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Heat-induced increases in endothelial NO synthase expression and activity and endothelial NO release

M. Brennan Harris,[1,2] Michele A. Blackstone,[1] Hong Ju,[1] Virginia J. Venema,[1] and Richard C. Venema[1,2,3]

[1]*Vascular Biology Center,* [2]*Department of Pediatrics,* [3]*Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912-2500*

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Harris, M. Brennan, Michele A. Blackstone, Hong Ju, Virginia J. Venema, and Richard C. Venema. Heat-induced increases in endothelial NO synthase expression and activity and endothelial NO release. *Am J Physiol Heart Circ Physiol* 285: H333–H340, 2003. First published March 27, 2003; 10.1152/ajpheart.00726.2002.—Endothelial nitric oxide (NO) synthase (eNOS) is regulated by heat shock protein 90 (HSP90), a heat-inducible protein; however, the effect of heat shock on eNOS expression and eNO release is unknown. Bovine aortic endothelial cells were incubated for 1 h at 37°C, 42°C, or 45°C and cell lysates were evaluated with the use of Western blotting. We observed a 2.1 ± 0.1-fold increase in eNOS protein content, but no change in HSP90 content, HSP70 content, or HSP90/eNOS association, 24 h after heat shock at 42°C. We also observed a 7.7 ± 1.5-fold increase in HSP70 protein content, but did not observe a change in eNOS or HSP90 24 h after heat shock at 45°C. eNOS activity and maximal bradykinin-stimulated NO release was significantly increased 24 h after heat shock at 42°C. Heat shock in rats (core temperature: 42°C, 15 min) resulted in a significant increase in aortic eNOS, HSP90, and HSP70 protein content. The aorta from heat-shocked rats exhibited a decreased maximal contractile response to phenylephrine, which was abolished by preincubation with N^G-nitro-L-arginine. We conclude that prior heat shock is a physical stimulus of increased eNOS expression and is associated with an increase in eNOS activity, agonist-stimulated NO release, and a decreased vasoconstrictor response.

endothelium; heat shock; rat; heat shock proteins

ENDOTHELIAL NITRIC OXIDE (NO) synthase (eNOS) is the primary source of NO production in the cardiovascular system and is a key regulator of systemic blood pressure (11), blood vessel proliferation (24), and vascular lesion formation (16, 26). eNOS is regulated by numerous mechanisms, including subcellular location, phosphorylation, and protein-protein interactions. Previously, we (10) and others (4, 7, 8) have demonstrated that eNOS is regulated by and binds directly to heat shock protein 90 (HSP90). Specifically, García-Cardeña et al. (8) demonstrated that the activity of purified eNOS was increased as much as 2.5-fold in vitro when increasing amounts of HSP90 were added to the reaction. Although HSP90 is one of the most abundant cytosolic proteins, accounting for ~1% of all soluble protein (36), Brouet et al. (4) recently demonstrated that overexpression of HSP90 in human umbilical vein endothelial cells increases Akt-mediated phosphorylation of eNOS, an important regulatory mechanism. These studies suggest that increasing expression of HSP90 in the vascular endothelium would likely result in increased eNOS activity and, therefore, increased endothelial-derived NO release. Prior heat shock has been shown to result in increased expression of HSP90 in various mammalian cell lines (37). Although several studies (13, 29, 34) have examined the effects of heat shock on HSP90 expression in vascular endothelium, the results vary depending on the vascular bed, passage, and degree of heat shock (13). Therefore, in light of the effects of HSP90 on eNOS, it is important to determine changes in vascular HSP90 expression in response to a specific heat shock treatment.

In addition to HSP90, it is well documented that HSP70 increases in response to heat shock in the cardiovascular system (3). HSP70 rapidly accumulates after heat shock and can increase as much as eightfold in rat hearts after whole animal heat shock (32). In fact, Amrani et al. (1) have suggested that the increase in rat hearts after whole animal heat shock occurs primarily in the vascular endothelium, which is associated with improved recovery of endothelial function from cardioplegic arrest. With the use of confocal microscopy, Leger et al. (18) indicated that the primary site of HSP70 induction after whole animal heat shock is in the blood vessels; however, these authors did not distinguish between vascular smooth muscle and the endothelium. Studies using transfection of HSP70 into endothelial cell cultures have demonstrated improved protection of the endothelium from hypoxia (18, 30) or H_2O_2 (9, 18). However, functional recovery of these cells or their ability to release NO was not determined.

Whereas numerous studies have examined changes in expression of the traditional HSPs like HSP90 and HSP70 after heat shock, no previous studies have examined the effects of prior heat shock on eNOS expression and function. However, it is interesting to note

Address for reprint requests and other correspondence: M. Brennan Harris, Vascular Biology Center, Medical College of Georgia, 1459 Laney Walker Blvd., CB3207, Augusta, GA 30912-2500 (E-mail: bharris@mail.mcg.edu).

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that eNOS expression can be increased under other stressful conditions. For instance, hypoxia, which induces HSP70 expression (2), can also increase eNOS expression by as much as 2.5-fold (12). The increase in eNOS expression after hypoxia corresponds to increased NO release (12) and vascular reactivity (27). In addition, shear stress has also been shown to increase eNOS expression (5). Furthermore, endothelial eNOS expression has also been shown to increase in response to hydrogen peroxide by a different mechanism than shear stress (6). Therefore, it is possible that other stressors like heat shock may also increase eNOS expression and result in changes in vascular reactivity. Therefore, we hypothesized that heat shock would result in increased endothelial HSP90, HSP70, and eNOS expression 24 h after heat shock in both cell cultures and whole animals. In addition, we hypothesized that prior heat shock would alter endothelial-derived NO release and vasoreactivity.

METHODS

Cell culture. Bovine aortic endothelial cells (BAECs) were passaged from primary cultures and used for experiments during *passages 2–6*. Cultures were maintained in water-jacketed incubators at 37°C and 5% CO_2 and in medium 199 supplemented with 10% fetal bovine serum, 5% iron-supplemented calf serum, 20 μg/ml L-glutamine, 1× minimal essential medium, 0.6 μg/ml thymidine, 500 IU/ml penicillin, and 500 μg/ml streptomycin. For heat shock, cells were placed in water-jacketed incubators at 37°, 42°, or 45°C and 5% CO_2 for 1 h and then returned to a water-jacketed incubator at 37°C and 5% CO_2 for 24 h. Rat aortic smooth muscle cells were also passaged from primary cultures and used in experiments during *passages 2–5*. Cultures were maintained in DMEM containing 10% fetal bovine serum, 500 IU/ml penicillin, and 500 μg/ml streptomycin.

Trypan blue. Trypan blue exclusion was used to determine the viability of the BAECs. BAECs were resuspended 24 h after heat shock and incubated with 0.4% trypan blue dye for 2 min. BAECs were observed with the use of a hemocytometer under a light microscope. Cells that were able to exclude the stain were considered viable and the percentage of non-blue cells over total cells was used as an index of viability.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting of BAECs were carried out as previously described (19). Briefly, 24 h after heat shock, BAECs were washed twice with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4. Cells were then lysed in ice cold buffer containing 20 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and 1% phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 10,000 *g* for 20 min to remove insoluble material. For immunoprecipitation, samples were precleared by the addition of protein A/G agarose (sc-2003, Santa Cruz Biotechnology). Anti-eNOS (N30020, Transduction Laboratories) or anti-HSP90 (SPA-845, StressGen Biotechnologies) was then added to the supernatant and incubated overnight at 4°C. Protein A/G agarose (sc-2003, Santa Cruz Biotechnology) was then added, and samples were incubated for an additional 3 h at 4°C. The immunoprecipitated proteins bound to the agarose beads were washed twice, and proteins were eluted from the beads by boiling the samples in SDS sample buffer. Agarose beads were pelleted by centrifugation, and protein supernatants were used for immunoblotting as described below.

For immunoblotting, cell lysates or protein supernatants from immunoprecipitation were loaded and run on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, which was subsequently immunoblotted with either an anti-HSP90 (H38220, Transduction Laboratories), anti-HSP70 (SPA-810, StressGen Biotechnologies), or anti-eNOS antibody (N30020, Transduction Laboratories).

Immunoblotting of rat aorta was carried out as follows. Previously frozen aortas were thawed on ice and placed in RIPA buffer (1:15 wt/vol). The aortas were homogenized using a glass-glass homogenizer and were then centrifuged at 10,000 *g* for 10 min at 4°C. Protein concentrations were determined with the use of the Bio-Rad detergent-compatible protein assay. Volumes were adjusted to equalize the concentration of the samples. Fifty microliters of each sample were then loaded and run on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, which was subsequently immunoblotted with either anti-HSP90 or anti-HSP70 antibodies. For eNOS, 80 μl of a 2′,5′-ADP Sepharose slurry were added to 1 ml of the cleared homogenate and incubated at 4°C for 2 h. The beads were then washed three times with ice-cold PBS. A SDS sample buffer (100 μl) was then added to the beads, and the samples were boiled for 5 min to elute the proteins from the beads. The samples were centrifuged, and the supernatant was subjected to immunoblotting as described above with an anti-eNOS antibody.

Endothelial NO release. NO release from BAECs was measured 24 h after heat shock, as previously described (10). Briefly, 24 h after heat shock, BAEC cultures were switched to serum-free medium overnight and then equilibrated in Locke's buffer composed of (in mM) 154.0 NaCl, 5.6 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 3.6 $NaHCO_3$, 5.6 glucose, and 10.0 HEPES (pH 7.4), 0.3 isobutyl methylxanthine, and 20 U/ml superoxide dismutase. The cells were treated with 1 μM bradykinin for 0, 1, 5, 10, 15, or 30 min before being transferred to the bathing medium to confluent rat aortic smooth muscle cells in six-well plates. After the transfer, the rat aortic smooth muscle cells were incubated for 3 min and then lysed in ice-cold sodium acetate (20 mM, pH 4.0). Lysates were frozen at −20°C until assayed for cGMP concentrations with the use of an enzyme-immunoassay kit (RPN226, Amersham Biosciences). cGMP concentrations in the lysates were quantified and reflect NO released by the endothelial cells.

eNOS activity. Cell lysates from control and heat-shocked BAECs were prepared as described for immunoblotting. eNOS activity in cell lysates was then determined by monitoring the rate of formation of L-[^{14}C]citrulline from L-[^{14}C]arginine under the conditions previously described (27, 35).

Animals. Experiments were approved by the Institutional Committee for Animal Use in Research and Education and adhered to the American Physiological Society standards of humane animal experimentation. Male Sprague-Dawley rats (~300 g) were anesthetized with pentobarbital sodium (40 mg/kg ip) and placed on a thermal blanket to raise core temperature (T_c) to 42°C. T_c was then maintained at 42.0 ± 0.2°C for 15 min and then allowed to return to normal. Controls were also anesthetized and placed on a thermal blanket to maintain T_c at 37°C. T_c was monitored with a digital thermometer inserted 6 cm into the rectum.

Vascular reactivity. Twenty-four hours after heat shock, the thoracic aortas were removed from control and heat-shocked animals and placed in physiological salt solution composed of (in mM) 130 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4·7H_2O$, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 5.5 dextrose, and

0.03 Na_2EDTA. The vessels were cleaned of adherent fat and connective tissue and cut into four 4-mm rings. The remainder of the aorta was frozen in liquid nitrogen and used for immunoblotting. The rings were mounted on stainless steel holders in muscle baths for isometric force recording. Muscle baths were filled with physiological salt solution heated to 37°C and aerated with 95% O_2-5% CO_2. The rings were stretched to an optimal resting tension (3 g) and allowed to stabilize for 1 h. After equilibration, aortic rings were made to contract in response to the cumulative addition of phenylephrine (10^{-9}-10^{-5} M) in the presence or absence of 1 mM N^G-nitro-L-arginine (L-NNA). The endothelium was left intact, and validation of its presence was assessed by relaxation in response to acetylcholine (1 μM) subsequent to contraction with phenylephrine (1 μM). All experiments were performed in the presence of 1 μM indomethacin to block cyclooxygenase.

Statistics. Descriptive data (means ± SD and SE) were calculated for each dependent variable. Overall differences between groups were analyzed with the use of two-way ANOVA with Student-Newman-Keuls post hoc analysis for determining differences between the means when more than two groups were compared. An independent *t*-test was used when only two groups were compared. In all tests, a probability level of $P < 0.05$ was used as the decision rule for significance testing.

RESULTS

Cell viability. Cell viability was determined as the percentage of nonblue cells versus total cells with the use of trypan blue exclusion 24 h after heat shock at 42°C and 45°C or no heat shock at 37°C. Cell viability was not statistically different ($P > 0.05$) 24 h after treatment at 42°C (93.7 ± 1.0%) or 45°C (96.3 ± 0.5%) compared with controls, 37°C (95.2 ± 0.6%).

eNOS, HSP90, and HSP70 expression in BAECs. eNOS, HSP90, and HSP70 protein levels were determined in BAECs 24 h after heat shock at 42°C and 45°C using Western blot analysis. The results are shown in Fig. 1 and are expressed as a percentage of control (37°C). eNOS protein content was significantly ($P < 0.05$) increased by 2.1 ± 0.1-fold in heat-stressed BAECs compared with controls. No significant difference ($P > 0.05$) in eNOS expression was observed in BAECs heat shocked for 1 h at 45°C (Fig. 1*A*). HSP90 protein content was not significantly increased ($P > 0.05$) after heat shock at 42°C or 45°C (Fig. 1*B*). HSP70 protein content was not significantly increased after heat shock at 42°C, but was significantly ($P < 0.05$) increased by 7.7 ± 1.5-fold after heat shock at 45°C (Fig. 1*C*).

eNOS and HSP90 complex formation. eNOS and HSP90 complex formation was determined in BAECs 24 h after heat shock at 42°C for 1 h by immunoprecipitation of HSP90 and subsequent immunoblotting of eNOS, and by immunoprecipitation of eNOS with subsequent immunoblotting of HSP90. The results, shown in Fig. 2, *A* and *B*, demonstrated that no change in eNOS-HSP90 complex formation was observed after heat shock of BAECs at 42°C.

NO release in BAECs after heat shock. Bradykinin-stimulated NO release was determined 24 h after heat shock of BAECs at 42°C and 45°C using a reporter cell assay for cGMP. The results are shown in Fig. 3 compared with controls (37°C). Heat shock at 42°C resulted in a significant ($P < 0.05$) increase in the maximal bradykinin-stimulated NO release 24 h later. Furthermore, heat shock at 42°C and 45°C resulted in a more

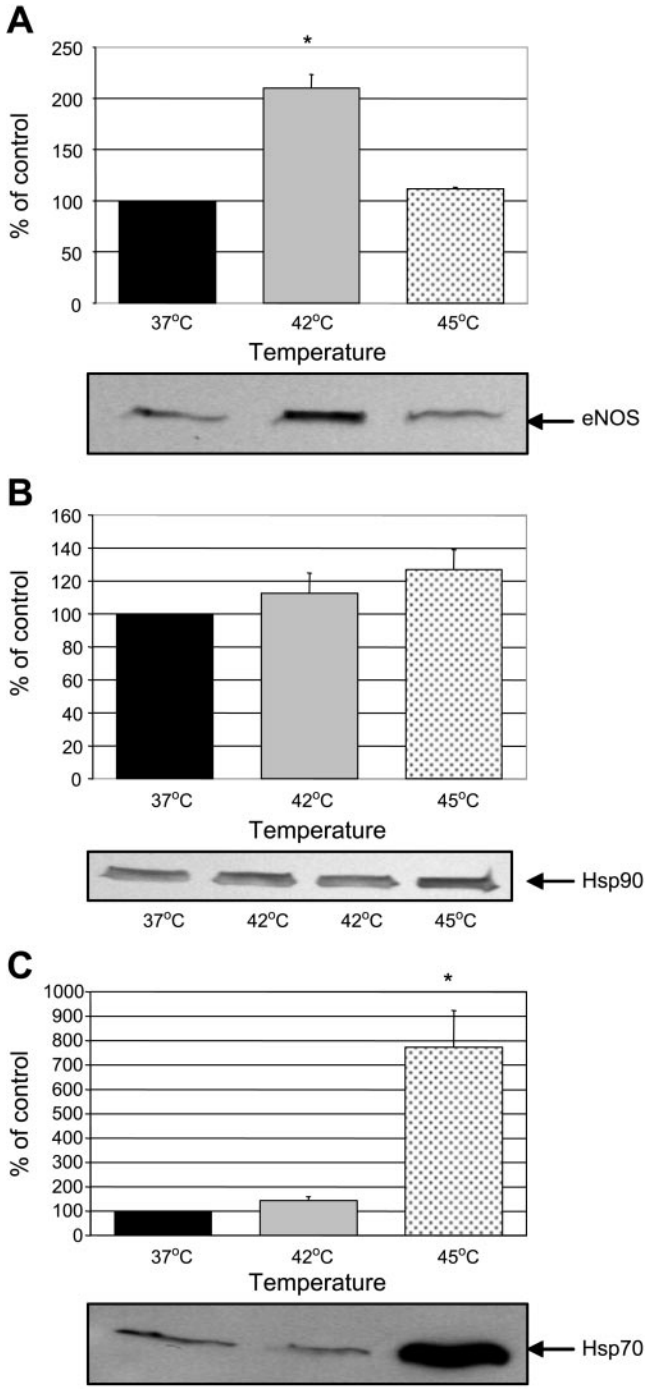

Fig. 1. Endothelial nitric oxide (NO) synthase (eNOS), heat shock protein 90 (HSP90), and HSP70 protein content in bovine aortic endothelial cells (BAECs) 24 h after heat shock for 1 h at 42°C and 45°C. *A*: eNOS content; *B*: HSP90 content; *C*: HSP70 content. A representative Western blot for each group is shown immediately below histogram. Values are means ± SE; $n = 3$. *Significantly different from control (37°C).

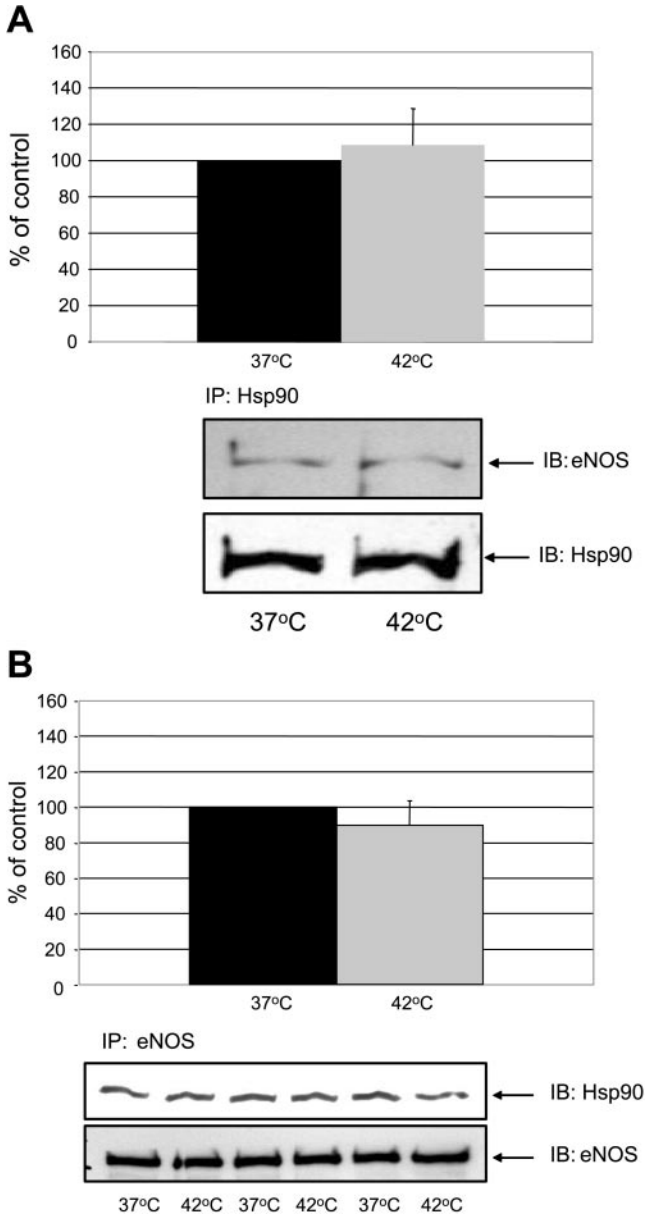

Fig. 2. HSP90/eNOS complex formation in BAECs 24 h after heat shock for 1 h at 42°C. *A*: immunoprecipitation (IP) with anti-HSP90 and immunoblotting (IB) with anti-eNOS and anti-HSP90 antibodies. *B*: IP with anti-eNOS antibody and immunoblotting with anti-HSP90 and anti-eNOS antibodies. A representative Western blot for each group is shown immediately below histogram. Values are means ± SE; $n = 6$.

prolonged increase in bradykinin-stimulated NO release at 10 and 15 min and returned to control levels by 30 min.

eNOS activity in BAECs after heat shock. eNOS activity in BAECs was determined 24 h after heat shock by measuring the rate of conversion of L-[^{14}C]citrulline from L-[^{14}C]arginine. Results are shown in Fig. 4 and are expressed as fmol L-citrulline·min^{-1}·mg^{-1}. A significant ($P < 0.05$) increase in eNOS activity was observed 24 h after heat shock of BAECs for 1 h at 42°C.

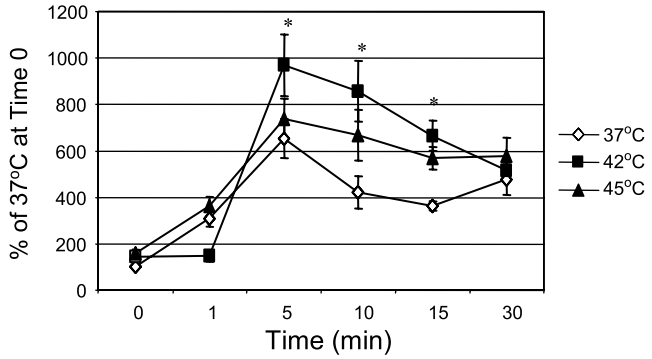

Fig. 3. Bradykinin-stimulated NO release in BAECs 24 h after heat shock for 1 h at 42°C and 45°C. Values are means ± SE; $n = 6$. *Significantly different from control (37°C).

eNOS, HSP90, and HSP70 expression in rat aorta. eNOS, HSP90, and HSP70 protein levels were determined in rat aortas 24 h after whole animal heat shock ($T_c = 42.0 \pm 0.5$°C, 15 min) using Western blot analysis. A significant ($P < 0.05$) increase in eNOS protein content in the aorta (40 ± 9%) was observed (Fig. 5*A*). In addition, HSP90 protein content was significantly ($P < 0.05$) increased 2.4 ± 0.4-fold (Fig. 5*B*) and HSP70 content was increased 3.8 ± 0.5-fold (Fig. 5*C*).

Vascular reactivity. Contractile responses to phenylephrine were determined in rat aortic rings 24 h after whole animal heat shock ($T_c = 42.0 \pm 0.5$°C, 15 min). Maximal vasoconstriction to phenylephrine (10^{-5} M) in aortic rings from control and heat-shocked animals is shown in Fig. 6 and expressed as a percentage of the maximal force produced in controls. Heat shock resulted in an attenuation of the maximal phenylephrine-induced vasoconstriction compared with controls ($P < 0.05$). Preincubation of the aortic rings with 1 mM L-NNA abolished this difference.

DISCUSSION

Previously, several studies (4, 7, 8, 10) have demonstrated that HSP90, part of the heat-inducible class of proteins, directly interacts with and regulates eNOS. However, no studies have examined the effects of prior

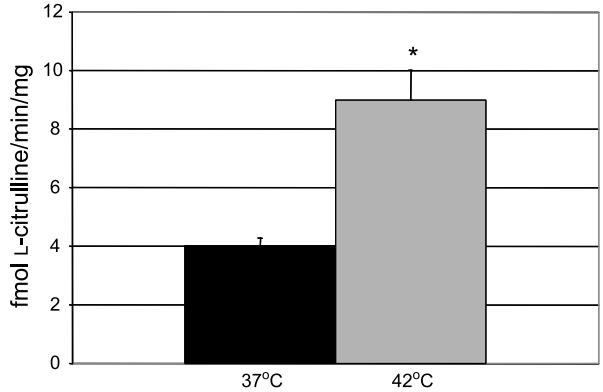

Fig. 4. eNOS activity in BAECs 24 h after heat shock for 1 h at 42°C. Values are means ± SE; $n = 3$. *Significantly different from control (37°C).

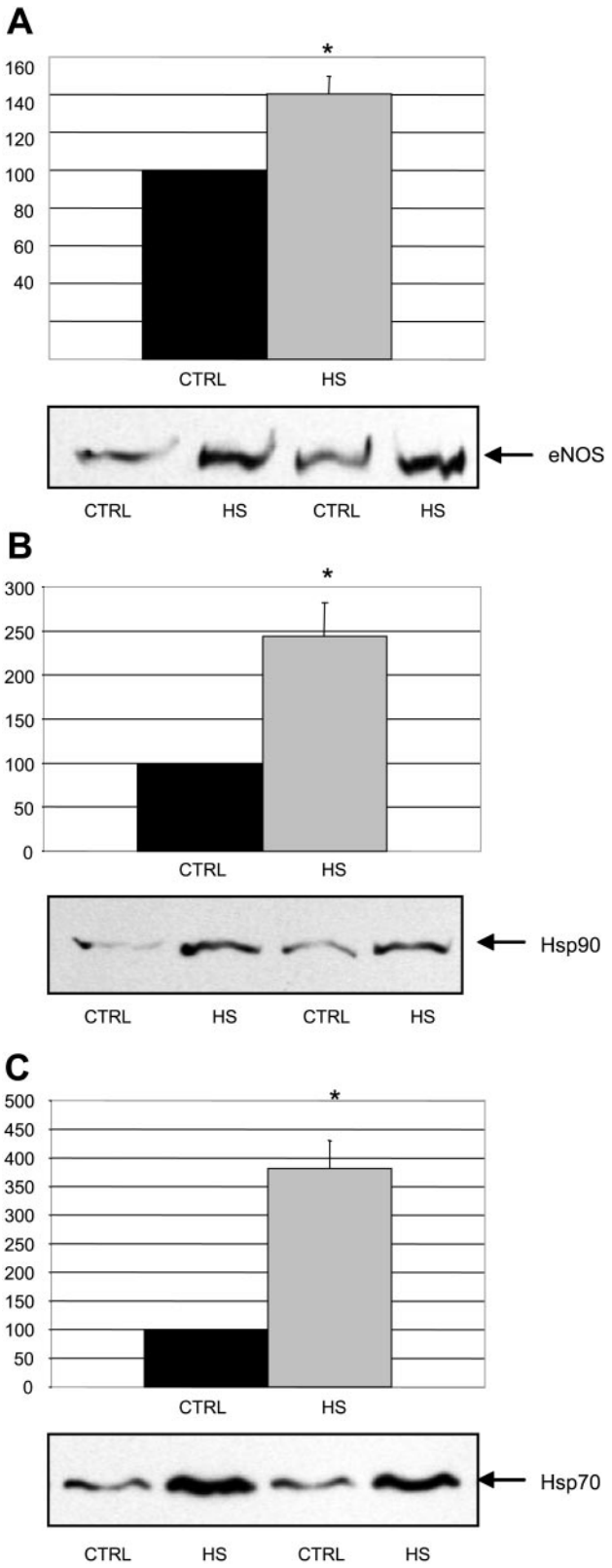

Fig. 5. *A*: eNOS content; *B*: HSP90 content; *C*: HSP70 content. eNOS, HSP90, and HSP70 protein content in rat aorta 24 h after heat shock (HS) at 42°C for 15 min. A representative Western blot for each group is shown immediately below histogram. Values are means ± SE; $n = 6$. *Significantly different from control (CTRL) (37°C).

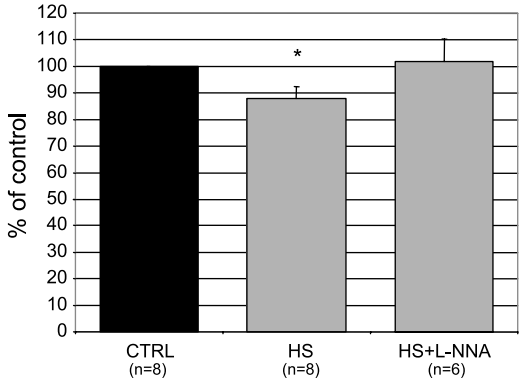

Fig. 6. Maximal phenylephrine-induced vasoconstriction in rat aortic rings 24 h after heat shock at 42°C for 15 min. L-NNA, N^G-nitro-L-arginine. Values are means ± SE. *n*, Number of animals. *Significantly different from control.

heat shock on HSP90 and eNOS expression in endothelial cells or the subsequent effects on endothelial-derived NO release. In the present study, we report that mild heat shock (42°C, 1 h) results in increased eNOS expression in cultured BAECs, which is associated with an increase in eNOS activity and in the maximal bradykinin-stimulated NO release 24 h later. Furthermore, we demonstrate that 24 h after whole animal heat shock in rats (42°C, 15 min) there is an increase in HSP90, HSP70, and eNOS expression in the aorta that is associated with a decrease in maximal phenylephrine-induced vasoconstriction. This change in vasoconstriction is reversed by the eNOS inhibitor L-NNA. These data suggest that heat shock can have a significant effect on vascular homeostasis, resulting in increased eNOS expression and endothelial-derived NO release.

In the first series of experiments, heat shock at 42°C for 1 h in BAEC resulted in an increase in eNOS protein content 24 h later, but no change in HSP90 and HSP70 protein content. These results, however, failed to confirm our original hypothesis that heat shock would result in increased HSP90 and HSP70 content. Differential expression of heat shock proteins in the vascular endothelium has previously been reported and was found to be dependent on the vascular bed, passage, and degree of heat shock (13). We also demonstrate that heat shock at a higher temperature (45°C, 1 h) does result in an increase in HSP70, indicating that the 42°C treatment is too mild to produce the classic heat shock response. Interestingly, eNOS can be induced by other stressors, such as hypoxia (12), hydrogen peroxide (6), and shear stress (5). Drummond et al. (6) suggest that the change in eNOS expression after hydrogen peroxide treatment of endothelial cells may be the result of a change in redox status of the cell. Future studies should evaluate the possibility that the heat-induced change in eNOS expression may occur via the same mechanism. Previous studies (3) have also shown HSPs to be redox-sensitive proteins, and, taken together with the current study, these observations suggest that eNOS should also be considered a heat shock or stress protein.

Despite the increase in eNOS protein content, no change in eNOS-HSP90 complex association was observed in BAECs after heat shock at 42°C. The lack of change in eNOS-HSP90 association is likely due to the abundance of HSP90 (36) and indicates that no further increase in HSP90 expression is necessary to maintain the regulatory interaction of HSP90 with eNOS. These data, taken together with the observation that neither HSP90 nor HSP70 levels changed in BAECs, indicate that changes in eNOS activity and endothelial-derived NO release after mild heat shock are due primarily to the changes in eNOS content. This observation was contrary to our original hypothesis that heat shock would result in an increase in HSP90 and HSP70 expression and alter endothelial NO release. Although more severe heat shock of BAECs (45°C for 1 h) did result in an increase in HSP70, this was not associated with a change in bradykinin-stimulated NO release at the time points measured. Therefore, it appears that the heat-induced changes in eNOS activity and NO release in this study were not dependent on either HSP90 or HSP70.

The increase in eNOS protein content was associated with a significant increase in the maximal bradykinin-stimulated NO release, whereas the increase in HSP70 after the more intense heat shock treatment was not associated with a significant increase in bradykinin-stimulated NO release. These results are similar to those obtained by Justice et al. (12), in which hypoxia-induced increased eNOS expression resulted in increased A-23187-stimulated NO release from cultured endothelial cells. No change in the basal release of NO was observed after heat shock in serum-starved BAECs in the present study. However, under serum starvation, basal NO release is greatly reduced, and it is likely that heat-induced changes in basal NO release under serum starvation were too small to be detected by the technique employed. Under serum conditions, however, we did observe an increase in eNOS activity using the arginine-citrulline assay, which parallels the observed increase in eNOS content. Therefore, the heat-induced increase in eNOS protein content is associated with an increase in endothelial-derived NO release.

Whole animal heat shock in rats results in a 40 ± 9% increase in eNOS content in aortic homogenates, thus confirming our findings in cell culture. Differences in the degree of eNOS content between the cell culture experiment and the rat experiments may be due to differences in the severity of the heat shock (42°C for 1 h vs. 42°C for 15 min) as well as possible modulation of the response in rats by humoral factors. Although mild heat shock of cultured endothelial cells that results in increased eNOS expression does not result in increased HSP70 and HSP90 content, heat shock in rats does increase HSP90 and HSP70 levels in rat aorta (Fig. 4, *B* and *C*). This difference is likely because the aortic homogenates included both endothelial and smooth muscle cells. Heat shock (42°C, 1 h) of both rat and bovine aortic smooth muscle cells in culture results in significant increases in both HSP90 and HSP70 (M. B. Harris and R. C. Venema, unpublished observations). These data combined with the cell culture data also suggest that eNOS is a heat shock/stress protein.

In the present study, we also demonstrate that whole animal heat shock alters vasoreactivity 24 h later. We show that the maximal phenylephrine-induced vasoconstriction is attenuated by prior heat shock. Furthermore, we show that this decrease in vasoconstriction can be reversed with the NOS inhibitor L-NNA suggesting that the difference is due to increased NO production. Although no studies have examined changes in vasoreactivity 24 h after whole animal heat shock, a few studies (15, 20–23) have examined changes in vascular reactivity during heat stress. In whole animal hyperthermia, an overall increase in the basal mean arterial pressure was observed as well as a blunted hemodynamic response to various vasoconstrictors (22, 23). However, no change in norepinephrine-induced vasoconstriction and enhanced potassium chloride and calcium-induced vasoconstriction was observed in hyperthermic vascular rings (20). In general, these studies suggest that there is an increase in vasoconstriction during hyperthermia or heat shock. The increase in eNOS expression and NO release 24 h after heat shock in the present study may therefore represent a compensatory response to the increase in vascular tone caused by heat shock.

In addition to studies examining changes in vascular reactivity during heat shock, one study by Kim et al. (14) has examined the effects of prior heat shock (42°C for 15 or 45 min) of isolated rat aortic rings on vascular reactivity. These authors reported an increase in contractility in response to potassium chloride 8 h after heat shock for 45 min. These results were observed regardless of the presence of endothelium, suggesting that eNOS does not play a part (14). The difference between the study by Kim et al. and the present study may be explained by the shorter time course for recovery as well as the difference between heat shock of isolated vessels compared with whole animals. In addition, heat shock disrupts protein synthesis and is followed by the rapid and preferential synthesis of the traditional HSPs after heat shock (31). It is possible, therefore, that increased eNOS expression and the subsequent increase in NO release were not manifested at 8 h after heat shock, but were evident at 24 h after heat shock.

In summary, the results of the present study indicate that moderate heat shock results in increased vascular eNOS expression, which is associated with increased eNOS activity and endothelial-derived NO release as well as NO-mediated attenuation of vasoconstriction in isolated vessels. Furthermore, we demonstrate that more severe heat shock of endothelial cells results in the classic heat shock response, i.e., increase HSP70 expression, without an increase in eNOS expression or NO release. The present study, therefore, provides a framework for future studies evaluating the degree of heat shock necessary for eNOS induction and the subsequent increase in NO release. Previous studies (3) have already shown that

prior heat shock in both whole animals and isolated vessels provides protection against ischemia-reperfusion injury. Although most of these studies have focused on HSP70 expression, it is possible that increased eNOS expression may account for the improved recovery of endothelial function. Furthermore, studies (17, 28) have shown that exercise results in increased eNOS expression, which these authors suggest is likely due to mechanical or shear stress. However, it is possible that the increase in eNOS may also be due to increased T_c that occurs during exercise. The results of this study also suggest that increased eNOS expression might play a role in the increased NO-mediated hypotension that occurs during hyperthermia in humans under conditions such as septic shock (25, 33). In conclusion, heat shock, like shear stress, is another physical stimulus of increased eNOS expression, which should be considered when evaluating situations where T_c may be elevated.

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REFERENCES

1. **Amrani M, Latif N, Morrison K, Gray CC, Jayakumar J, Corbett J, Goodwin AT, Dunn MJ, and Yacoub MH.** Relative induction of heat shock protein in coronary endothelial cells and cardiomyocytes: implications for myocardial protection. *J Thorac Cardiovasc Surg* 115: 200–209, 1998.
2. **Benjamin IJ, Kroger B, and Williams RS.** Activation of the heat shock transcription factor by hypoxia in mammalian cells. *Proc Natl Acad Sci USA* 87: 6263–6267, 1990.
3. **Benjamin IJ and McMillan DR.** Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83: 117–32, 1998.
4. **Brouet A, Sonveaux P, Dessy C, Balligand JL, and Feron O.** HSP90 ensures the transition from the early Ca^{2+}-dependent to the late phosphorylation-dependent activation of the endothelial nitric oxide synthase (eNOS) in VEGF-exposed endothelial cells. *J Biol Chem* 276: 32663–32669, 2001.
5. **Davis ME, Cai H, Drummond GR, and Harrison DG.** Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circ Res* 89: 1073–1080, 2001.
6. **Drummond GR, Cai H, Davis ME, Ramasamy S, and Harrison DG.** Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res* 86: 347–354, 2000.
7. **Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, and Sessa WC.** Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* 90: 866–873, 2002.
8. **Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, and Sessa WC.** Dynamic activation of endothelial nitric oxide synthase by HSP90. *Nature* 392: 821–824, 1998.
9. **Gill RR, Gbur CJ Jr, Fisher BJ, Hess ML, Fowler AA III, Kukreja RC, and Sholley MM.** Heat shock provides delayed protection against oxidative injury in cultured human umbilical vein endothelial cells. *J Mol Cell Cardiol* 30: 2739–2749, 1998.
10. **Harris MB, Ju H, Venema VJ, Blackstone M, and Venema RC.** Role of heat shock protein 90 in bradykinin-stimulated endothelial nitric oxide release. *Gen Pharmacol* 35: 165–170, 2000.
11. **Ignarro LJ.** Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res* 65: 1–21, 1989.
12. **Justice JM, Tanner MA, and Myers PR.** Endothelial cell regulation of nitric oxide production during hypoxia in coronary microvessels and epicardial arteries. *J Cell Physiol* 182: 359–365, 2000.
13. **Ketis NV, Hoover RL, and Karnovsky MJ.** Effects of hyperthermia on cell survival and patterns of protein synthesis in endothelial cells from different origins. *Cancer Res* 48: 2101–2106, 1988.
14. **Kim Y, Kim J, Kim M, Baek W, and Kim I.** Effect of heat shock on the vascular contractility in isolated rat aorta. *J Pharmacol Toxicol Methods* 42: 171–174, 1999.
15. **Kregel KC, Kenney MJ, Massett MP, Morgan DA, and Lewis SJ.** Role of nitrosyl factors in the hemodynamic adjustments to heat stress in the rat. *Am J Physiol Heart Circ Physiol* 273: H1537–H1543, 1997.
16. **Kubes P, Suzuki M, and Granger DN.** Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 88: 4651–4655, 1991.
17. **Laughlin MH, Pollock JS, Amann JF, Hollis ML, Woodman CR, and Price EM.** Training induces nonuniform increases in eNOS content along the coronary arterial tree. *J Appl Physiol* 90: 501–510, 2001.
18. **Leger JP, Smith FM, and Currie RW.** Confocal microscopic localization of constitutive and heat shock-induced proteins HSP70 and HSP27 in the rat heart. *Circulation* 102: 1703–1709, 2000.
19. **Marrero MB, Venema VJ, Ju H, He H, Liang H, Caldwell RB, and Venema RC.** Endothelial nitric oxide synthase interactions with G-protein-coupled receptors. *Biochem J* 343: 335–340, 1999.
20. **Massett MP, Lewis SJ, Bates JN, and Kregel KC.** Effect of heating on vascular reactivity in rat mesenteric arteries. *J Appl Physiol* 85: 701–708, 1998.
21. **Massett MP, Lewis SJ, Bates JN, and Kregel KC.** Modulation of temperature-induced tone by vasoconstrictor agents. *J Appl Physiol* 86: 963–969, 1999.
22. **Massett MP, Lewis SJ, and Kregel KC.** Effect of heating on the hemodynamic responses to vasoactive agents. *Am J Physiol Regul Integr Comp Physiol* 275: R844–R853, 1998.
23. **Massett MP, Lewis SJ, Stauss HM, and Kregel KC.** Vascular reactivity and baroreflex function during hyperthermia in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1282–R1289, 2000.
24. **Papapetropoulos A, Garcia-Cardena G, Madri JA, and Sessa WC.** Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 100: 3131–3139, 1997.
25. **Petros A, Bennett D, and Vallance P.** Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 338: 1557–1558, 1991.
26. **Radomski MW, Palmer RMJ, and Moncada S.** An L-arginine-nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* 87: 5193–5197, 1990.
27. **Resta TC, Chicoine LG, Omdahl JL, and Walker BR.** Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia. *Am J Physiol Heart Circ Physiol* 276: H699–H708, 1999.
28. **Sessa WC, Pritchard K, Seyedi N, Wang J, and Hintze TH.** Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res* 74: 349–353, 1994.
29. **Shivers RR, Pollock M, Bowman PD, and Atkinson BG.** The effect of heat shock on primary cultures of brain capillary endothelium: inhibition of assembly of zonulae occludentes and the synthesis of heat-shock proteins. *Eur J Cell Biol* 46: 181–195, 1988.
30. **Suzuki K, Sawa Y, Kaneda Y, Ichikawa H, Shirakura R, and Matsuda H.** Overexpressed heat shock protein 70 attenuates hypoxic injury in coronary endothelial cells. *J Mol Cell Cardiol* 30: 1129–1136, 1998.

31. **Tanguay RM.** Genetic regulation during heat shock and function of heat-shock proteins: a review. *Can J Biochem Cell Biol* 61: 387–394, 1983.
32. **Taylor RP, Harris MB, and Starnes JW.** Acute exercise can improve cardioprotection without increasing heat-shock protein. *Am J Physiol Heart Circ Physiol* 276: H1098–H1102, 1999.
33. **Titheradge MA.** Nitric oxide in septic shock. *Biochim Biophys Acta* 1411: 437–455, 1999.
34. **Tomasovic SP, Ramagli LS, Simonette RA, Wilson MJ, and Rodriguez LV.** Heat-stress proteins of rat lung endothelial and mammary adenocarcinoma cells. *Radiat Res* 110: 45–60, 1987.
35. **Venema RC, Sayegh HS, Arnal J, and Harrison DG.** Role of the enzyme calmodulin-binding domain in membrane association and phospholipid inhibition of endothelial nitric oxide synthase. *J Biol Chem* 270: 14705–14711, 1995.
36. **Welch WJ and Feramisco JR.** Purification of the major mammalian heat shock proteins. *J Biol Chem* 257: 14949–14959, 1982.
37. **Welch WJ, Garrels JI, Thomas GP, Lin JJ, and Feramisco JR.** Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose- and Ca^{2+}-ionophore-regulated proteins. *J Biol Chem* 258: 7102–7111, 1983.