Heat Shock Protein 70 Confers Cardiovascular Protection During Endotoxemia via Inhibition of Nuclear Factor-κB Activation and Inducible Nitric Oxide Synthase Expression in the Rostral Ventrolateral Medulla

Julie Y.H. Chan, PhD; Chen-Chun Ou, MS; Ling-Lin Wang, MS; Samuel H.H. Chan, PhD

Background—Overproduction of nitric oxide (NO) by inducible NO synthase (iNOS) in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons are located, plays a pivotal role in the manifestation of fatal cardiovascular depression during endotoxemia. The iNOS gene is regulated transcriptionally by nuclear factor-κB (NF-κB) activation. The present study tested the hypothesis that heat shock protein 70 (HSP70) may confer protection against sepsis-induced circulatory fatality via inhibition of iNOS gene expression in the RVLM through prevention of NF-κB activation.

Methods and Results—Adult male Sprague-Dawley rats subjected to a brief hyperthermic heat shock (42°C for 15 minutes) exhibited significant upregulation of HSP70 in the RVLM. Brief heat shock preconditioning also significantly suppressed iNOS mRNA or protein surge and alleviated hypotension, bradycardia, and reduction in neurogenic sympathetic vasomotor activity manifested during experimental endotoxemia induced by intravenous administration of Escherichia coli lipopolysaccharide. An increase in DNA binding activity and nuclear translocation of transcription factor NF-κB were detected during endotoxemia. Heat shock preconditioning significantly decreased DNA binding activity of NF-κB, which was reversed by microinjection of an hsp70 antisense oligonucleotide bilaterally into the RVLM. Heat shock preconditioning also blocked inhibitory κB (IκB) kinase activity or degradation of IκB in the RVLM during endotoxemia.

Conclusions—We conclude that HSP70 confers protection against sepsis-related circulatory fatality via inhibition of iNOS gene expression in the RVLM through prevention of NF-κB activation.

Key Words: blood pressure cardiovascular diseases nitric oxide synthase shock signal transduction

Cardiovascular depression during sepsis remains a significant cause of morbidity and mortality.1 One well-known mediator of sepsis-induced circulatory failure at the peripheral vasculature is nitric oxide (NO) produced via activation of inducible NO synthase (iNOS) in macrophages.2,3 In the central nervous system, we demonstrated recently that by eliciting a reduction in sympathetic vasomotor outflow and arterial pressure,4,5 overproduction of NO by iNOS and formation of peroxynitrite by reacting NO with superoxide anion6 in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons are located,7 play a pivotal role in the fatal cardiovascular depression associated with endotoxemia.

The iNOS gene is regulated transcriptionally in part by nuclear factor-κB (NF-κB) activation.8 NF-κB is sequestered in the cytoplasm in an inactive state because of its association with inhibitory κB (IκB).9 Under stress conditions,10,11 phosphorylation of IκB by IκB kinase (IKK) occurs, which leads to degradation of IκB and disruption of the NF-κB/IκB complex. The dissociated NF-κB subsequently translocates from the cytoplasm to the nucleus, where this transcription factor binds to the κB promoter region of target genes, including iNOS.8,12 Accordingly, prevention of NF-κB activation inhibits iNOS expression.13–15

Heat shock (HS) response consists of expression of a family of highly conserved proteins known as heat shock proteins (HSPs).16 Thermal preconditioning induces expression of HSP70 and reduces iNOS expression elicited by bacterial endotoxin in various cell types.15,17,18 HS inhibits iNOS gene expression by transcriptional mechanisms that involve the NF-κB/IκB pathway.15,19,20 It follows that HSP70

Received May 15, 2004; revision received July 22, 2004; accepted July 27, 2004.

From the Department of Medical Education and Research (J.Y.H.C., C.C.O., L.L.W.), Kaohsiung Veterans General Hospital, Kaohsiung, and Center for Neuroscience (S.H.H.C.), National Sun Yat-sen University, Kaohsiung, Taiwan, Republic of China.

The online-only Data Supplement, which contains additional information about Methods, is available with this article at http://www.circulationaha.org. Correspondence to Samuel H.H. Chan, PhD, Center for Neuroscience, National Sun Yat-sen University, Kaohsiung 80424, Taiwan, Republic of China. E-mail schan@mail.nsysu.edu.tw

© 2004 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000143082.63063.33

3560
induced by HS may confer protection against sepsis-related circulatory depression via inhibition of iNOS gene expression in the RVLM through prevention of NF-κB activation. This hypothesis was validated in the present study. We further demonstrated that HSP70-induced cardiovascular protection is related to stabilization of IκBα, possibly through prevention of IKK activation and inhibition of IκBα degradation.

Methods

A brief summary of the experimental strategy is provided. Detailed information on materials and methods can be found in the online Data Supplement.

HSP70 Expression in Ventrolateral Medulla After HS

Adult Sprague-Dawley rats anesthetized with pentobarbital (50 mg/kg) were subjected to hyperthermic HS by maintenance of the core temperature of animals at 42±0.5°C for 15 minutes. The ventrolateral medulla that contained bilateral RVLM was removed, and Western blot analysis was performed to detect HSP70 expression at various time intervals after HS.

Effect of HS on Cardiovascular Responses During Experimental Endotoxemia

The effect of HS preconditioning on temporal changes in mean systemic arterial pressure (MSAP), heart rate (HR), or power density of vasomotor components of systemic arterial pressure (SAP) signals after intravenous administration of lipopolysaccharide (LPS; 20 mg/kg, serotype 0111:B4; Sigma) was routinely monitored for 6 hours in animals maintained under propofol (30 mg · kg⁻¹ · h⁻¹) anesthesia.23 We confirmed the presence of a causative relationship between HS-induced HSP70 expression and cardiovascular protection during experimental endotoxemia by bilateral microinjection of an antisense oligonucleotide that targets against the coding region (nt 61 to 78) of hsp70 gene into the RVLM immediately after HS.22

Effect of HS on iNOS mRNA or Protein Expression in Ventrolateral Medulla During Experimental Endotoxemia

Expression of iNOS mRNA or protein in the ventrolateral medulla from LPS-treated animals that received HS before endotoxemia was measured by reverse transcription-polymerase chain reaction or Western blot analysis.6

Activation of NF-κB in Ventrolateral Medulla During Experimental Endotoxemia and Its Modulation by HS

We measured NF-κB DNA binding activity in nuclear protein from ventrolateral medulla during endotoxemia and its modulation by HS using electrophoresis mobility shift assay. Antiserum against NF-κB p65, p50, or c-Rel subunit was used in supershift assay to study translocation of NF-κB subunits to the nucleus. We again confirmed the presence of a causative relationship between HS-induced HSP70 expression and nuclear translocation of NF-κB by antisense hsp70 oligonucleotide treatment. We also evaluated the significance of the activated NF-κB in cardiovascular responses and iNOS expression during endotoxemia by blocking the κB element in the nucleus with bilateral microinjection of the double-stranded κB decoy DNA into the RVLM.24

Mechanisms Underlying Prevention of NF-κB Nuclear Translocation by HS

Two approaches were used to delineate the mechanisms that underlie HS-induced prevention of NF-κB nuclear translocation during endotoxemia. An immune complex kinase assay was used to determine the effects of HS on the activity of IKK, which phosphorylates IκB, leading to dissociation of NF-κB from NF-κB/IκB complex. Western blot analysis was performed to detect the degradation and reexpression of IκB in the ventrolateral medulla after phosphorylation by IKK.

Results

Hyperthermic HS Induced Temporal Changes in HSP70 Expression in the Ventrolateral Medulla That Were Reversed by Antisense hsp70 Oligonucleotide Treatment

Western blot analysis (Figure 1A) revealed that HSP70 in the ventrolateral medulla underwent a significant increase at 8 hours, followed by a progressive augmentation that peaked at
24 hours, and a return to baseline by 48 hours after exposure of animals to a brief HS. In normothermic (NT) controls, in which animals were similarly anesthetized but without subsequent hyperthermic treatment, HSP70 expression was comparable to that of sham controls at all time intervals examined (data not shown). Bilateral microinjection of an antisense hsp70 oligonucleotide (50 pmol) into the RVLM significantly attenuated the upregulation of HSP70 expression in the ventrolateral medulla 16 or 24 hours after HS (Figure 1B). Treatment with sense (50 pmol) or scrambled (50 pmol) hsp70 oligonucleotide was ineffective.

Hyperthermic HS Alleviated Cardiovascular Depression During Experimental Endotoxemia and Its Antagonism by Antisense hsp70 Oligonucleotide Treatment

Similar to our previous findings,6 systemic administration of LPS (20 mg/kg) characteristically promoted hypotension, bradycardia, and a reduction in the power density of the vasomotor component of the SAP spectrum, our experimental index for neurogenic sympathetic vasomotor tone.23 On the basis of the temporal changes in these hemodynamic parameters (Figure 2), experimental endotoxemia was divided into 3 phases.6 Phase I endotoxemia manifested an immediate and significant reduction in MSAP and the power density of the vasomotor components of the SAP spectrum. Phase II exhibited a reversal of hypotension, along with a significant increase in sympathetic vasomotor outflow. Phase III was characterized by a significant secondary decrease in MSAP, HR, and the power density of the vasomotor components of SAP signals.

Twenty-four hours after HS, when upregulation of HSP70 expression in the ventrolateral medulla was optimal, the cardiovascular depression during phases II and III of endotoxemia was almost completely blunted. The same pretreatment, on the other hand, had no discernible effect on phase I
endotoxemia. Interestingly, whereas bilateral microinjection of an antisense hsp70 oligonucleotide (50 pmol) into the RVLM significantly blunted the cardiovascular protection conferred by prior HS on phases II and III endotoxemia, treatment with sense or scrambled hsp70 oligonucleotide (50 pmol) was ineffective (Figure 3).

Hyperthermic HS Attenuated iNOS Upregulation in Ventrolateral Medulla During Experimental Endotoxemia

As exemplified by observations during phase III endotoxemia, the markedly upregulated iNOS mRNA (Figure 4A) or protein (Figure 4B) in the ventrolateral medulla was significantly attenuated 16 or 24 hours after HS, during which HSP70 expression was optimal. Comparable results were obtained during phase II endotoxemia (data not shown).

kB Decoy DNA Reversed Cardiovascular Depression or iNOS Upregulation in Ventrolateral Medulla During Experimental Endotoxemia

Microinjection of the double-stranded kB decoy DNA (10 µg) bilaterally into the RVLM 24 hours before LPS treatment significantly reversed hypotension, bradycardia, and the decrease in power density of vasomotor components of the SAP spectrum (Figure 5A) or antagonized the upregulated iNOS mRNA (Figure 5B) or protein (Figure 5C) during phase II or III endotoxemia. Control microinjection of double-stranded or scrambled DNA (10 µg) into RVLM had no discernible effect on basal MSAP, HR, sympathetic vasomotor tone, or iNOS mRNA or protein expression.
Activation of NF-κB in Ventrolateral Medulla During Experimental Endotoxemia and Its Modulation by Hyperthermic HS

Electrophoresis mobility shift assay (Figure 6A, left panel) showed, contrary to saline, a significant increase in the association of NF-κB with its consensus DNA oligonucleotide in nuclear extracts from the ventrolateral medulla 120 minutes after LPS treatment (phase II endotoxemia). Similar results were obtained during phase III endotoxemia (data not shown). Supershift experiments further revealed that the major NF-κB family member activated during phase II or III endotoxemia was the p65/p50 heterodimer, because p65 or p50 but not c-Rel subunit of NF-κB (A, right panel). Temporally changes in cytosolic and nuclear content of NF-κB in ventrolateral medulla of animals after LPS treatment 24 hours after NT or HS are shown, respectively, in C and D. Values are mean ± SEM of quadruplicate analyses on samples pooled from 4 to 5 animals in each group. *P<0.05 vs corresponding sham-control group in Scheffé multiple range analysis.

Nuclear Translocation of NF-κB During Experimental Endotoxemia and Its Modulation by Hyperthermic HS

Immunoblot analysis revealed that expression of NF-κB p65 protein in the cytosolic fraction of the ventrolateral medulla sample exhibited a significant decline 60 minutes after LPS administration, reaching 44±6% (n=5, P<0.05) of its initial value within 120 minutes (Figure 6C). Concomitantly, the progressively elevated NF-κB p65 protein in the nuclear fraction reached 148±10% (n=6, P<0.05) of its initial value by prior HS (Figure 6A, left panel). This inhibition of an LPS-promoted increase in DNA binding activity of NF-κB 24 hours after HS was reversed (Figure 6B) in animals that received treatment with antisense but not sense or scrambled hsp70 oligonucleotide (50 pmol).
Figure 7. Representative gels showing IKK activity, presented as level of phospho-IκBα, in ventrolateral medulla of LPS-treated animals, 24 hours after NT or HS (A). Also shown are representative Western blots of IκBα (inset) and amount of IκBα protein relative to preinjection control detected from ventrolateral medulla of LPS-treated animals 24 hours after NT or HS (B). Values are mean±SEM of quadruplicate analyses on samples pooled from 4 to 5 animals in each group. *P<0.05 vs sham-control group in Scheffé multiple range analysis. C indicates sham control.

120 minutes after induction of experimental endotoxemia. Both the decrease in cytosolic and increase in nuclear fraction of NF-κB p65 expression detected 60 to 120 minutes after LPS treatment were attenuated 24 hours after HS treatment (Figure 6D).

IKK Activity or Degradation of IκBα in the Ventrolateral Medulla During Experimental Endotoxemia and Its Modulation by Hyperthermic HS

Figure 7A shows that LPS treatment led to an increase in IKK activity in the ventrolateral medulla during early phase II endotoxemia, as manifested by an increase in IκBα phosphorylation. HS, delivered to animals 24 hours before endotoxemia, discernibly depressed such an increase in IKK enzyme activity. There was nonetheless no discernible change in IKKα protein expression under either NT or HS conditions. Western blot analysis also demonstrated that IκBα protein in the ventrolateral medulla was degraded by 36±5% (n=6, P<0.05) within 30 minutes, followed by a complete reexpression 120 minutes after LPS treatment (Figure 7B). This degradation of IκBα protein in the ventrolateral medulla was again significantly antagonized by prior HS.

Discussion

Our laboratory reported previously that overproduction of NO after activation of iNOS3 and formation of peroxynitrite6 in the RVLM play a pivotal role in the reduction of sympathetic vasomotor outflow and arterial pressure during experimental endotoxemia. The present study further showed that HSP70 induced by hyperthermia HS conferred protection against this induced circulatory depression via an antagonism against iNOS mRNA and protein upregulation in the RVLM. We additionally demonstrated that this protective action of HSP70 may be related to suppression of NF-κB activation through stabilization of IκBα via prevention of IKK activation and inhibition of IκBα degradation.

We demonstrated previously that HS-induced HSP70 confers cardiovascular protection against heatstroke by potentiating the baroreceptor reflex response21 via upregulation of glutamate receptors22 in the nucleus tractus solitarii, the terminal site of baroreceptor afferents in the caudal medulla.25 The present study further demonstrated that HS also elicits cardiovascular protection against experimental endotoxemia by upregulation of HSP70 in the RVLM, the final integration site for neural regulation of sympathetic vasomotor activity.5 Together, these observations suggest an important role for HSP70 in central autonomic control of circulation. It is intriguing that our results indicate that HS-induced HSP70 promoted cardiovascular protection by suppressing the surge in iNOS mRNA and protein expression during fatal endotoxemia. These results suggest that in addition to its actions as a molecular chaperone,26 HSP70 may promote circulatory protection during endotoxemia via transcriptional regulation of iNOS gene induction.

The present results provide the first in vivo demonstration of the functional significance of NF-κB activation in the manifestation of cardiovascular depression and its modulation by HS-induced HSP70 during endotoxemia. We found in the present study that both protein level and DNA binding activity of NF-κB in the RVLM were significantly augmented during endotoxemia. That the major subunit activated in the RVLM revealed by supershift analysis was the p65/p50 heterodimer is consistent with reports24,27 that p65/p50 complex is the predominant form of NF-κB in the central nervous system. More importantly, the present results suggest that suppression of iNOS mRNA expression by HSP70 in the RVLM during endotoxemia may result from an attenuation of NF-κB activation. We observed that prior HS treatment retarded nuclear translocation of the p65 subunit of NF-κB and its DNA binding activity during endotoxemia in the RVLM. Observations with antisense hsp70 oligonucleotide treatment further established a causative relationship between HS-induced HSP70 expressed in the ventrolateral medulla and blockade of NF-κB DNA binding activity. Functionally, blockade by the double-stranded κB decoy DNA of NF-κB binding to its cognate site, κB element, in the RVLM also significantly attenuated the iNOS surge and cardiovascular depression during endotoxemia.

The present results revealed 2 possible mechanisms via which HS preconditioning may suppress NF-κB activation during experimental endotoxemia. One mechanism involves inhibition of IKK activation, which was proposed28,29 to underlie the inhibition by HS of NF-κB activation in peripheral tissues during inflammatory responses. The present results also showed that hyperthermic HS significantly attenuated the increase in IKK activity at the ventrolateral medulla...
during endotoxemia that preceded nuclear translocation of NF-kB. The second mechanism entails inhibition of cytoplasmic 1kBo degradation.30 HS preconditioning significantly blunted the transient reduction in 1kBo level at the ventrolateral medulla during experimental endotoxemia. However, because HSP70 also enters the nucleus,31 the possibility of impeding NF-kB nuclear translocation simply by competing for nuclear pore complexes during endotoxemia cannot be excluded.

HSP70 expression was detected in the ventrolateral medulla under basal conditions. We reasoned that this was not due to nonspecific stress to the animals, because HSP70 was not detected by the same antisem in the nucleus tractus solitarii in sham-control or NT rats.32 We also noted that endogenously expressed HSP70 has been identified in normal rodent brain by Western blot32 or proteomic33 analysis.

In conclusion, the present study revealed that upregulation of HSP70 by hyperthermic HS in the RVLM conferred protection against cardiovascular depression during experimental endotoxemia. We further demonstrated that this cardiovascular protective effect was exerted via blockade of NF-kB activation, possibly through prevention of IKK activation and inhibition of 1kBo degradation, leading to inhibition of iNOS upregulation in the ventrolateral medulla. We recognize that HSP70 is not the only HSP induced after thermal preconditioning or the only change to a live organism. As such, other HS-induced cellular mechanisms that may elicit cardiovascular protection during endotoxemia should not be overlooked.

Acknowledgments

This study was supported by research grant VGHKS93–16 (Dr Julie Chan) from the Kaohsiung Veterans General Hospital and the Academic Excellence Program (89-B-FAO8-1-4) from the Ministry of Education, and Sun Yat-sen Research Chair Professor, appointed by National Sun Yat-sen University, Taiwan.

References

Heat Shock Protein 70 Confers Cardiovascular Protection During Endotoxemia via Inhibition of Nuclear Factor-κB Activation and Inducible Nitric Oxide Synthase Expression in the Rostral Ventrolateral Medulla

Circulation. 2004;110:3560-3566; originally published online November 22, 2004; doi: 10.1161/01.CIR.0000143082.63063.33
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/23/3560

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2004/12/06/01.CIR.0000143082.63063.33.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/