

Heat Shock Protein 70 (HSP70), but not HSP90, in the Rostral Ventrolateral Medulla
Confers Neuroprotection Against Fatal Endotoxemia Via Augmentation of
Nitric Oxide Synthase I (NOS I)/Protein Kinase G Signaling Pathway
and Inhibition of NOS II/Peroxynitrite Cascade

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Neuroprotection by heat shock protein 70 in Endotoxemia

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ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; HR, heart rate; HSP70, heat shock protein 70; HSP90, heat shock protein 90; LF, low-frequency; LPS, lipopolysaccharide; MSAP, mean systemic arterial pressure; NO, nitric oxide, NOS, nitric oxide synthase; NTS, nucleus tractus solitarii; PKG, protein kinase G; RVLM, rostral ventrolateral medulla; SAP, systemic arterial pressure; VLF, very low-frequency.

ABSTRACT

Heat-shock proteins (HSPs) represent a group of highly conserved intracellular proteins that participate in protective adaptation against cellular stress. We evaluated the neuroprotective role of HSP70 and HSP90 at the rostral ventrolateral medulla (RVLM), the medullary origin of sympathetic vasomotor tone, during fatal endotoxemia. In Sprague-Dawley rats maintained under propofol anesthesia, *Escherichia coli* lipopolysaccharide (30 mg/kg, i.v.) induced a decrease (Phase I), followed by an increase (Phase II; “Pro-life” phase) and a secondary decrease (Phase III; “Pro-death” phase) in the power density of the vasomotor component of systemic arterial pressure spectrum, along with progressive hypotension or bradycardia. Proteomic and Western blot analyses revealed that whereas HSP70 expression in the RVLM was significantly augmented during Phases I and II and returned to baseline during Phase III endotoxemia, HSP90 protein expression remained constant. The increase in HSP70 level was significantly blunted on pretreatment with microinjection of the transcription inhibitor, actinomycin D or protein synthesis inhibitor, cycloheximide into the bilateral RVLM. Functional blockade of HSP70 in the RVLM by an anti-HSP70 antiserum or prevention of synthesis by an antisense *hsp70* oligonucleotide exacerbated mortality or potentiated the cardiovascular depression during experimental endotoxemia, alongside significantly reduced NOS I or PKG level or augmented NOS II or peroxynitrite level in the RVLM. We conclude that whereas HSP90 is ineffective, *de novo* synthesis of HSP70 in the RVLM may confer neuroprotection during fatal endotoxemia by preventing cardiovascular depression via enhancing the sympathoexcitatory NOS I/PKG signaling pathway and inhibiting the sympathoinhibitory NOS II/peroxynitrite cascade in the RVLM.

The heat shock proteins (HSPs) represent a group of intracellular proteins that are highly conserved across species and are thought to participate in protective adaptation that spares cells from otherwise lethal consequences of exposure to heat, toxins, infection, seizure, trauma, ischemia or other cellular stresses (Lindquist and Craig, 1988; Welch, 1992; Morimoto and Santoro, 1998). Because of their critical roles in intracellular processing, synthesis, transportation and degradation of proteins, HSPs have been termed molecular chaperones. The cellular protective mechanisms of HSPs are believed to be related to these chaperone functions, which lead to the prevention of protein denaturation and promotion of refolding of damaged proteins after stress. In addition, HSP chaperones may sustain proteins in the productive folding pathway or maintain newly synthesized proteins in an unfolded conformation suitable for translocation across membranes (Welch, 1992; Morimoto and Santoro, 1998).

A majority of studies on the *in vivo* neuroprotective role of HSPs centers on protection against cerebral ischemia during heatstroke (Yang and Lin, 1999). HSP70 induced by a brief hyperthermic heat shock also confers cardiovascular protection during heatstroke via potentiation of baroreceptor reflex response (Li et al., 2001) by up-regulation of glutamatergic neurotransmission (Chan et al., 2002a) at the nucleus tractus solitarii. Other investigators reported (Yenari et al., 1998) that HSP70 enhances neuronal survival during transient focal cerebral ischemia or excitotoxin-induced seizures. On the other hand, the best-studied cellular action of HSPs by far is protection against apoptosis, based primarily on studies using cell lines under non-pathological conditions. Within the HSP family, HSP70 and HSP90 are two members that are often reported to be anti-apoptotic (Meriin et al., 1998; Robertson et al., 1999; Lee et al., 2001).

Cardiovascular depression during sepsis remains a significant cause of morbidity and mortality (Parrillo, 1993). Our laboratory (Chan et al., 2001a,b) demonstrated previously that, by eliciting a reduction in sympathetic vasomotor outflow and arterial pressure, overproduction of nitric oxide (NO) by NO synthase II (NOS II or iNOS) and formation of peroxynitrite by reacting NO with superoxide anion (Chan et al.,

2002b; 2005) in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons are located (Ross et al., 1984), plays a pivotal role in the fatal cardiovascular depression associated with endotoxemia. Based on this rat model of experimental endotoxemia, which mimics clinically the systemic inflammatory response syndrome, the present study was undertaken to assess the hypothesis that HSP70 and HSP90 in the RVLM confers neuroprotection against fatal endotoxemia. This hypothesis was partially validated based on combined physiological, pharmacological and biochemical results. We demonstrated that whereas an augmented expression of HSP70 resulted from *de novo* synthesis in the RVLM plays a neuroprotective role in fatal endotoxemia; HSP90 is essentially not involved. We further showed that the cellular mechanisms that underlie this neuroprotective action of HSP70 include prevention of cardiovascular depression by an enhancement of the NOS I (or nNOS)/protein kinase G (PKG) signaling pathway and an inhibition of the NOS II/peroxynitrite cascade in the RVLM.

Materials and Methods

Adult male Sprague-Dawley rats (288-352 g, n = 278) 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, and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Recording and Power Spectral Analysis of Systemic Arterial Pressure Signals.

Animals received cannulation of both femoral artery and vein and tracheal intubation under an initial pentobarbital sodium anesthesia (50 mg/kg, i.p.). They received thereafter intravenous infusion of propofol (Zeneca, Macclesfield, UK) at 20-25

mg/kg/h. This scheme provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation (Yang et al., 1995). Systemic arterial pressure (SAP) signals recorded from the femoral artery were simultaneously subject to on-line power spectral analysis (Chan et al., 2001a,b; Li et al., 2001; Chan et al., 2002b; Chang et al., 2003; Chan et al., 2004, 2005). We were particularly interested in the very low-frequency (VLF; 0-0.25 Hz) and low-frequency (LF; 0.25-0.8 Hz) components of SAP signals. Our laboratory demonstrated previously (Kuo et al., 1997) that these spectral components of SAP signals take origin from the RVLM, and their power density reflects the prevailing sympathetic neurogenic vasomotor tone. Heart rate (HR) was derived instantaneously from SAP signals. The SAP spectra and power density of the two spectral components were displayed during the experiment, alongside pulsatile SAP, mean SAP (MSAP) and HR, in an on-line and real-time manner. During the recording session, body temperature of the animals was maintained at 37°C with a heating pad, and animals were allowed to breathe spontaneously with room air via the intubated trachea.

Experimental Endotoxemia. *Escherichia coli* lipopolysaccharide (LPS; serotype 0111:B4; Sigma-Aldrich, St. Louis, MO) was administered intravenously over 1-2 min at 30 mg/kg (Chan et al., 2001a). Temporal changes in MSAP, HR or power density of LF or VLF component of the SAP signals were routinely followed for 240 min, or until the animal succumbed to endotoxemia. The survival rate within 240 min was also recorded.

Collection of Ventrolateral Medullary Tissue Samples. As we reported previously (Chan et al., 2001a, 2004), the sequence of cardiovascular events during LPS-induced endotoxemia can be divided into three phases. At the peak of each of these phases of experimental endotoxemia (LPS group) or 30, 150 or 240 min after intravenous injection of saline (saline group), rats were perfused intracardiacly with 100 cc of warm (37°C) saline that contains heparin (100 U/ml). The brain was

removed rapidly and placed on dry ice. Tissues on both sides of the ventrolateral part of medulla oblongata, at the level of RVLM (0.5-2.5 mm rostral to the obex), were collected and processed (Chan et al., 2001a,b, 2002b; Chang et al., 2003; Chan et al., 2004, 2005) for subsequent proteomic or Western blot analysis. Tissues obtained from animals that were anesthetized and received preparatory surgery served as our sham-controls. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL).

Proteomic Analysis. Proteomic analysis of proteins extracted from the ventrolateral medulla was carried out as detailed previously (Huang et al., 2002). The silver-stained 2-dimensional electrophoresis gels in the domain of pI: 3-10 and Mr: 14.4-94 kDa were scanned by an ImageScanner (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein spots were quantified and numbered using the ImageMaster 2D Elite software (Amersham Pharmacia Biotech), and were checked manually to eliminate artifacts due to gel distortion, abnormal silver staining or poorly detectable spots. The protein level of each spot was expressed as a percentage of total spot volume in the 2-dimensional electrophoresis gel. Protein spots of interests excised from corresponding Coomassie brilliant blue-stained gels were further subject to in-gel digestion, and analyzed by MALDI-TOF mass spectrometry (Applied Biosystems Voyager ED-PRO, Foster City, CA). To identify proteins, the measured mono-isotopic masses of peptides were routinely analyzed using both MS-Fit (Protein Prospector, UCSF Mass Spectrometry Facility, San Francisco, CA) and MASCOT (Matrix Science, Boston, MA) search programs.

Western Blot Analysis. Western blot analysis (Chan et al., 2002a,b; Chang et al., 2003; Chan et al., 2004, 2005) was carried out on proteins extracted from the ventrolateral medulla for HSP70, HSP90, NOS I, NOS II, PKG, nitrotyrosine, an experimental index for peroxynitrite activity (Chan et al., 2002b; 2005), or β -actin. The primary antisera used included mouse monoclonal antiserum against HSP70

(SPA-810; Stressgen, Victoria, BC, Canada), HSP90 (SPA-830; Stressgen), nitrotyrosine (05-233; Upstate, Lake Placid, NY) or β -actin (MAB-1501; Chemicon, Temecula, CA); or rabbit polyclonal antiserum against NOS I (sc-648; Santa Cruz, Santa Cruz, CA), NOS II (sc-650; Santa Cruz) or PKG (539729; Calbiochem, San Diego, CA). The secondary antisera used included horseradish peroxidase-conjugated sheep anti-mouse IgG (NA931; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) for HSP70, HSP 90, nitrotyrosine or β -actin; or donkey anti-rabbit IgG (NA934; Amersham Biosciences) for NOS I, NOS II or PKG. Specific antibody-antigen complex was detected by an enhanced chemiluminescence Western blot detection system (NEN, Boston, MA). The amount of protein was quantified by the ImageMaster software (Amersham Pharmacia Biotech), and was expressed as the ratio relative to β -actin protein.

Microinjection of Test Agents. Microinjection bilaterally of test agents into the RVLM was carried out stereotaxically and sequentially at a volume of 50 nL. The coordinates used 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 (Chan et al., 2001a,b, 2002b; Chang et al., 2003; Chan et al., 2004, 2005). Test agents used included a transcription inhibitor (Fernando et al., 2000; Chang et al., 2004), actinomycin D (Tocris Cookson, Bristol, UK); a translation inhibitor (Fernando et al., 2000; Chang et al., 2004), cycloheximide (Tocris Cookson); normal mouse serum (Sigma-Aldrich, St. Louis, MO), mouse monoclonal antiserum against HSP70 (Stressgen) or HSP90 (Stressgen); or sense, antisense or scrambled oligodeoxynucleotide (Genemed Synthesis, South San Francisco, CA) against *hsp70* (Robertson et al., 1999; Chan et al., 2004) or *hsp90* (Zucchi et al., 2002) gene (Table 1). The dose and treatment regimen were adopted from previous reports (Fernando et al., 2000; Zucchi et al., 2000; Li et al., 2001; Chan et al., 2004; Chang et al., 2004) that used those test agents for the same purpose as in this study. Actinomycin D was prepared with 0.1% DMSO, and cycloheximide with 50% EtOH. We added 0.02% Triton X-100 (Sigma-Aldrich) to anti-HSP70 or HSP90

antiserum to facilitate its transport across the cell membrane (Li et al., 2001; Chan et al., 2002a). All oligonucleotides were phosphorothioated in all positions and were diluted in artificial cerebrospinal fluid (aCSF) at pH 7.4. Microinjection of 0.1% DMSO, 50% EtOH, normal mouse serum plus Triton X-100 or aCSF served as our vehicle and volume control.

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- μ m frozen sections stained with Neutral red.

Statistical Analysis. All values are expressed as mean \pm SE. The averaged value of MSAP or HR calculated every 20 min after administration of test agents or vehicle, the sum total of power density for LF or VLF component in the SAP spectra over 20 min, or the protein expression level in the ventrolateral medulla during each phase of experimental endotoxemia, were used for statistical analysis. One-way or two-way analysis of variance with repeated measures was used, as appropriate, to assess group means, followed by the Scheffé multiple range test for post hoc assessment of individual means. $p < 0.05$ was considered statistically significant.

Results

Both HSP70 and HSP90 are Present in the Proteomic Map of RVLM. One basic premise for HSP70 or HSP90 to play a functional role in the RVLM during experimental endotoxemia is for those HSPs to be expressed in this medullary site. For this purpose, we generated proteomic maps (Fig. 1) for samples of the ventrolateral medulla obtained immediately after animals were anesthetized with

pentobarbital sodium. MALDI-TOF mass spectrometric analysis (Fig. 2) was subsequently used to identify protein spots in the domain of pI: 3-10 and Mr: 67-94 kDa in the 2-dimensional electrophoresis gels. Based on complementary search results on samples from 5 different animals using MS-Fit and MASCOT programs, which detect matched peptides in the tryptic peptide spectrum with observed m/z values and tryptic cutting sites (Table 2), we found that HSP70 (SWISS Prot accession No P48721) or HSP90 (SWISS Prot accession No P34058) is consistently present in the RVLM. The relative contribution of the two HSPs to the total spot volume in the proteomic map (pI: 3-10 and Mr: 14.4-94 kDa) of the RVLM was $0.05 \pm 0.02\%$ for HSP70 or $0.4 \pm 0.05\%$ for HSP90 (mean \pm SE of duplicate analyses on samples from 5 different animals).

Three Phases of Cardiovascular Responses are Present During Endotoxemia.

As reported previously (Chan et al., 2001a), based on the decrease, increase, and a secondary decrease in the power density of the LF or VLF component in the SAP spectrum, the sequence of cardiovascular responses induced by intravenous administration of LPS (30 mg/kg) can be divided into three phases (Figs. 3 and 4). Both SAP and HR underwent typically a significant decrease and a rebound during Phase I, to be followed by progressive hypotension or bradycardia during Phases II and III endotoxemia.

HSP70, but not HSP90, Expression in the Ventrolateral Medulla is Augmented During Endotoxemia. Analysis of changes in spot volume of HSP70 or HSP90 in the proteomic map of RVLM in sham-controls (Fig. 5A) or during Phases I (Fig. 5B), II (Fig. 5C) or III (Fig. 5D) endotoxemia revealed that the two HSPs manifested differential expression patterns. From a low level of presence (Chan et al., 2004), the HSP70 expression in the ventrolateral medulla underwent a significant and progressive elevation during Phases I and II endotoxemia, followed by a return to

baseline during Phase III (Fig. 5E). On the other hand, HSP90 expression remained relatively constant at all phases of experimental endotoxemia (Fig. 5F). The level of HSP70 or HSP90 in the RVLM of saline-controls was stable, and was comparable to sham-controls. Western blot analysis using an antiserum that recognizes selectively the inducible form of HSP70, but not HSC70, or an anti-HSP90 antiserum that recognizes both HSP86 and HSP84, essentially confirmed these observations (Fig. 6). There was a progressive and significant augmentation of HSP70 expression in the ventrolateral medulla during Phases I and II endotoxemia, followed by a decline towards baseline during Phase III. The level of HSP90 again remained constant during the entire course of experimental endotoxemia.

The Augmented HSP70 Expression in the Ventrolateral Medulla During Endotoxemia Involves De Novo Synthesis. To delineate whether the augmented HSP70 expression in the ventrolateral medulla during experimental endotoxemia entailed *de novo* synthesis, animals were pretreated with microinjection of the transcription inhibitor, actinomycin D (5 nmol) or the translation inhibitor, cycloheximide (20 nmol) bilaterally into the RVLM, 1 h prior to intravenous administration of LPS (30 mg/kg). Western blot analysis showed that animals that received 0.1% DMSO or 50% EtOH pretreatment exhibited typically the progressive augmentation in HSP70 protein level in the ventrolateral medulla during Phases I and II endotoxemia (Fig. 7A). However, this increase in HSP70 expression was significantly blunted on pretreatment with actinomycin D (Fig. 7A). Animals that were pretreated with cycloheximide succumbed to endotoxemia within 10 min after LPS administration without manifestation of the phasic cardiovascular responses or the surge in HSP70 expression in the ventrolateral medulla (Fig. 7B). Both pretreatments or their vehicle controls did not affect the HSP90 expression in the ventrolateral medulla in saline- or LPS-treated animals (Fig. 7C,D). Likewise, neither

actinomycin D, cycloheximide (data not shown) or their respective solvent (Fig. 7A,C) affected HSP70 or HSP90 level in the ventrolateral medulla of saline-control rats.

The Augmented HSP70 Expression in the Ventrolateral Medulla Confers Neuroprotection During Endotoxemia. We employed two loss-of-function manipulations (immuno-neutralization and gene knockdown) to establish a causative relationship between the augmented HSP70 expression in the ventrolateral medulla and survival from fatal experimental endotoxemia. At the intravenous dose (30 mg/kg) we used, LPS elicited approximately 60% fatality within 240 min after administration (Fig. 8). Pretreatment with microinjection into the bilateral RVLM of an anti-HSP70 antiserum (1:20) resulted in 100% mortality by 150 min after injection of LPS (Fig. 8A). Similarly, all animals pretreated with local application into the bilateral RVLM of an antisense *hsp70* oligonucleotide (50 pmol), which knocks down selectively *hsp70*, but not *hsc70* gene (Robertson et al., 1999), died 160-170 min after the induction of endotoxemia; along with an antagonism of the augmented HSP70 expression in the ventrolateral medulla during Phases I and II endotoxemia (Fig. 9). On the other hand, pretreatments with normal mouse serum, or sense or scrambled *hsp70* oligonucleotide (50 pmol) were ineffective (Fig. 8A). Likewise, immuno-neutralization using the same anti-HSP90 antiserum employed in Western blot analysis, or gene knockdown using an antisense *hsp90* oligonucleotide that acts selectively on HSP84 (Zucchi et al., 2002), in the RVLM, similar to normal mouse serum (1:20), or sense or scrambled *hsp90* oligonucleotide (50 pmol), did not affect the LPS-induced mortality rate (Fig. 8B).

The Augmented HSP70 Expression in the Ventrolateral Medulla Confers Cardiovascular Protection During Endotoxemia. We recently reported (Chan et al., 2004) that HSP70 induced in the RVLM by a prior hyperthermic heat shock confers

cardiovascular protection during experimental endotoxemia. It thus is of interest to delineate whether the augmented HSP70 expression in the ventrolateral medulla during endotoxemia may also antagonize the LPS-induced cardiovascular depression, leading to our observed neuroprotection against fatality. Based again on a loss-of-function approach, we observed that pretreatment by microinjection into the bilateral RVLM of an anti-HSP70 antiserum (1:20; Fig. 3, left column) or an antisense *hsp70* oligonucleotide (50 pmol; Fig. 3, right column) significantly potentiated the elicited hypotension or bradycardia, and blunted the increase in power density of LF or VLF component of the SAP signals during the significantly shortened Phase II endotoxemia. It is intriguing to note that, comparable to survival rate (Fig. 8), all these animals succumbed to fatal endotoxemia 140-160 min after LPS administration. Again, pretreatments with normal mouse serum (1:20; Fig. 3, left column) or sense or scrambled *hsp70* oligonucleotide (50 pmol; Fig. 3, right column) were ineffective. Immuno-neutralization or gene knockdown of HSP90 in the RVLM also did not affect the LPS-induced cardiovascular depressions (Fig. 4).

The Augmented HSP70 Expression in the Ventrolateral Medulla Enhances NOS I/PKG Signaling Pathway During Endotoxemia. We reported previously that the tonically active NOS I in the RVLM is responsible for the sympathoexcitatory cardiovascular actions of the endogenous NO at this medullary site (Chan et al., 2001b), and confers cardiovascular protection possibly via activation of the soluble guanylyl cyclase/cGMP/PKG cascade (Chan et al., 2005). It is thus intriguing to note that whereas the HSP70 level in the ventrolateral medulla underwent a progressive augmentation during Phases I and II, and a decline towards baseline during Phase III endotoxemia (Figs. 5 and 6), NOS I expression in the RVLM was previously found (Chan et al., 2001a) to be maintained until its significant reduction during Phase III endotoxemia. Whether these temporally correlated changes in HSP70 and NOS I in the ventrolateral medulla are causatively related were evaluated in the present study

(Fig. 9). We first ascertained that the temporal changes of HSP70 and NOS I expression in the same sample of ventrolateral medulla indeed took place during experimental endotoxemia. Western blot analysis revealed that animals that received pretreatment via microinjection of aCSF bilaterally into the RVLM exhibited a progressive augmentation of HSP70 expression, alongside maintained NOS I or PKG level in the ventrolateral medulla during Phases I and II, to be followed by a significant reduction in HSP70, NOS I or PKG expression during Phase III endotoxemia. Pretreating animals by microinjection bilaterally into the RVLM of an antisense *hsp70* oligonucleotide (50 pmol) blunted significantly the LPS-induced surge in HSP70 level at the ventrolateral medulla. Intriguingly, this pretreatment also elicited a significant and progressive down-regulation of NOS I or PKG in the ventrolateral medulla that commenced at Phase I endotoxemia (Fig. 9). With the exception of a significantly reduced baseline expression, pretreatment with an antisense *hsp90* oligonucleotide (50 pmol) exerted no discernible effects on the temporal changes in HSP70, NOS I or PKG levels in the ventrolateral medulla during endotoxemia. Likewise, pretreatments with sense or scrambled *hsp70* or *hsp90* oligonucleotide (50 pmol) were ineffective against NOS I or PKG level (data not shown), and antisense *hsp70* oligonucleotide (50 pmol) pretreatment did not affect HSP90 expression (Fig. 9).

The Augmented HSP70 Expression in the Ventrolateral Medulla Also Inhibits NOS II/Peroxynitrite Cascade During Endotoxemia. We reported previously that experimental endotoxemia is accompanied by a progressive augmentation in both molecular synthesis and functional expression of NOS II (Chan et al., 2001a), followed by the formation of the cytotoxic substance, peroxynitrite via a reaction between NO and superoxide anion (Chan et al., 2002b) in the RVLM. Subsequent experiments (Chan et al., 2004) showed that HSP70 induced in the RVLM by a prior heat shock confers cardiovascular protection by down-regulating NOS II expression in this medullary site. It thus is of interest to delineate whether the augmented HSP70

expression in the ventrolateral medulla during endotoxemia may also antagonize the LPS-induced up-regulation of NOS II. Figure 9 demonstrated that, in animals that were pretreated by microinjection of an antisense *hsp70* oligonucleotide (50 pmol) into the bilateral RVLM, the progressive elevations of NOS II or nitrotyrosine (marker for peroxynitrite) in the ventrolateral medulla over the course of experimental endotoxemia was significantly potentiated. Again, pretreatment with an antisense *hsp90* oligonucleotide (50 pmol) exerted no discernible effects on the temporal changes in HSP70, NOS II or nitrotyrosine levels in the ventrolateral medulla during experimental endotoxemia. Likewise, pretreatments with sense or scrambled *hsp70* or *hsp90* oligonucleotide (50 pmol) were ineffective against NOS II or nitrotyrosine level (data not shown), and antisense *hsp70* oligonucleotide (50 pmol) pretreatment did not affect HSP90 expression (Fig. 9).

Discussion

Based on a rat model that closely resembles the clinical conditions of systemic inflammatory response syndrome, the present study provided the novel demonstration of an augmented protein level of HSP70 in the ventrolateral medulla during Phases I and II endotoxemia. We further showed that this up-regulated HSP70, which resulted from *de novo* synthesis, conferred neuroprotection by preventing cardiovascular depression via enhancing the NOS I/PKG signaling pathway and inhibiting the NOS II/peroxynitrite cascade in the RVLM. On the other hand, HSP90 did not alter its expression in the ventrolateral medulla during the course of experimental endotoxemia, nor play a protective role in this model of endotoxemia.

One notion derived from the conflicting role of HSP70 or HSP90 in apoptosis (Galea-Lauri et al., 1996; Meriin et al., 1998; Robertson et al., 1999; Wagstaff et al., 1999; Lee et al., 2001; Lopez-Maderuelo et al., 2001) is that whether a HSP family member is pro- or anti-apoptotic depends on the cell type or the stressor. The present

study provided credence to this notion by demonstrating a differential neuroprotective role for HSP70 and HSP90 in the RVLM against fatal endotoxemia. We found that whereas both HSPs are present in the proteomic map of the ventrolateral medulla, complementary data from proteomic and Western Blot analyses revealed that only HSP70 manifested a progressive augmentation in its expression level during Phases I and II endotoxemia. More importantly, despite its short duration (160-180 min), the induced HSP70 expression in the RVLM conferred neuroprotection against fatal endotoxemia. Our results showed that functional blockade of HSP70 in the RVLM by immuno-neutralization using an anti-HSP70 antiserum that recognizes selectively the inducible form of HSP70 (Chan et al., 2002a), or prevention of synthesis by gene knockdown using an antisense *hsp70* oligonucleotide that acts selectively on the *hsp70* gene (Robertson et al., 1999), exacerbated mortality and potentiated the cardiovascular depression during experimental endotoxemia. It is intriguing to note that augmentation of HSP70 expression in the ventrolateral medulla that peaks 24 h after exposing animals to a brief hyperthermic heat shock is also causatively and temporally related to antagonism of the circulatory suppression during endotoxemia (Chan et al., 2004). Together, these observations indicated that regardless of the means of induction or the duration of expression, an augmented HSP70 level in the ventrolateral medulla confers neuroprotection against fatal endotoxemia via prevention of cardiovascular depression.

That HSP90 did not play a neuroprotective role in our model of endotoxemia may not be confounded by the non-selectivity of the anti-HSP90 antiserum or antisense *hsp90* oligonucleotide used is supported by two observations. First, both proteomic and Western blot analyses demonstrated that HSP90 expression in the ventrolateral medulla remained constant during the course of experimental endotoxemia. Second, pretreatment with an anti-HSP90 antiserum that recognizes both HSP86 and HSP84, or an antisense *hsp90* oligonucleotide that knocks down selectively HSP84 (Zucchi et al., 2002), produced complementary results on cardiovascular depression or mortality during fatal endotoxemia.

Our observations with actinomycin D or cycloheximide pretreatment showed that the elevated HSP70 expression during Phases I and II was the result of *de novo* synthesis. The ability of a cell to rapidly change its gene expression pattern in response to extracellular signals usually involves modulation of the activity of pre-existing transcription factors, a major mechanism of which is protein phosphorylation (Hunter, 2000). In this regard, heat shock transcription factor 1 (HSF1) is known to regulate *hsp* induction in response to stress, and HSP70 expression is attributable to phosphorylation of HSF1 at serine 230 (Holmberg et al., 2001, Pirkkala et al., 2001). Overexpression of HSP70 inhibits phosphorylation of HSF1 in the nucleus at serine residues (Mosser et al., 1993; Pirkkala et al., 2001). 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 (Mosser et al., 1993), may also account for our observation that HSP70 expression returned to baseline during Phase III endotoxemia.

Our laboratory demonstrated recently (Chang et al., 2003) that whereas all three NOS isoforms are expressed in the ventrolateral medulla at both mRNA and protein levels, only NOS I and II are present in RVLM neurons. Furthermore, physiological regulation of sympathetic vasomotor outflow by the endogenous NO at the RVLM is determined by a balance between the tonically active NOS I and NOS II (Chan et al., 2001b). We propose that, under physiological conditions, the prevalence of NOS I over NOS II activity at the RVLM and the associated dominance of sympathoexcitation over sympathoinhibition underlie the maintenance of sympathetic vasomotor outflow and stable SAP by the endogenous NO in the RVLM (Chan et al., 2001b). On the other hand, a tilt towards the progressive augmentation in molecular synthesis and functional expression of NOS II in the RVLM underlies the cardiovascular depression seen during pathological conditions such as endotoxemia (Chan et al., 2001a). It is therefore intriguing that the present study demonstrated that HSP70 may confer neuroprotection by regulating cardiovascular functions during endotoxemia via modulating NOS I or II expression in the RVLM.

Low concentration of NO generated by NOS I in the RVLM increases sympathetic vasomotor outflow (Chan et al., 2001b) by exciting sympathetic premotor neurons in this medullary site via a cGMP/PKG-dependent facilitation of presynaptic glutamate release (Huang et al., 2003). It is therefore interesting to observe that the progressive augmentation of HSP70 expression during Phases I and II endotoxemia was associated with maintained NOS I or PKG level in the ventrolateral medulla, alongside an increase in vasomotor tone during Phase II. Significant reduction in NOS I or PKG expression took place only during Phase III when HSP70 returned to baseline level and when significant and severe cardiovascular depression ensued. Results from antisense *hsp70* oligonucleotide pretreatment further confirmed that these temporally correlated cellular (HSP70, NOS I or PKG expression; Fig. 9) and cardiovascular events (Fig. 3) are causatively related. It follows that the up-regulated HSP70 may confer neuroprotection against fatal endotoxemia by preventing cardiovascular depression via enhancement of the NOS I/PKG signaling pathway in the RVLM. The potential candidates whereby HSP70 exerts this regulatory effects on NOS I gene expression include the Oct-2 transcription factor (Deans et al., 1996) and members of the Sp and ZNF families of transcription factors (Saur et al., 2002).

High concentration of NO generated by NOS II decreases sympathetic vasomotor outflow (Chan et al., 2001b) by inhibiting RVLM neurons via a peroxynitrite-mediated reduction of presynaptic glutamate release (Huang et al., 2004). Intriguingly, the present study showed that whereas HSP70 level returned to baseline during Phase III endotoxemia, the NOS II and nitrotyrosine levels were further enhanced, alongside significant reduction in SAP, HR or sympathetic vasomotor outflow. Results from our antisense *hsp70* oligonucleotide pretreatment again confirmed that these temporally correlated cellular (HSP70, NOS II or nitrotyrosine expression; Fig. 9) and cardiovascular events (Fig. 3) are causatively related. HSP70 inhibits NOS II gene expression by transcriptional mechanisms that involve the NF- κ B/I κ B pathway (Feinstein et al., 1996; Chan et al., 2004). HSP70 also protects macrophages against peroxynitrite cytotoxicity (Hirvonen et al., 1996; Szabo et al.,

1996). It follows that the up-regulated HSP70 may also confer neuroprotection by preventing cardiovascular depression via holding NOS II expression and peroxynitrite formation at the RVLM in check. The return of HSP70 level to baseline, the reduction in NOS I/PKG expression and augmentation of the NOS II/peroxynitrite level in the RVLM, the significant circulatory depression and the ensuing fatality during Phase III endotoxemia, together with the exacerbated mortality and potentiated cardiovascular depression by loss-of-function manipulations of HSP70, provided ample credence to this notion.

Brain death is associated with the permanent termination of essentially all brain functions, particularly the autonomic cardiovascular regulatory mechanisms in the brain stem (Anonymous, 1981). It is thus intriguing that the neuroprotective action of HSP70 against fatal endotoxemia was elicited on the RVLM, a neural substrate that is intimately related to sympathetic vasomotor tone (Ross et al., 1984) and whose neuronal activity is closely associated with a “life-and-death” signal (Kuo et al., 1997) that is drastically reduced or lost before patients succumbed to systemic inflammatory response syndrome (Yien et al., 1997). Since death represents the end of existence for an individual organism, we propose that multiple “pro-life” and “pro-death” programs must be engaged during the progression towards brain stem death. The present study provided novel findings to indicate that HSP70 in the RVLM may be one of those “pro-life” programs. We demonstrated that *de novo* synthesis of HSP70 in the RVLM during experimental endotoxemia confers neuroprotection against fatality by preventing cardiovascular depression via enhancing the NOS I/PKG signaling pathway and inhibiting the NOS II/peroxynitrite cascade in this crucial neural substrate. We also showed that, although present in the RVLM, HSP90 does not appear to play a protective role in endotoxemia. This information should provide further insights on the etiology of brain stem death, and offer new directions for the development of therapeutic strategy against sepsis.

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Fig. 1. Representative silver-stained 2-dimensional electrophoresis gel of the ventrolateral medulla showing the location of the protein spot for HSP70 and HSP90. Results are representative of five individual samples obtained from the ventrolateral medulla under basal conditions.

Fig. 2. Tryptic peptide spectrum of HSP70 or HSP90 generated by MALDI-TOF mass spectrometry.

Fig. 3. Temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR), or power density of the low-frequency (LF) or very low-frequency (VLF) component of SAP signals in rats that received pretreatment by microinjection into the bilateral RVLM of aCSF, normal mouse serum (NEM; 1:20) or anti-HSP70 antiserum (HSP70Ab; 1:20) (left column); or aCSF, scrambled (SC; 50 pmol), sense (SON; 50 pmol) or antisense (ASON; 50 pmol) *hsp70* oligonucleotide (right column); followed by i.v. administration of saline or LPS (30 mg/kg). Values are mean \pm SE, n = 7-8 animals per experimental group. * $p < 0.05$ vs aCSF+saline group and $^+p < 0.05$ vs aCSF+LPS group at corresponding time-points in the Scheffé multiple-range test.

Fig. 4. Temporal changes in MSAP, HR, or power density of the LF or VLF component of SAP signals in rats that received pretreatment by microinjection into the bilateral RVLM of aCSF, NEM (1:20) or anti-HSP90 antiserum (HSP90Ab; 1:20) (left column); or aCSF, scrambled (SC; 50 pmol), sense (SON; 50 pmol) or antisense (ASON; 50 pmol) *hsp90* oligonucleotide (right column); followed by i.v. administration of saline or LPS (30 mg/kg). Values are mean \pm SE, n = 7-8 animals per experimental group. * p < 0.05 vs aCSF+saline group at corresponding time-points in the Scheffé multiple-range test.

Fig. 5. Representative silver-stained 2-dimensional electrophoresis gels of the ventrolateral medulla obtained from sham-control animals (A) or during Phases I (B), II (C) or III (D) endotoxemia; or the relative contribution of HSP70 (E) or HSP 90 (F) to the total spot volume in the proteomic map (pI: 3-10 and Mr: 14.4-94 kDa) of the RVLM in sham-control animals, or during the 3 phases of endotoxemia or corresponding time intervals after animals received i.v. administration of LPS (30 mg/kg) or saline. Values are mean \pm SE, n = 7-8 animals per experimental group. * p < 0.05 vs sham-control group (B) or corresponding time-interval in saline group.

Fig. 6. Representative gels (inset) or temporal changes in the percentage of HSP70 or HSP90 relative to β -actin protein, detected in the ventrolateral medulla of sham-control animals, or during the 3 phases of endotoxemia or corresponding time intervals after animals received i.v. administration of LPS (30 mg/kg) or saline. Values are mean \pm SE of quadruplicate analyses from 7-8 animals per experimental group. * p < 0.05 vs sham-control group (B) or corresponding time-interval in saline group.

Fig. 7. Representative gels (inset) or temporal changes in the percentage of HSP70 (A,B) or HSP90 (C,D) relative to β -actin protein, detected in the ventrolateral medulla of rats that received pretreatment by microinjection into the bilateral RVLM of actinomycin D (ActD; 5 nmol) (A,C) or cycloheximide (CHX; 20 nmol) (B,D); followed by i.v. administration of saline or LPS (30 mg/kg). Since comparable results were obtained from 0.1% DMSO or 50% EtOH pretreatment, those data are denoted as vehicle (Veh) group for clarity. Note also that animals pretreated with cycloheximide succumbed rapidly to endotoxemia without manifestation of the phasic cardiovascular responses. Values are mean \pm SE of quadruplicate analyses from 7-8 animals per experimental group. * P < 0.05 vs Veh+saline group, and ⁺ p < 0.05 vs Veh+LPS group at corresponding time-intervals in the Scheffé multiple-range test.

Fig. 8. Survival rate of rats that received pretreatment by microinjection into the bilateral RVLM of aCSF, NMS (1:20), HSP70Ab (1:20), scrambled (SC; 50 pmol), sense (SON; 50 pmol) or antisense (ASON; 50 pmol) *hsp70* oligonucleotide (A); or aCSF, NMS (1:20), HSP90Ab (1:20), scrambled (SC; 50 pmol), sense (SON; 50 pmol) or antisense (ASON; 50 pmol) *hsp90* oligonucleotide (B); followed by i.v. administration of saline or LPS (30 mg/kg). Each group contains 7-8 animals at the beginning of the experiment.

Fig. 9. Representative gels (inset) or temporal changes in the percentage of HSP70, HSP90, NOS I, PKG, NOS II or nitrotyrosine (NT) relative to β -actin protein, detected in the ventrolateral medulla of rats that received pretreatment by microinjection into the bilateral RVLM of aCSF, or antisense (ASON; 50 pmol) *hsp70* or *hsp90* oligonucleotide; followed by i.v. administration of LPS (30 mg/kg). Results from medullary tissues in sham-control animals, or 150 min after animals received i.v. administration of saline were also included for comparison. Values are mean \pm SE of quadruplicate analyses from 7-8 animals per experimental group. * $p < 0.05$ vs sham-control (B) or saline group, and $^+p < 0.05$ vs aCSF+LPS group at corresponding time-intervals in the Schaffer multiple-range test. ND denotes below detection limit.

Table 1. Sequences of antisense (AODN), sense (SODN) or scrambled (SC) *hsp70* or *hsp90* oligonucleotides used in the present study.

<i>hsp70</i>	AODN: 5'-CACCTTGCCGTGCTGGAA-3'
	SODN: 5'-TTCCAGCACGGCAAGGTG-3'
	SC: 5'-TGGATCCGACATGTCAGT-3'
<i>hsp90</i>	AODN: 5'-CTCCACCTCTTCCTCTCCAT-3'
	SODN: 5'-ATGGAGAGGAAGAGGTGGAG-3'
	SC: 5'-TCTCACCCCTTCCCTCATTC-3'

Table 2. Identification of HSP70 or HSP90 based on MS-Fit and MASCOT search programs.

Shown are peptides with observed m/z values and tryptic cutting sites that matched the tryptic peptide spectra of these two HSPs. The amino acid sequence of all matched peptides in HSP70 (left column) or HSP90 (right column) is listed, or underlined in the internal sequence.

HSP70

MS-Fit Search Results

Submitted	m/z	MH ⁺	Delta	Start	End	Missed	Database Sequence
Matched			ppm			Cleavages	
1029.4768	1029.5443	-66	647	654	1	(K) LFEMAYKK (M)	
1242.6325	1242.6806	-39	207	218	0	(K) DAGQISGLNVLNR (V)	
1290.6298	1290.6806	-39	395	405	0	(K) VQQTVDLFGGR (A)	
1446.7309	1446.7626	-22	378	391	0	(K) SDIGEVILVGGMTR (M)	
1476.6941	1476.7334	-27	86	99	0	(R) TTPSVVAFTPDGER (L)	
1489.7609	1489.8379	-52	349	361	1	(R) AQFEGIVTDLIKR (T)	
1568.7232	1568.7709	-30	108	121	0	(R) QAVTNPNTFYATK (R)	
1592.8530	1592.7743	49	174	187	1	(K) MKETAENYLGHATAK (N)	
1592.8530	1592.9528	-63	499	513	0	(K) LLQGFITLIGPPAPR (G)	
1645.7476	1645.8801	-81	219	234	0	(R) VINEPTAAALAYGLDK (S)	
1681.7564	1681.7835	-16	26	39	0	(R) HQDGWNGLSHEVFR (F)	
1690.7909	1690.8434	-31	293	307	1	(R) ETGVDLTKDNMALQR (V)	
1694.7971	1694.8502	-31	188	202	0	(K) NAVITVPAYFNDNSQR (Q)	
1706.7246	1706.8383	-67	293	307	1	(R) ETGVDLTKDNMALQR (V)	
1707.7745	1707.8455	-42	108	122	1	(R) QAVTNPNTFYATKR (L)	
1724.8105	1724.8720	-36	108	122	1	(R) QAVTNPNTFYATKR (L)	
1724.8105	1724.8720	-36	107	121	1	(K) RQAVTNPNTFYATK (R)	
1808.8442	1808.9030	-33	469	485	0	(K) SQVFSTAADGQTQVEIK (K)	
2055.9307	2055.9623	-15	266	284	0	(K) SQVFSTAADGQTQVEIK (K)	
2418.1721	2418.2186	-19	542	563	1	(R) SQVFSTAADGQTQVEIK (R)	

Mascot Search Results

Start	End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
86	-	1476.69	1475.69	1475.73	-0.04	0	TTPSVVAFTPDGER
108	-	1568.72	1567.72	1567.76	-0.05	0	QAVTNPNTFYATK
108	-	1724.81	1723.80	1723.86	-0.06	1	QAVTNPNTFYATKR
174	-	1592.85	1591.85	1591.77	0.08	1	MKETAENYLGHATAK
188	-	1694.80	1693.79	1693.84	-0.05	0	NAVITVPAYFNDNSQR
207	-	1242.63	1241.63	1241.67	-0.05	0	DAGQISGLNVLNR
219	-	1645.75	1644.74	1644.87	-0.13	0	VINEPTAAALAYGLDK
266	-	2055.93	2054.92	2054.95	-0.03	0	STNGDTFLGGEDFDQALLR
293	-	1690.79	1689.78	1689.84	-0.05	1	ETGVDLTKDNMALQR
349	-	1489.76	1488.75	1488.83	-0.08	1	AQFEGIVTDLIKR
369	-	2406.18	2405.17	2405.19	-0.02	1	AMQDAEVSKSDIGEVILVGGMTR
378	-	1446.73	1445.72	1445.75	-0.03	0	SDIGEVILVGGMTR
395	-	1290.63	1289.62	1289.67	-0.05	0	VQQTVDLFGGR
469	-	1808.84	1807.84	1807.90	-0.06	0	SQVFSTAADGQTQVEIK
542	-	2418.17	2417.16	2417.21	-0.05	1	EQVVIQSSGGLSKDDIENMVK
647	-	1029.48	1028.47	1028.54	-0.07	1	LFEMAYKK

No match to: 1251.68 1449.64 1475.70 1481.76 1487.65 1681.76
 1689.70 1691.75 1706.72 1707.77 1745.80 1981.92
 2056.90

Matched peptides are **underlined and shown in bold.**

1	MISASRAAAA	RLVGTASRS	PAAARHQDQW	NGLSHEAFRF	VSRRDYASEA
51	IKGAVVGDLD	GTTNSCVAVM	EKGQAKVLEN	AEGART TIPSV	VAFTPDGERL
101	VGMPAKROAV	TNPNTFYAT	KRLIGRRYDD	PEVQDKTKNV	PFKIVRASNG
151	DAWVEAHGKL	YSPQIGAFV	LMK MKETAEN	YLGHTAKNAV	ITVPAYFNDS
201	ORQATK DAGO	ISGLNVLRLV	NEPTAAALAY	GLDK SEDKVI	AVYDLGGGTF
251	DISILEIQKG	VFEVKSTNGD	FLGGEDFDQ	ALLRHIVKEF	KRETGVDLTK
301	DNMALOR VRE	AAEKAKCELS	SSVQTDINLP	YLTMDSVSGPK	HLNMLKTRAQ
351	FEIGIVTDLIK	RNIAPCQKAM	QDAEVSKSDI	GEVILVGGMT	EMPKVQOFTVQ
401	DLFGR APSKA	VNPDEAVIAG	AAIQGGVLAG	DVTDVLLLDV	TPLSLGIETL
451	GGVFTKLINR	NTTIPTKKSQ	VFSTAADGQT	QVEIK VCQGE	REMGADNKLL
501	QQFTLIGIPP	APRGPVQIEV	TFDIDANGIV	HVSAKDKGTG	REQOIVIOSS
551	GLSKDDIEN	MVK NAEKYAE	EDRRKERVE	AVNMAEGIIH	DTETKMEEFK
601	DQLPADECNK	LKEEISKMRE	LLARKDSETG	ENIRQAASSL	QQASLKLFEF
651	AVKK MASERE	GSSSSTGEQ	KEDQKEEKQ		

HSP90

MS-Fit Search Results

Submitted	m/z	MH ⁺	Delta	Start	End	Missed	Database Sequence
Matched			ppm			Cleavages	
829.4980	829.5300	-39	331	337	0	(R) ALLFIPR (R)	
901.4908	901.5260	-39	285	291	0	(K) TKPIWTR (N)	
1208.6078	1208.6315	-20	339	348	1	(R) APFDLFENK (K)	
1236.6202	1236.6377	-14	338	347	1	(R) RAPFDLFENK (K)	
1242.6057	1242.7058	-81	96	107	0	(K) ADLNNLGTIAK (S)	
1249.5990	1249.6177	-15	492	502	0	(K) EQVANSFAVER (V)	
1264.6347	1264.5012	106	613	623	0	(R) DNSTMGYMMAK (K)	
1311.5843	1311.5705	11	187	196	0	(K) EDQTEYLEER (R)	
1348.6628	1348.6650	-1.6	320	330	0	(K) HFSVEGQLEFR (A)	
1513.7943	1513.7862	5.3	379	392	0	(R) GVVSDPLNLSR (E)	
1527.7774	1527.7443	22	307	319	0	(K) SLTNDWEDHLAVK (H)	
1782.9536	1782.9503	1.9	625	639	0	(K) HLEINPDHPIVETLR (Q)	
1847.8093	1847.7976	6.4	292	306	0	(R) NPDDITQEYGEFYK (S)	
1911.0733	1911.0452	15	624	639	1	(K) HLEINPDHPIVETLR (Q)	
2015.0679	2015.0449	11	181	196	1	(K) VILHKEDQTEYLEER (R)	
2192.9568	2192.9406	7.4	457	475	0	(R) EQVANSFAVER (V)	
2255.9839	2255.9594	11	149	168	0	(K) HLEINPDHPIVETLR (Q)	

Mascot Search Results

Start	End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
96	-	1242.61	1241.60	1241.70	-0.10	0	ADLNNLGTIAK
149	-	2255.98	2254.98	2254.95	0.03	0	HNDDEQYAWESSAGGSFTVR
181	-	2015.07	2014.06	2014.04	0.02	1	VILHKEDQTEYLEER
187	-	1311.58	1310.58	1310.56	0.01	0	EDQTEYLEER
224	-	1589.86	1588.85	1588.67	0.18	1	EISDDEAEHEEKGEK
285	-	901.49	900.48	900.52	-0.03	0	TKPIWTR
292	-	1847.81	1846.80	1846.79	0.01	0	NPDDITQEYGEFYK
307	-	1527.78	1526.77	1526.74	0.03	0	SLTNDWEDHLAVK
320	-	1348.66	1347.66	1347.66	-0.00	0	HFSVEGQLEFR
331	-	829.50	828.49	828.52	-0.03	0	ALLFIPR
338	-	1236.62	1235.61	1235.63	-0.02	1	RAPFDLFENK
339	-	1208.61	1207.60	1207.62	-0.02	1	APFDLFENK
379	-	1513.79	1512.79	1512.78	0.01	0	GVVSDPLNLSR
457	-	2192.96	2191.95	2191.93	0.02	0	EQVANSFAVER
492	-	1249.60	1248.59	1248.61	-0.02	0	DNSTMGYMMAK
613	-	1264.63	1263.63	1263.49	0.13	0	EQVANSFAVER
624	-	1911.07	1910.07	1910.04	0.03	1	HLEINPDHPIVETLR
625	-	1782.95	1781.95	1781.94	0.00	0	HLEINPDHPIVETLR

No match to: 1235.61 1329.70 1364.72 1475.73 1541.75 1554.80
 1629.81 1645.72 1786.93 1794.82 1833.78 1909.99
 1915.05 1940.95 1951.96 2170.02 2211.12 2238.88
 2240.05

Matched peptides are **underlined and shown in bold.**

1	MPEEVHHGEE	EVETFAFQAE	IAQLMSLIN	TFYSNKEIFL	RELISNASDA
51	LDKIRYESLT	DPSKLDGSKG	LKDIHPNPQ	EATLTLVDTG	IGMT KADLN
101	NLGTIAK SGT	KAFMEALQAG	ADISMIQGFQ	VGFYSAYLVA	EKVVVITR HN
151	DDEQYAWESS	AGGSFTVRAD	HGEPIGRGTG	YLHFKEDQT	EYLEER RVKE
201	VVKKHSQFIG	YPTLYLEKE	RE EISDDEA	EEKGEK EEE	DKDEEEKPKI
251	EDVGSDEEDD	SGKDKKKTKT	KIKEKYIDQE	ELN KTKPIWT	RNPDDITQEE
301	YGEFYKSLTN	DWEDHLAVKH	FSGVEGQLEFR	ALLFIPRRAP	FDLFENK KKK
351	NNIKLYVRRV	FIMSDCDDL	PEYLNFRGV	VSDSDPLNLSR	SREML QQSK
401	LKVIKRNIVK	KCELEFSELA	EDKENYKIFY	EAFSKNLKLG	IHEDSTNRRR
451	LSLELLRYHTS	OSGDEMTSL	EYYSR MKETQ	KSIYITGES	KEQVANSFAV
501	ERVR KRGFEV	VYMTPEIDEY	CVQLKFEFDG	KLSVSVTKEG	LELPEDEEEK
551	KKMEESKARF	ENLCKLMKEI	LDKKVEKVTI	SNRLVSSPCC	IVTSTYGWTA
601	NMERIMKAQA	LRDNSTMGYM	MAKKHLEINP	DHPIVETLRQ	KAEADKNKKA
651	VKDLVLLFFE	TALSSLASHF	RRPKTHSNRI	YRMIKLGLGI	DEDEVTAEEP
701	SAAVPDEIPP	LEGDEDASRM	EEVD		

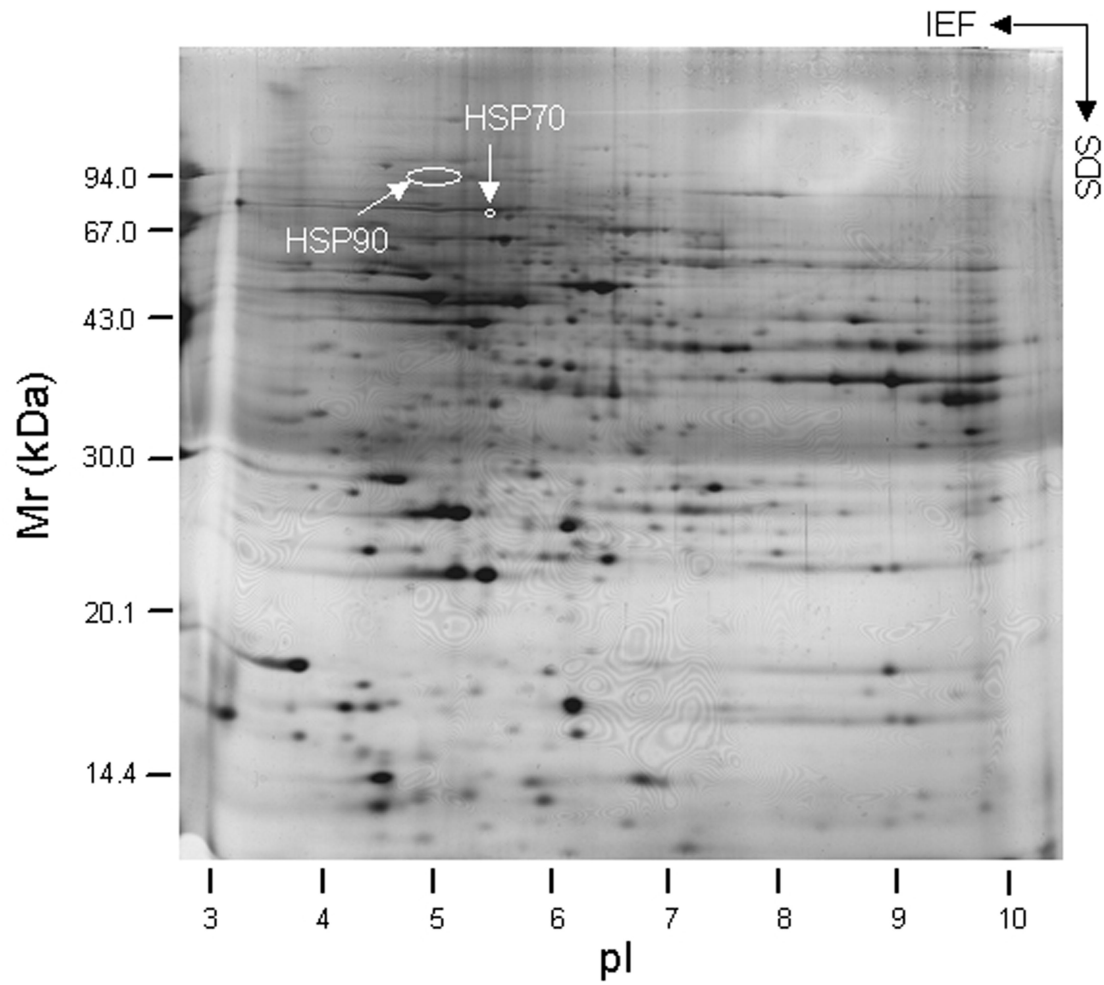


Figure 1

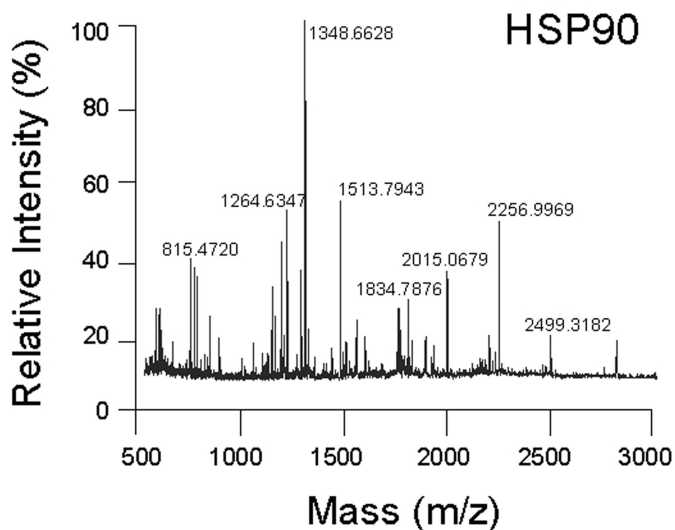
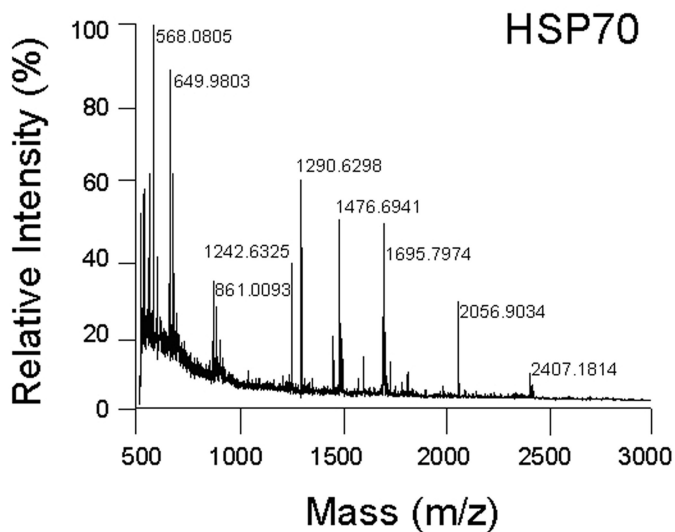


Figure 2

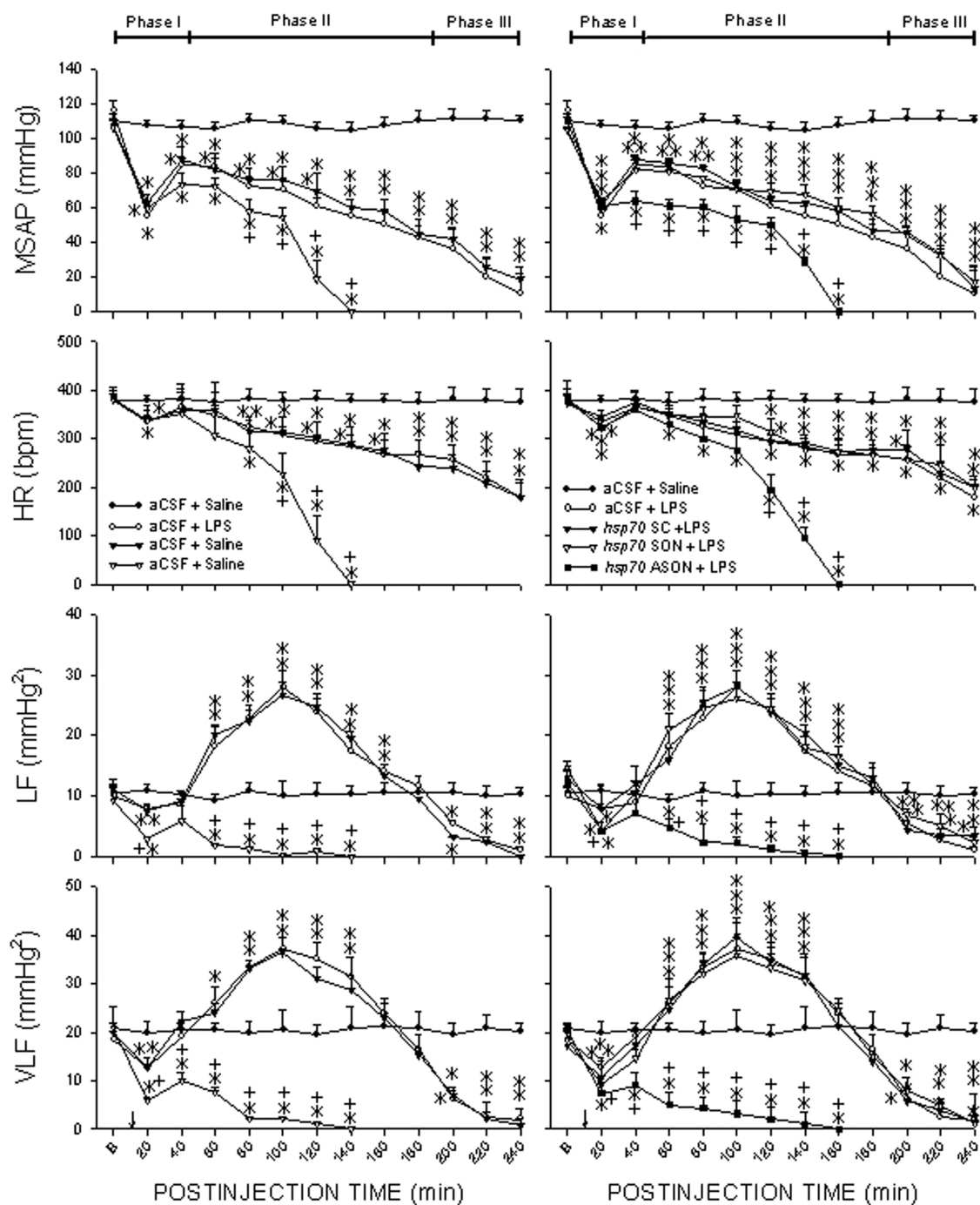


Figure 3

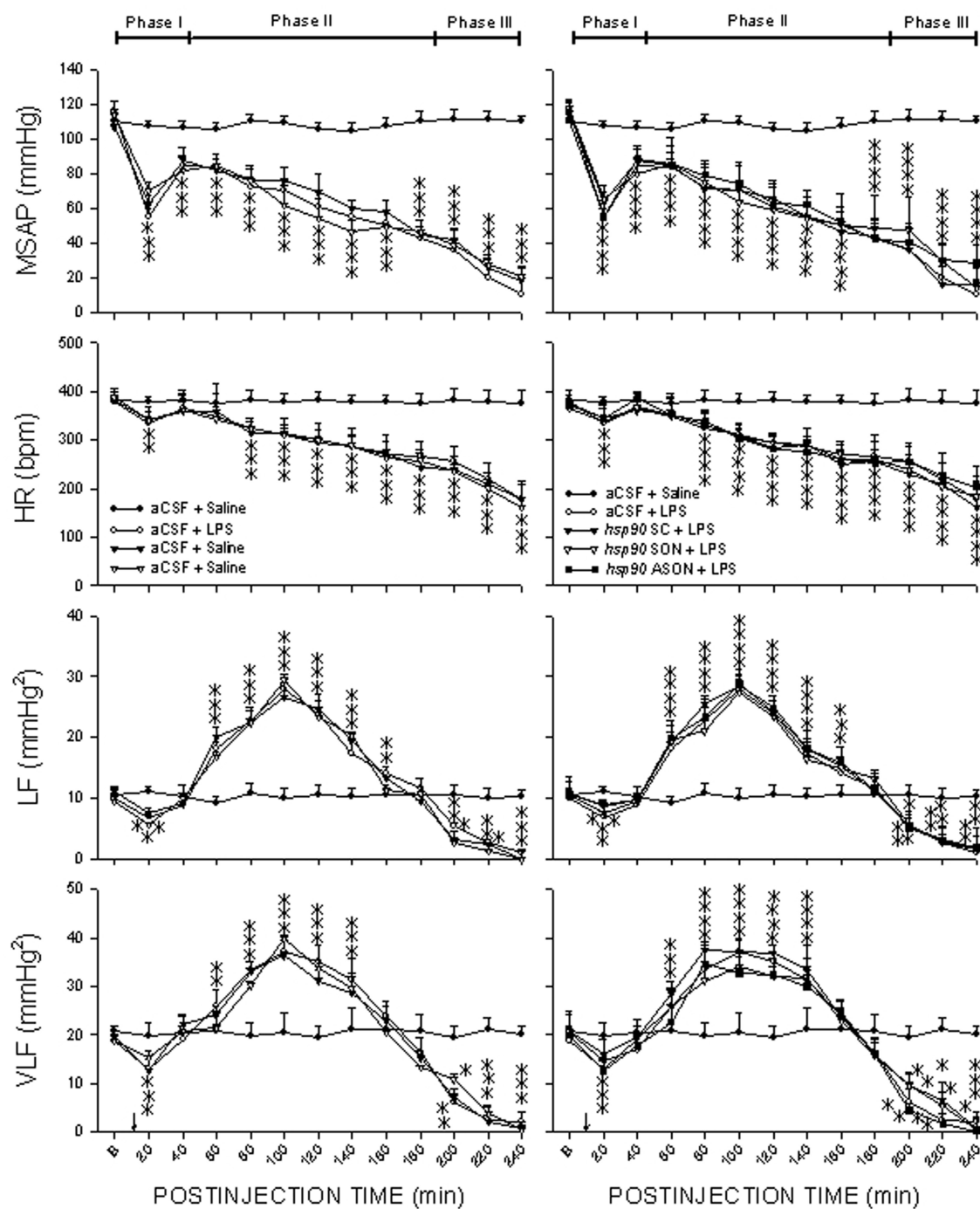


Figure 4

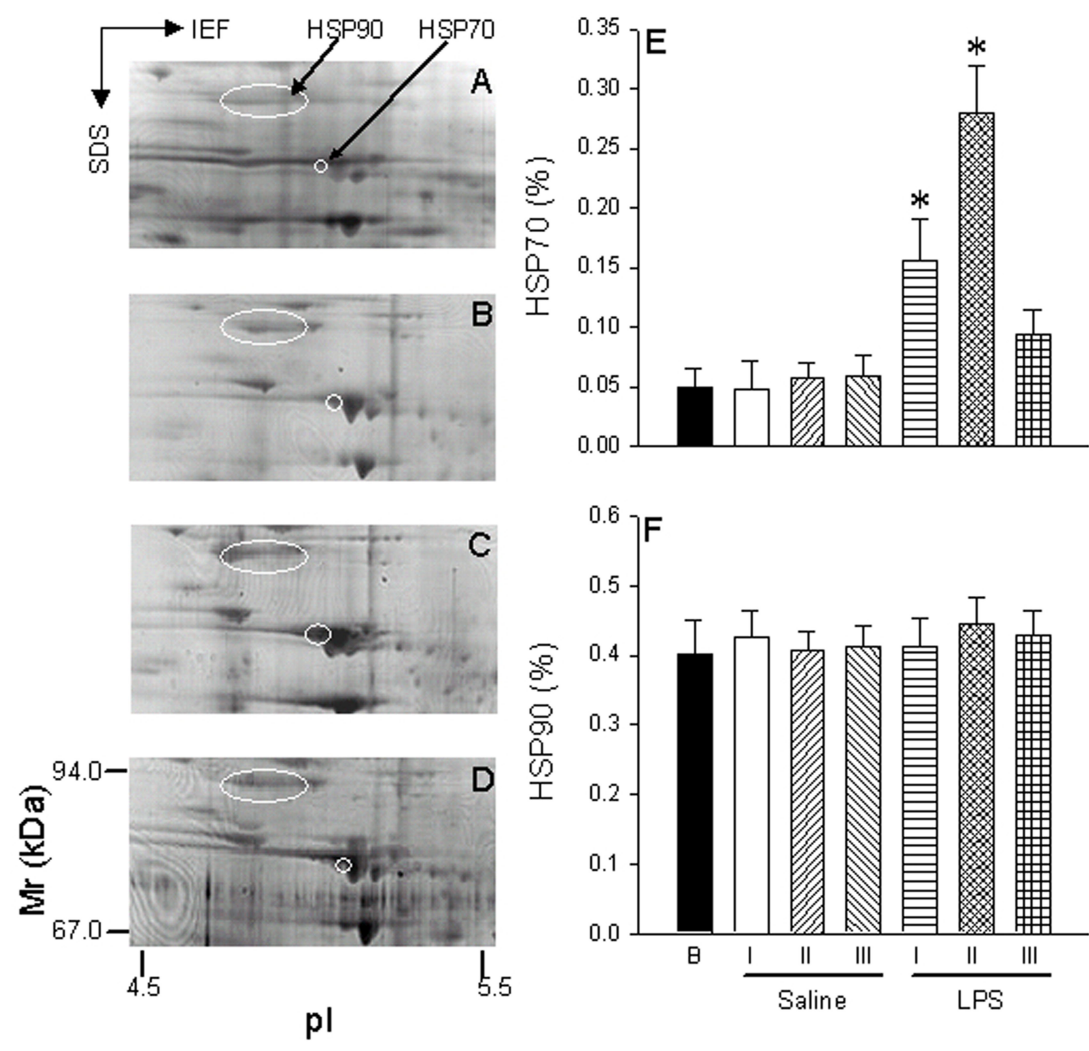


Figure 5

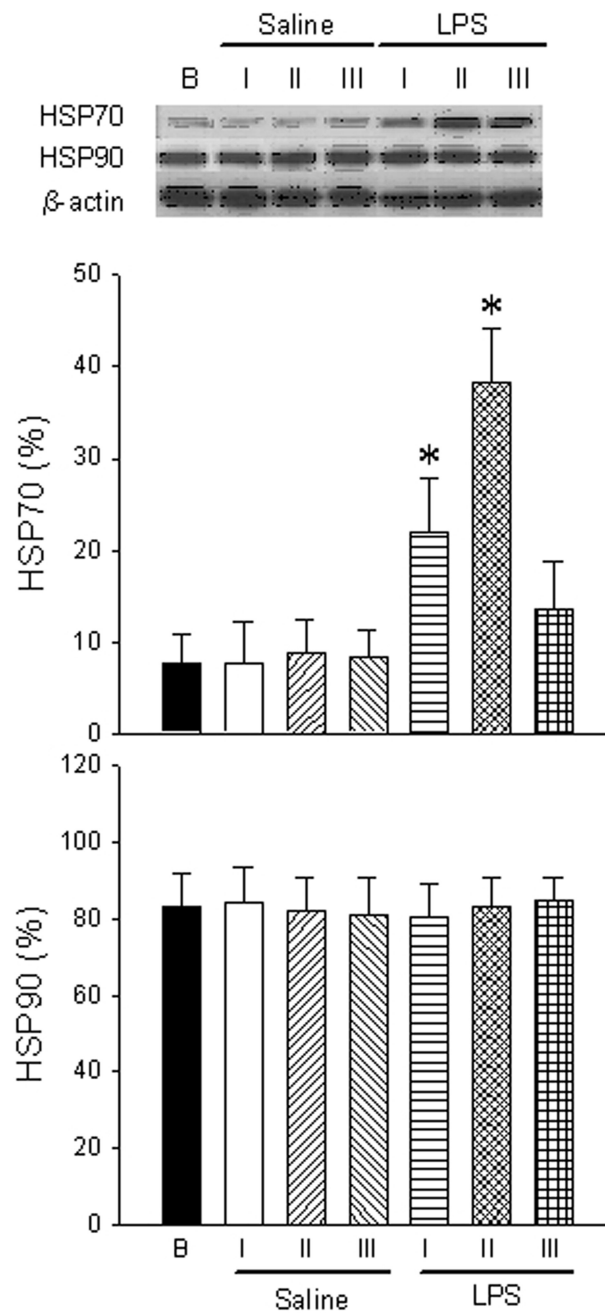


Figure 6

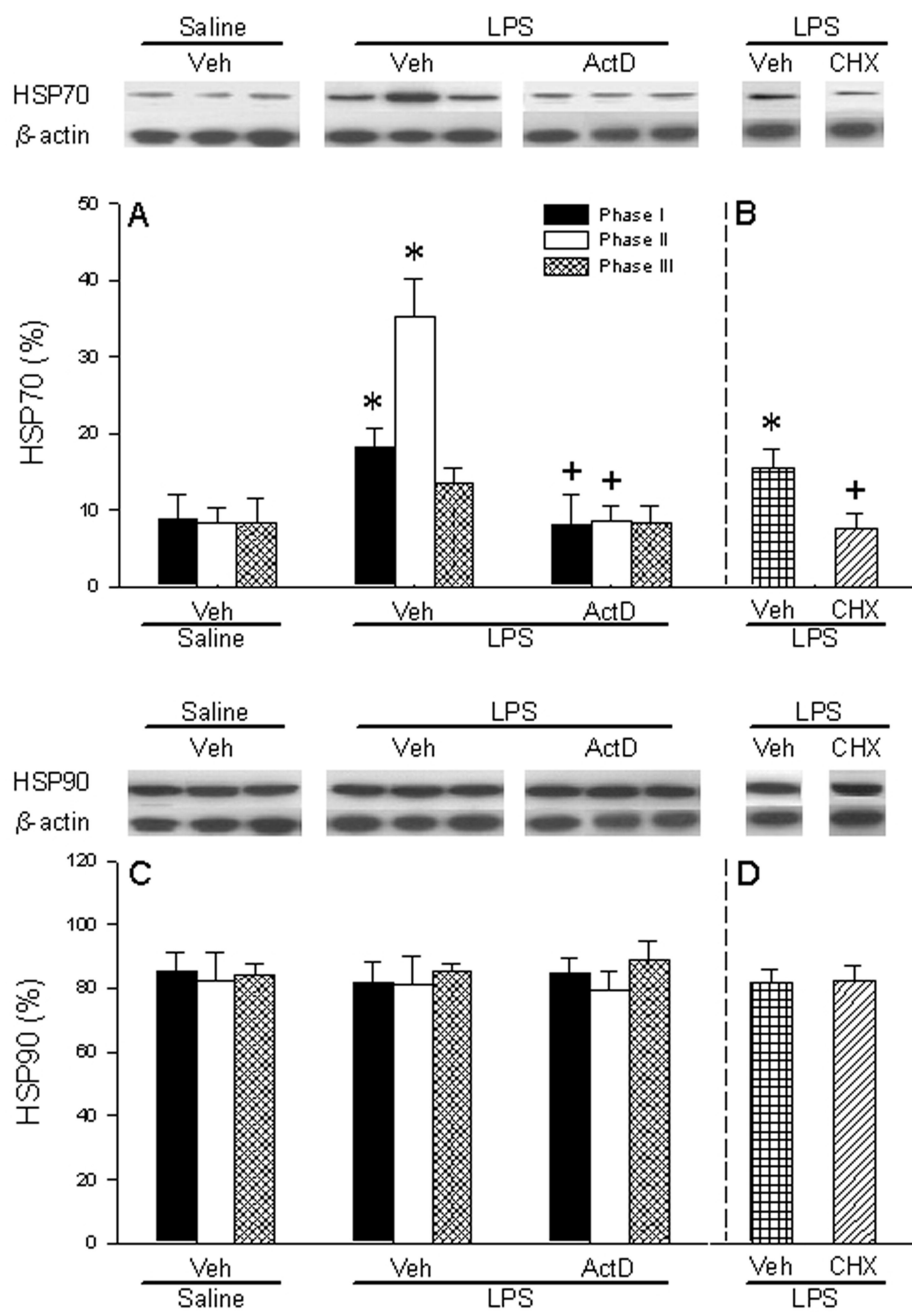


Figure 7

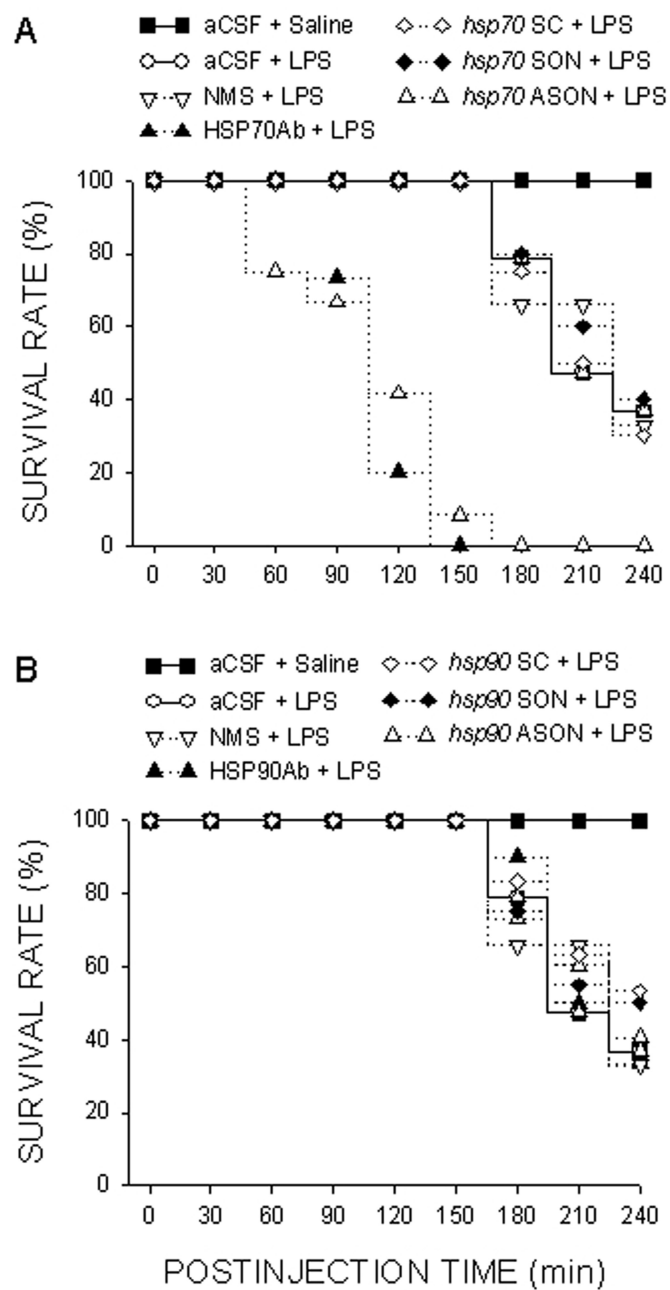


Figure 8

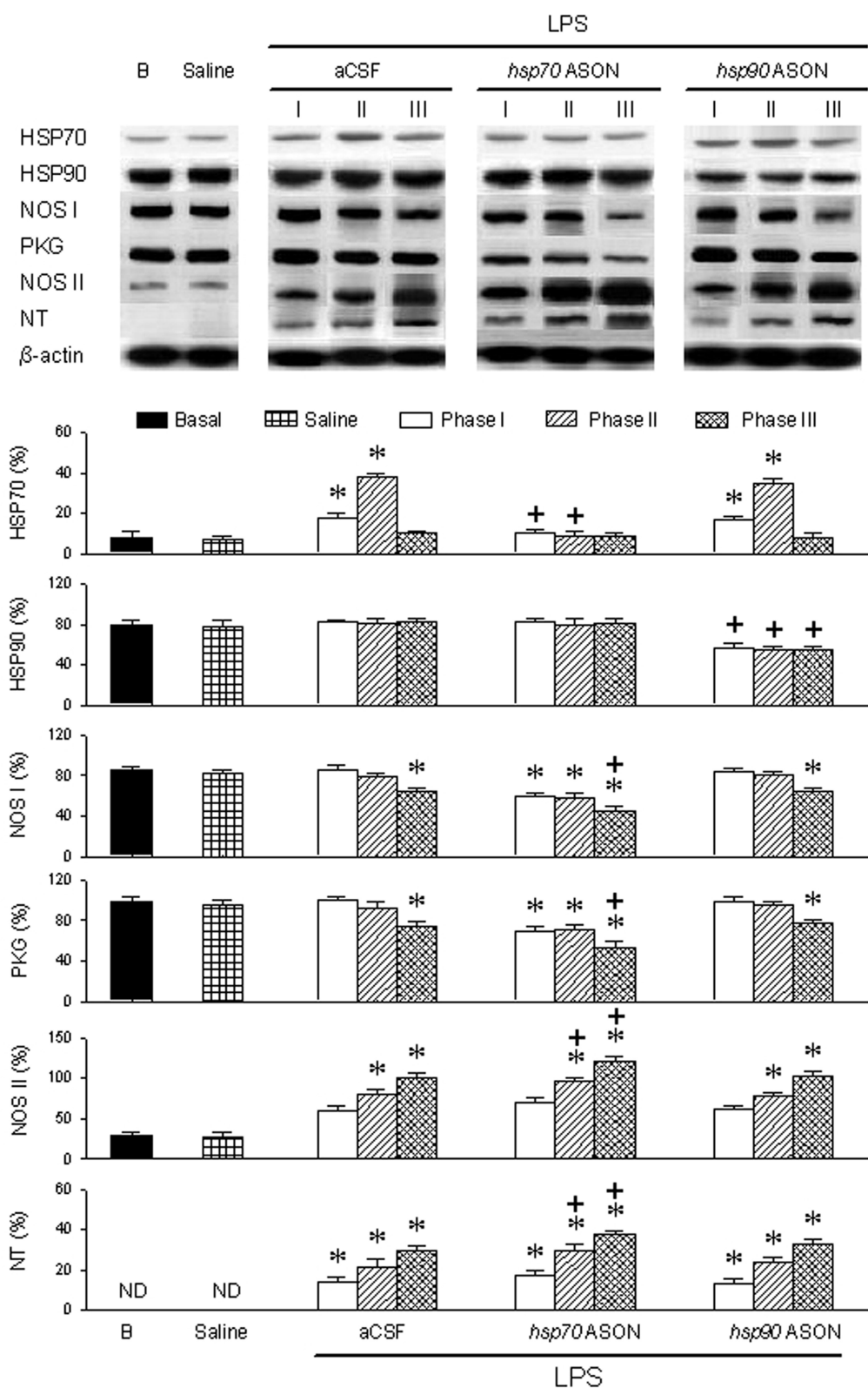


Figure 9