extracted from samples of the ventrolateral medulla, and nucleosomal DNA ladders were separated by electrophoresis on a 1% agarose gel (35), after amplification using a PCR kit for DNA ladder assay (Maxim Biotech, South San Francisco, CA) to enhance the detection sensitivity. To quantify apoptosis-related DNA fragmentation, a cell death enzyme-linked immunosorbent assay (Roche Applied Science) that detects apoptotic but not necrotic cell death (45) was used to assay the level of histone-associated DNA fragments in the cytoplasm (46). Absorbance was measured at 405 nm and referenced at 490 nm using an enzyme-linked immunosorbent assay microtiter plate reader (Anthros Labtec, Salzburg, Austria).

**Double Immunofluorescence Staining and Laser Confocal Microscopy**—The procedures for double immunofluorescence staining were modified from those reported previously (11, 36). In brief, free-floating 30-μm sections of the medulla oblongata were incubated with a rabbit polyclonal antiserum against activated caspase-3 (Cell Signaling), together with a mouse monoclonal antiserum directed against a specific neuron marker (47), neuron-specific nuclear protein (NeuN, Chemicon) or a mouse monoclonal antiserum against hsp90 (Hsp 80 or Hsp 70 (Stressgen). The sections were subsequently incubated concurrently with two appropriate secondary antisera (Molecular Probes, Eugene, OR), including a goat anti-rabbit IgG conjugated with Alexa Fluor 488 for activated caspase-3, a goat anti-mouse IgG conjugated with Alexa Fluor 568 for NeuN, or a goat anti-mouse IgG conjugated with Alexa Fluor 568 for Hsp60 or Hsp70. Viewed under a Fluorview FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan), immunoreactivity for NeuN, Hsp60, or Hsp70 exhibited red fluorescence and activated caspase-3 manifested green fluorescence. The exhibition of yellow fluorescence or co-localization of red and green fluorescence on merged images indicated the presence of activated caspase-3 immunoreactivity in neurons, or the relationship between cells that were immunoreactive to Hsp60 or Hsp70 and activated caspase-3.

**RESULTS**

**Pro-life and Pro-death Phase of Cardiovascular Responses in Mev Intoxication Model of Brain Stem Death**—As reported previously (10–12), microinjection of Mev (10 nmol) bilaterally into the RVLM elicited a progressive hypotension that became significant 100 min after application (Figs. 1A and 2), accompanied by an indiscernible change in HR. Concurrent changes in power density of the LF component of SAP signals revealed two distinct phases of Mev-induced cardiovascular responses. The pro-life Phase I entailed a significantly augmented LF power that endured 80–100 min. The pro-death Phase II, which lasted the remainder of our 180-min observation period, exhibited further and significant reduction in the power density of this spectral component to below baseline, which reflects failure of brain stem cardiovascular regulatory functions that precedes brain stem death (9). Because systemically administered Mev acts on the RVLM to elicit comparable cardiovascular responses (10), direct application of Mev to this brain stem site

**Immunoprecipitation and Immunoblot Analysis**—Protein extracts from either the mitochondrial or cytosolic fraction of samples from the ventrolateral medulla were immunoprecipitated at 4°C overnight with affinity-purified goat polyclonal anti-Hsp60, rabbit polyclonal anti-Bax, or mouse monoclonal anti-Bcl-2 antiserum conjugated with protein G-agarose beads (Santa Cruz Biotechnology). Western blot analysis of Bax, Bcl-2, or Hsp60 from proteins immunoprecipitated by anti-Hsp60 antiserum, Hsp60, or Bax from proteins immunoprecipitated by anti-Bax antiserum, or Hsp60, or Bcl-2 from proteins immunoprecipitated by anti-Bcl-2 antiserum was carried out as described above.

**Histology**—In some experiments, the brain stem was removed after the physiological experiment and fixed in 10% formaldehyde in 30% sucrose solution for at least 72 h. Histological verification of the microinjection site was performed on 25-μm frozen sections stained with Neutral red.

**Statistical Analysis**—All values are expressed as mean ± S.E. The effects of various treatments on the averaged value of MSAP or HR calculated every 20 min, the sum total of power density for LF components in the SAP spectra over 20 min, or the mRNA or protein expression level or the level of histone-associated DNA fragments in the ventrolateral medulla during each phase of Mev intoxication, were assessed using one-way or two-way analysis of variance with repeated measures, as appropriate, for group difference. The Scheffé multiple-range test was used for a posteriori comparison of individual means. \(p < 0.05\) was considered to be statistically significant.
has been routinely carried out (11–13) to produce site-specific actions in this brain stem death model.

HSP60 or HSP70, but Not HSP90, in RVLM Affects Differentially the Phasic Cardiovascular Responses during Mev Intoxication—Based on the stipulation that the magnitude and duration of phasic cardiovascular responses during Mev intoxication reflect the prevalence of the life-and-death signal, we first employed two loss-of-function manipulations (immuno-neutralization and gene knockdown) to evaluate whether a causal relationship exists between HSP60, HP70, or HSP90 in the RVLM and brain stem death in this animal model. Pretreatment with microinjection into the bilateral RVLM of an anti-HSP60 or anti-HSP70 antiserum (1:20), 1 h before local application of Mev (10 nmol), significantly enhanced the elicited hypotension or blunted the increase in power density of LF component of SAP signals during Phase I Mev intoxication, without affecting HR (Fig. 1A). This pretreatment also significantly shortened the pro-life phase to 25–35 min by shifting the prevailing phase of the 180-min observation period toward the pro-death phase (Fig. 1B). Interestingly, whereas anti-HSP60 antiserum pretreatment further potentiated the exhibited hypotension or reduced LF power during Phase II Mev intoxi-

**FIGURE 2.** Knockdown of *hsp60*, *hsp70*, or *hsp90* gene differentially affects the magnitude of phasic cardiovascular responses during Mev intoxication. Temporal changes in MSAP, HR, or power density of the LF component of SAP signals in rats that received knockdown of *hsp60*, *hsp70*, or *hsp90* gene as depicted in Fig. 1B, 24 h before microinjection bilaterally of Mev (10 nmol) or aCSF into the RVLM (at arrow). Values are mean ± S.E., n = 5–7 animals per experimental group. *, p < 0.05 versus the Veh + aCSF group for Veh + Mev, individual SC + Mev, individual SON + Mev, or *hsp90* ASON + Mev group, and †, p < 0.05 versus the Veh + Mev group for *hsp60* ASON + Mev or *hsp70* ASON + Mev group at corresponding time points in the Scheffe’ multiple-range test.

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cation, anti-HSP70 antiserum elicited no further action (Fig. 1A). On the other hand, pretreatment with an anti-HSP90 antiserum (1:20) was ineffective against either the magnitude or duration of the phasic cardiovascular responses to Mev. Those differential results were essentially duplicated on microinjection into the bilateral RVLM of an antisense oligonucleotide (50 pmol) against hsp60 (32), an antisense oligonucleotide (50 pmol) that knocks down selectively hsp70, but not hsc70 gene (14, 39), or an antisense hsp90 oligonucleotide (50 pmol) that acts selectively on hsp84 gene (14, 40), 24 h prior to the induction of Mev intoxication (Figs. 1B and 2). On the other hand, control pretreatments with the corresponding normal goat or mouse serum (1:20, Fig. 1) or sense or scrambled oligonucleotide (50 pmol, Figs. 1B and 2) did not discernibly affect the phasic cardiovascular responses during Mev intoxication. At the dose (10 nmol) we used, microinjection of Mev into the RVLM elicited minimal fatality during the 180-min observation (supplemental Table S1). Nonetheless, pretreatment with an anti-HSP60 or anti-HSP70 antiserum or an antisense hsp60 or hsp70 oligonucleotide exacerbated Mev lethality (supplemental Table S1). Pretreatment with an anti-HSP90 antiserum or antisense hsp90 oligonucleotide was again ineffective.

Differential and Phasic Changes in HSP60, HSP70, or HSP90 Protein Expression in Ventrolateral Medulla during Mev Intoxication—We next evaluated the possibility that the differential effects of HSP60, HSP70, or HSP90 in the RVLM on Mev-elicited phasic cardiovascular responses are coupled with differentially induced expression of these HSPs in the ventrolateral medulla. Proteomic analysis revealed the presence of HSP60 (Swiss-Prot accession P19226), HSP70 (Swiss-Prot accession P48721), or HSP90 (Swiss-Prot accession P34058) in the proteomic map (pI: 3–10; molecular mass: 14.4–94 kDa) of the RVLM (Fig. 3A). Intriguingly, whereas HSP60 expression in the ventrolateral medulla underwent a significant elevation during both phases of Mev intoxication (Fig. 3, B–E), HSP70 level exhibited only a significant
increase during Phase I and returned to baseline during Phase II (Fig. 3, B–D, and F). On the other hand, HSP90 expression in ventrolateral medulla remained relatively constant during both phases of Mev intoxication (Fig. 3, B–D, and G). The level of HSP60, HSP70, or HSP90 in the ventrolateral medulla of aCSF controls was comparable to sham controls. Western blot analysis using an antisera, which recognizes selectively HSP60, the inducible form of HSP70 but not HSC70, or both HSP86 and HSP84, essentially confirmed the differential and phasic changes in HSP60, HSP70, or HSP90 protein expression in the ventrolateral medulla during Mev intoxication (Fig. 4). The site specificity of those Mev effects was revealed when the differential and phasic expression of HSP isoforms was absent in tissue samples obtained from a region immediately adjacent to the anatomical confines of the RVLM and lateral to the nucleus ambiguus (supplemental Fig. S2).

**The Augmented HSP60 or HSP70 Expression in Ventrolateral Medulla during Mev Intoxication Involves de Novo Synthesis**—Whether the augmented HSP60 or HSP70 expression in the ventrolateral medulla during Mev intoxication entails de novo synthesis was delineated in our third series of experiments. Real-time PCR analysis (Fig. 5A) revealed that, although hsp60 mRNA underwent a progressive increase during Phases I and II, significantly augmented hsp70 mRNA took place only during Phase I Mev intoxication. On the other hand, hsp90 mRNA remained constant. Western blot analysis further showed that, although the respective vehicle (0.1% Me2SO or 50% EtOH) was ineffective, the phasic pattern of augmented HSP60 or HSP70 protein expression in the ventrolateral medulla was significantly blunted and HSP90 expression remained unaltered (Fig. 5B) in animals that received microinjection of the transcription inhibitor, actinomycin D (5 nmol), or the translation inhibitor, cycloheximide (20 nmol) into the bilateral RVLM 1 h prior to local application of Mev (10 nmol). Likewise, neither actinomycin D nor cycloheximide (data not shown), nor their respective solvent affected HSP60, HSP70, or HSP90 level in the ventrolateral medulla of aCSF control rats.

**HSP60 or HSP70 Reduces Apoptotic Cell Death in Ventrolateral Medulla during Mev Intoxication**—Animals that were pretreated with microinjection of normal goat or mouse serum (1:20) into the bilateral RVLM manifested apoptosis with a characteristic nucleosomal DNA ladder (Fig. 6A) or cytoplasmic histone-associated DNA fragments (Fig. 6B) in the ventrolateral medulla during Mev intoxication, with progressively heightened intensity. Interestingly, microinjection bilaterally of an anti-HSP60 or anti-HSP70 antiserum (1:20) into the RVLM significantly exacerbated the manifested DNA fragmentation induced by Mev in the ventrolateral medulla (Fig. 6), although anti-HSP60 antiserum was more efficacious. To confirm that these biochemical observations from protein extracts indeed take place at the cellular level, we examined the expression of another indicator for apoptosis (46), activated caspase-3, in the RVLM (Fig. 7A) during Mev intoxication using double immunofluorescence staining. Viewed under a laser scanning confocal microscope, activated caspase-3 was minimally present in RVLM cells in sham control animals that were immunoreactive to the neuronal marker, NeuN (Fig. 7B). In contrast, there was a progressive increase in the immunoreactivity of activated caspase-3 in RVLM neurons (Fig. 7, C and D) during the course of Mev intoxication in rats that were pretreated with normal goat or mouse serum. Of note, against the
clearly defined nucleus and nucleolus in RVLM neurons, immunoreactivity of activated caspase-3 was primarily confined to the cytoplasm. Intriguingly, the expression of this index of apoptotic cell death in RVLM neurons was further enhanced in animals pretreated with microinjection of an anti-HSP60 (Fig. 7, E and F) or anti-HSP70 (Fig. 7, G and H) antiserum into the bilateral RVLM. Again, anti-HSP60 antiserum was more efficacious.

Results from another series of double immunofluorescence staining further demonstrated the crucial inverse relationship between HSP60 (Fig. 7 I) or HSP70 (Fig. 7 J) expression and apoptosis. Thus, RVLM neurons that exhibited high levels of HSP60 or HSP70, respectively, during Phase II or Phase I Mev intoxication manifested nominal expression of activated caspase-3 immunoreactivity; and vice versa for cells that manifested nominal level of either HSP.

HSP60 or HSP70 Modulates NOS I/PKG Cascade and NOS II/ Peroxynitrite Signaling in Ventrolateral Medulla during Mev Intoxication—Our laboratory demonstrated previously (11, 12) that, whereas NOS I/PKG cascade in the RVLM is responsible for the pro-life phase, NOS II/peroxynitrite signaling underlies the pro-death phase of Mev intoxication. In addition, NO produced by NOS I or NOS II is, respectively, anti-apoptotic (48–50) or pro-apoptotic (35). It is therefore conceivable that HSP60 or HSP70 may confer neuroprotection by an anti-apoptotic action via modulating these two signaling pathways. Western blot analysis (Fig. 8) revealed in animals that received pretreatment with microinjection of aCSF into the bilateral RVLM a significant elevation of HSP60, NOS II, or nitrotyrosine (an experimental index for peroxynitrite) (12, 14, 35) in the ventrolateral medulla during both phases of Mev intoxication. On the other hand, HSP70, NOS I, or PKG exhibited only a significant increase during Phase I, followed by a return to baseline during Phase II.

Gene knockdown manipulation was subsequently used to ascertain that these temporally correlated biochemical changes are causally linked (Fig. 8). Pretreating animals by microinjection into the bilateral RVLM of an antisense hsp60 or hsp70 oligonucleotide (50 pmol) blunted significantly and selectively the respective Mev-induced phasic surge in HSP60 or HSP70 expression at the ventrolateral medulla. Intriguingly, both pretreatments also significantly blunted the augmented NOS I or PKG level but potentiated the increase in NOS II or nitrotyrosine expression during Phase I Mev intoxication. In addition, antisense hsp60 oligonucleotide pretreatment extended the potentiated NOS II or nitrotyrosine expression in the ventrolateral medulla to Phase II Mev intoxication. The specificity of these pretreatments was confirmed when antisense hsp60 exerted no discernible effects on HSP70 or HSP90 expression, or hsp70 oligonucleotide on HSP60 or HSP90 expression in the ventrolateral medulla. At the same time, pretreatments with sense or scrambled hsp60 or hsp70 oligonucleotide (50 pmol)
were ineffective against the phasic changes in HSP60, HSP70, NOS I, PKG, NOS II, or nitrotyrosine level in the ventrolateral medulla induced by microinjection bilaterally of Mev into the RVLM (Fig. 8).

HSP60 Also Interacts with the Bax/Bcl-2/Cytochrome c/Caspase-3 Apoptotic Machinery in Ventrolateral Medulla—
The observation, that anti-HSP60 antiserum was more potent than anti-HSP70 antiserum in potentiating DNA fragmentation in the ventrolateral medulla of rats that received immunoneutralization of HSP60 or HSP70 as depicted in Fig. 1A, 1 h before induction of Mev intoxication. Values in B are mean ± S.E., n = 5–7 animals per experimental group. *, p < 0.05 versus sham control (Basal) or at corresponding time intervals in the NS + aCSF group, and †, p < 0.05 versus the NS + Mev group at corresponding time intervals in the Scheffé multiple-range test.

FIGURE 6. HSP60 or HSP70 prevents DNA fragmentation in ventrolateral medulla during Mev intoxication. Qualitative (A) or quantitative (B) analysis of DNA fragmentation in the ventrolateral medulla of rats that received immunoneutralization of HSP60 or HSP70 as depicted in Fig. 1A, 1 h before induction of Mev intoxication. Values in B are mean ± S.E., n = 5–7 animals per experimental group. *, p < 0.05 versus sham control (Basal) or at corresponding time intervals in the NS + aCSF group, and †, p < 0.05 versus the NS + Mev group at corresponding time intervals in the Scheffé multiple-range test.

HSP60 or HSP70 Confers Neuroprotection in Brain Stem Death intubation observed with total protein (Figs. 3 and 4) actually involved a progressive decrease in the mitochondrial, alongside a gradual increase in the cytosolic fraction (Fig. 9). This change in compartmental expression pattern was also observed for Bcl-2, cytochrome c, and activated caspase-3. On the other hand, Bax expression manifested a progressive increase in the mitochondrial fraction and a decline in the cytosolic fraction over the course of Mev intoxication. Of interest was that the differential temporal changes in mitochondrial or cytosolic levels of Bcl-2, Bax, cytochrome c, or activated caspase-3 were significantly augmented after pretreatment with microinjection of an anti-HSP60 antisem (1:20) into the bilateral RVLM (Fig. 9). These differential results were essentially duplicated (supplemental Fig. S3) upon pretreatment with an antisense hsp60 oligonucleotide.
However, pretreatment with an anti-HSP70 antiserum (Fig. 9) or an antisense hsp70 oligonucleotide (supplemental Fig. S3), similar to normal serum, was ineffective against the Mev-induced changes in Bax/Bcl-2/cytochrome c cascade; as was anti-HSP60 or anti-HSP70 antiserum in aCSF control rats (data not shown).

We also determined whether alterations in interactions between HSP60 and Bax or Bcl-2 occur in the mitochondrial or cytosolic fractions. Immunoblot analysis of proteins immunoprecipitated from the ventrolateral medulla by an anti-HSP60 antiserum (Fig. 10A) revealed a progressive augmentation in the complex formed between HSP60 and Bax or Bcl-2 in the mitochondrial fraction during Mev intoxication. Whereas the association between HSP60 and Bax in the cytosolic fraction also manifested a gradual increase, the complex between HSP60 and Bcl-2 exhibited a progressive reduction. The validity of these observations was confirmed when co-immunoprecipitation experiments pairing Bax and HSP60 or Bcl-2 and HSP60 essentially duplicated those findings (Fig. 10A). To ascertain the specificity of proteins brought down by immunoprecipitation, we found that HSP60 identified (Fig. 10B) in co-immunoprecipitation experiments pairing HSP60 and HSP60 exhibited a temporal pattern of gradual decline in the mitochondrial or progressive elevation in the cytosolic fraction during Mev intoxication that was comparable to that shown in Figs. 9 and S3. Parallel co-immunoprecipitation procedures pairing Bax and Bax or Bcl-2 and Bcl-2 also yielded results that were similar to those in Figs. 9 and S3.

**DISCUSSION**

Working in conjunction with a neural substrate whose activity reflects the failure of brain stem cardiovascular regulation during brain stem death, the present study provided novel demonstrations that, although HSP60, HSP70, and HSP90 are present in the proteomic map of the RVLM, only the 60-kDa or 70-kDa HSP plays a neuroprotective role via an amelioration of the induced cardiovascular depression in an experimental Mev intoxication model of brain stem death (9), which closely
resembles the progression toward death in patients hospitalized for organophosphate poisoning (6). Our loss-of-function manipulations of HSP60 or HSP70 activity or hsp60 or hsp70 gene expression in the RVLM significantly shortened the prolife phase, augmented lethality, potentiated hypotension, or reduced the power density of the LF component of SAP signals during Mev intoxication. The phasic augmentation of the protein level of HSP60 or HSP70 in the ventrolateral medulla during Mev intoxication revealed by complementary proteomic and Western blot analyses further corroborated these physiological results. In this regard, rat lung mitochondria exhibit a prolonged increase in HSP60 expression when exposed to paraquat, which prevents the progression of pulmonary fibrosis induced by this organophosphate poison (53). Augmentation of HSP70 expression in the ventrolateral medulla is causally and temporally related to its antagonism of circulatory suppression during endotoxemia (14, 20).

Two observations validated our conclusion that HSP90 does not play a neuroprotective role in our experimental model of brain stem death. First, our results may not be confounded by the non-specificity of the antiserum or antisense oligonucleotide used in our experiments. Pretreatment with an anti-HSP90 antiserum that recognizes both HSP86 and HSP84 or an antisense hsp90 oligonucleotide that knocks down selectively hsp84 gene (40) produced complementary results. Second, both proteomic and Western blot analyses demonstrated that HSP90 expression in the ventrolateral medulla remained constant during the course of Mev intoxication.

Our observations with real-time PCR assay and Western blot analysis coupled with actinomycin D or cycloheximide pretreatment showed that the elevated HSP60 or HSP70 expression in the RVLM during Mev intoxication results from de novo synthesis. In response to stress, heat shock transcription factor 1 (HSF1) is known to regulate hsp induction (54), and HSP70 expression is attributable to phosphorylation of HSF1 at serine 230 (55). Overexpression of HSP70 inhibits phosphorylation of HSF1 in the nucleus at serine residues (55, 56). This negative feedback mechanism, which was proposed to explain the well established reduction in HSP70 expression after repetitive exposure of cells or animals to heat shock (56), may also account

**FIGURE 9.** HSP60 modulates the Bax/Bcl-2/cytochrome c/caspase-3 apoptotic machinery in ventrolateral medulla during Mev intoxication. Illustrative gels (A) or temporal changes of HSP60, Bcl-2, Bax, cytochrome c, or activated caspase-3 (caspase-3*) in the mitochondrial (in % relative to prohibitin) or cytosolic (in % relative to β-actin) fraction (B) of samples collected from the ventrolateral medulla of rats that received immunoneutralization of HSP60 as depicted in Fig. 1A, 1 h before induction of Mev intoxication. Values are S.E., n = 5–7 animals per experimental group. *, p < 0.05 versus sham control (Basal) or NS+aCSF group, and †, p < 0.05 versus the NS+Mev group at corresponding time intervals in the Scheffe multiple-range test. Note that the activated caspase-3 in the mitochondrial fraction or prohibitin in the cytosolic fraction was below detection limit.
for our observation that HSP70 expression returned to baseline during Phase II Mev intoxication. Being a nuclear-coded protein (57, 58) with heat shock element present in the promoter region (59), it is conceivable that hsp60 gene is also regulated by HSF1. Accumulation of unfolded proteins within the mitochondrial matrix also results in selective transcriptional activation of the mitochondrial stress response element in the hsp60 gene (60). That this mitochondrial stress response does not up-regulate HSP70 (61) may account for the expression pattern of HSP60 in the ventrolateral medulla during Mev intoxication that is different from that of HSP70.

The present study unveiled that an intracellular process that underlies the neuroprotection conferred by HSP60 or HSP70 in the RVLM against cardiovascular depression during Mev intoxication entails enhancing the NOS I/PKG signaling pathway and inhibiting the NOS II/peroxynitrite cascade in the RVLM. Our laboratory demonstrated previously (36) that, although all three NOS isoforms are expressed in the RVLM at both mRNA and protein levels, only NOS I and II are present in RVLM neurons. Furthermore, a balance between the tonically active NOS I and NOS II determines the regulation of sympathetic vasomotor outflow by the endogenous NO at the RVLM (37). Low concentration of NO generated by NOS I in the RVLM increases vasomotor outflow (37) by exciting sympathetic premotor neurons via a cGMP/PKG-dependent facilitation of presynaptic glutamate release (62). High concentration of NO produced by NOS II decreases vasomotor outflow (37) by inhibiting RVLM neurons via a peroxynitrite-mediated reduction of presynaptic glutamate release (63). This NOS-dependent bi-directional role of NO suitably befits the notion (12) that, whereas NO generated by NOS I in the RVLM, followed by activation of the cGMP/PKG cascade, is responsible for the pro-life phase, peroxynitrite formed by a reaction of NO derived from NOS II with superoxide anion underlies the pro-death phase of Mev intoxication. It is therefore intriguing that the present study demonstrated that HSP60 or HSP70 may confer neuroprotection via enhancing NOS I/PKG cascade or depressing NOS II/peroxynitrite signaling in the RVLM.

The NOS I gene is the first example of gene activation in neuronal cells by the Oct-2 transcription factor (64). Nine distinct first exons of NOS I mRNA are identified (65), and...
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exon 1c is thought to be the predominant NOS I mRNA variant (66). Because exon 1c promoter activity is regulated by different members of the Sp and ZNF families of transcription factors (66), it is possible that these transcription factors, together with Oct-2, are potential candidates via which HSP60 or HSP70 regulates NOS I gene expression. HSP60 (21, 22) or HSP70 (23–25) reportedly inhibits NOS II gene expression by transcriptional mechanisms that involve the NF-κB/inhibitory κB (IκB) pathway. In the RVLM, our laboratory (20) demonstrated that suppression of NOS II mRNA expression by HSP70 in an experimental endotoxemia model of brain stem death (9) also results from an attenuation of NF-κB activation via inhibiting IκB kinase activation or degradation of cytoplasmic IκBα.

Complementary results from biochemical and immunofluorescence experiments revealed that HSP60 or HSP70 ameliorates Mev-induced cardiovascular depression via an anti-apoptotic action in the RVLM. We found that the same loss-of-function manipulations of HSP60 or HSP70 in the RVLM that potentiated Mev-induced circulatory inhibition also intensified the elicited nucleosomal DNA fragmentation, or elevated cytoplasmic histone-associated DNA fragments in the ventrolateral medulla, or augmented the induced expression of activated caspase-3 in RVLM neurons. Individual RVLM cells also manifested an inverse relationship between HSP60 or HSP70 expression and activated caspase-3 level during the peak of Mev intoxication. NO derived from NOS I has been given an anti-apoptotic role via suppression of Bax, caspase-3, and caspase-9 activation in dorsal root ganglion neurons (48), activation of cAMP-responsive element-binding protein (CREB) in neuroblastoma cells (49), or regulation of cGMP-dependent CREB phosphorylation and Bcl-2 expression in cerebellar neurons (50). On the other hand, marked increase in NO produced in brain by organophosphates (67) or excessive NO induced by endotoxin and various pro-inflammatory cytokines (68, 69) affects oxidative phosphorylation by inhibiting the mitochondrial respiratory enzymes. The resultant mitochondrial dysfunction induces apoptosis (70, 71) by triggering downstream caspase cascades via the release of pro-apoptotic factors, including cytochrome c, to the cytosol (72). Intriguingly, a crucial cross-talking cellular consequence between NO produced by NOS II, reduction in mitochondrial respiratory enzyme Complexes I and IV activities, and cardiovascular depression during endotoxemia is the induction of apoptotic cell death in the RVLM (35). A rapid onset of dysfunction of mitochondrial respiratory Complexes I and IV also takes place in the RVLM during Mev intoxication (13). Together with observations from the present study, it is conceivable that enhancement of NOS I/PKG signaling or depression of NOS II/peroxynitrite cascade also underlies the anti-apoptotic action of HSP60 or HSP70 in RVLM neurons in this experimental model of brain stem death.

The augmented HSP60 may additionally exert its anti-apoptotic effects via blunting Mev-induced activation of the Bax/cytochrome c/caspase-3 cascade. Superimposed on the augmentation of HSP60 expression in the RVLM during both phases of Mev intoxication was a progressive decline in mitochondrial or elevation in cytosolic HSP60 in the ventrolateral medulla. We reason that this re-distribution of HSP60 may arise from a higher cytosolic presence of the newly synthesized HSP60 (73) or as a result of mitochondrial to cytosolic translocation (31). More importantly, our novel results implicate that the re-distributed HSP60 in the RVLM exerts its anti-apoptotic action by an enhanced interaction between HSP60 and mitochondrial or cytosolic Bax or Bcl-2. In cardiac myocytes, the cytosolic HSP60 plays an anti-apoptotic role by complexing with Bax (32, 33). Results from our immunoprecipitation and immunoblot analysis suggest that, by increasing its interaction with Bax, the enhanced cytosolic HSP60 functions to prevent translocation of this pro-apoptotic factor to the mitochondria (32, 74). The intensified association between HSP60 and Bax in the mitochondria during Mev intoxication may conceivably prevent oligomerization and insertion of Bax into the mitochondrial membrane, two essential steps for triggering cytochrome c release (75). That pretreatment with an anti-HSP60 antiserum or an antisense hsp60 oligonucleotide augmented the mitochondrial level but reduced the cytosolic presence of Bax in the RVLM during Mev intoxication provided ample credence to these notions. On the other hand, it is unlikely that the enhanced Bax-HSP60 binding in the mitochondrial fraction is due simply to an increase in Bax, because there was a concomitant decrease in mitochondrial HSP60 level. By enhancing progressively its interaction with Bcl-2 in the mitochondria, the gradually reduced mitochondrial HSP60 may prevent apoptotic cell death by strengthening the capability of Bcl-2 to retard the release of cytochrome c to the cytosol. On the other hand, Bcl-2 dissociated from HSP60 in the cytosol may reinforce its anti-apoptotic action by preventing the translocation of Bax to the mitochondria (32, 74). Again, that pretreatment with an anti-HSP60 antiserum or an antisense hsp60 oligonucleotide further decreased the mitochondrial level but increased the cytosolic level of Bcl-2 in the RVLM during Mev intoxication provided ample support for these notions. However, the lack of effects of pretreatment with an HSP70 antiserum or an antisense hsp70 oligonucleotide suggests that HSP70 does not modulate the Mev-induced activation of the Bax/Bcl-2/cytochrome c cascade in the RVLM.

The cellular and molecular basis of brain stem death remains an enigma. Because death represents the end of existence for an individual organism, we propose recently that multiple pro-life and pro-death programs must be activated during the progression toward brain stem death (9). The present study provided novel findings to indicate that HSP60 or HSP70 in the RVLM may be one of those pro-life programs. We demonstrated that de novo synthesis of HSP60 or HSP70 confers neuroprotection in the Mev intoxication model of brain stem death by ameliorating cardiovascular depression via an anti-apoptotic action in RVLM neurons. We also unveiled that the underlying intracellular processes include enhancing the NOS I/PKG signaling pathway and inhibiting the NOS II/peroxynitrite cascade in the RVLM. The augmented HSP60 additionally exerts its anti-apoptotic effects via reducing activation of the Bax/cytochrome
c/caspase-3 cascade by an enhanced interaction with mitochondrial or cytosolic Bax or Bcl-2. On the other hand, although present in the RVLM, HSP90 does not appear to play a protective role in this model of brain stem death. This information should provide further insights on the etiology of brain stem death and shed light on new directions for clinical management of life and death.

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