
Endothelium-Dependent Effects of Heat Shock on Vasomotor Tone: Role of eNOS and HSP70

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Abstract

Heat shock-mediated delayed protection against myocardial and cerebral ischemia has been attributed to induction of heat shock proteins (HSP), especially HSP70, in myocytes and neurons. However, heat shock preconditioning only induces HSP70 in endothelium. The purpose of this study was to examine the effect of heat shock on endothelium-dependent vasomotor tone.

Isolated mouse aortas were heated to 43.5°C for 15 min and returned to 37°C for 18 h. They were then mounted in a wire myograph to assess vasomotor responses to vasoconstrictors and vasodilators. Intact heat shocked aortas showed reduction in phenylephrine- or 5-hydroxytryptamine-induced vasoconstriction, while no difference in vascular reactivity was detected in heat shocked endothelium-denuded aortas. Reduced vasoconstriction in heat shocked aortas was restored to control level by incubation with L-NAME, a nitric oxide synthase (NOS) inhibitor, but not by geldanamycin, a HSP90 inhibitor. Furthermore, the effect of heat shock was not observed in aortas from eNOS null mice, suggesting that vasomotor responsiveness reduced by heat shock was related to increased basal eNOS activity. Immunoblot of lysates from heat shocked aortas did not show upregulation of eNOS or other NOS isoforms, while showing robust expression of HSP70; immunoprecipitation using eNOS polyclonal or monoclonal antibodies demonstrated HSP70 interaction with eNOS after heat shock. In enzymatic assay

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(conversion of L-arginine to L-citrulline), HSP70 increased enzymatic activity of recombinant eNOS.

We conclude that heat shock decreases vasomotor responses to vasoconstrictors by enhancing eNOS basal activity, through interaction with HSP70, that is robustly induced. Such an increase of eNOS might be related to protective effect of heat shock against ischemia reported *in vivo*. Finally, interaction between HSP70 and eNOS opens the intriguing possibility that HSP70 could have vasoregulatory properties in addition to its cytoprotective functions.

Keywords: preconditioning, heat shock, endothelium, eNOS, HSP70.

Introduction

Heat shock preconditioning enhances myocardial resistance against ischemia by reducing incidence of arrhythmias and infarct volume, as well as by improving post-ischemic contractile recovery [1, 2]. This protective effect closely correlates with the induction of a stress response characterized by *de novo* expression of the inducible 70-kD heat shock protein, HSP70 [3]. In addition to its protein chaperone activity, HSP70 has also shown clear cytoprotective properties in cultured myocytes, neurons and other cell types where it confers resistance to caspase-mediated (apoptotic) cell death [4, 5] and necrotic cell death induced by oxidative stress or simulated ischemia [6]. The protective potential of HSP70 against myocardial and cerebral ischemia has also been established *in vivo* using gene transfer [7] and transgenic mice [8-10]. Constitutive expression of either human or rat hsp70 gene reduces myocardial and cerebral ischemic injury and improves functional recovery [8-10]. From this evidence, it has been suggested that heat shock preconditioning *in vivo* induces HSP70 accumulation in myocytes or neurons, thereby increasing cellular resistance to ischemic injury. This simple explanation has been challenged by reports demonstrating that heat shock preconditioning *in vivo* does not induce a stress response (measured by HSP70 expression) in myocytes [11] or neurons [12] but rather in endothelial cells [11]. These observations therefore suggest that additional protective mechanism(s) of heat shock preconditioning should be explored within the endothelium and endothelium-related functions.

Besides heat shock preconditioning, induction of a stress response in blood vessels also occurs during balloon angioplasty [13] and in several pathophysiological conditions, such as stroke [14] or atherosclerosis [15, 16]. However, the precise impact of HSP70 and other stress-induced proteins to endothelial patho-physiology is still poorly understood.

Nitric oxide production has a central role in ischemic preconditioning [17-19]. Furthermore, mice deficient in endothelial nitric oxide synthase (eNOS or NOS type III) [20] or rats treated with eNOS inhibitors [21] have larger cerebral infarctions when submitted to permanent occlusion of middle cerebral artery [20]. In contrast, increased endothelial NO production by upregulation of eNOS (by administration of statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA) [22], or by enhanced eNOS activity (by infusion of eNOS substrate L-arginine [23, 24], reduces the cerebral area at risk after middle cerebral artery occlusion. A key step in eNOS activation relies on eNOS subcellular localization and interaction with scaffold/chaperone proteins, such as caveolins. Caveolins localize in membrane invaginations, called caveolae, forming cellular compartments enriched

with proteins involved in signal transduction. In endothelial cells, caveolin-1 binds to eNOS, preventing its interaction with calmodulin and its subsequent activation [25, 26]. Upon stimulation (either following acetylcholine treatment or shear stress), caveolin-1 inhibitory binding with eNOS is displaced by the 90-kDa heat shock protein (HSP90) which favors interaction with calmodulin and increases eNOS activity [27].

Using isolated mouse aortas, here we examined the effect of induction of a stress response on endothelial function. More specifically, we investigated the effect of heat shock and heat shock proteins on NO-mediated vasomotor tone. We observed that heat shock preconditioning decreases the response to vasoconstrictor challenges, increases basal eNOS activity in a HSP90-independent manner and promotes eNOS-HSP70 interaction.

Methods

Animals

The animal protocols have been reviewed and approved by the Massachusetts General Hospital Committee on research, Subcommittee on research Animal Care (SRAC). All mice (6-8 week-old) used in this study had C57Bl/6J genetic background, including eNOS^{-/-} mice (Charles River Laboratories, MA). Mice were fed regular chow, and water was available ad libitum.

Reagents

Antibodies against heat shock proteins (HSP70 and HSP90) and recombinant human HSP70 were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Anti-NOS III (anti-eNOS) and anti-caveolin polyclonal antibodies were obtained from Transduction Laboratories (Lexington, NY, USA), anti-iNOS (anti-NOS II; sc-650) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-nNOS (anti-NOS I; 61-700) and anti-eNOS (anti-NOS III monoclonal antibody; 33-4600) antibodies from Zymed (Oxnard, CA, USA). All additional reagents including phenylephrine, acetylcholine, N(G)-nitro-L-arginine methyl ester, sodium nitroprusside and geldanamycin were purchased from Sigma (St Louis, MO, USA).

In Vitro Preparation and Heat Shock Treatment

Mice were killed by decapitation. Aortas were removed and dissected under a binocular microscope into 1.5-2 mm long rings. Aortic rings were placed in 100µl phosphate buffered saline (PBS; pH 7.4) and incubated at room temperature, 42°C or 43.5°C for 15 min, using a PCR thermal cycler (PTC-200, MJ Research, Waltham, MA, USA). After treatment, rings were transferred to 5 ml DMEM supplemented with 10% fetal bovine serum and antibiotics and maintained 18 h in a humidified atmosphere of 5% CO₂ at 37°C. After incubation, rings were rinsed in physiological solution before mounting in a wire myograph.

Measurement of Contractile Tension in Isolated Aortas

Aortic rings were immersed in physiological solution (composition, mM: NaCl, 118; KCl, 4.6; NaHCO₃, 25; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 1.25; glucose, 10; EDTA, 0.025; pH 7.4 at 37°C), threaded onto stainless steel wires (40 µm diameter) and mounted in an isometric myograph (610M, Danish Myo Technology, Aarhus, Denmark) in physiological solution, maintained at 37°C and aerated with a gas mixture of 95% O₂ - 5% CO₂. After equilibration, the normalized passive resting force and the corresponding diameter were determined for each preparation from its own length-pressure curve, according to Mulvany and Halpern [28]. In this aim, the preparation was distended stepwise while recording micrometer reading and force. The stepwise distension was stopped when the effective transmural pressure exceeded 100 mm Hg (13.3 kPa). An exponential curve was then fitted to the internal circumference pressure data and the point of the curve corresponding to 100 mm Hg was determined (IC₁₀₀). The preparation was then relaxed to a circumference equal to 0.9 IC₁₀₀ and held at this degree of stretch for the remainder of the experiment. Contractile responses were recorded into a computer, by using a data acquisition and recording software (Mydaq and Myodata, Danish Myo Technology).

After normalization and 30-min equilibration in physiological solution, the preparations were stimulated with a 100 mM KCl-depolarizing solution (composition, mM: NaCl, 22.6; KCl, 98.8; NaHCO₃, 25; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 1.25; glucose, 10; EDTA, 0.025, pH 7.4 at 37 °C). After washout and 30-min recovery, they were exposed to cumulative concentrations of phenylephrine (PE, 1 nM - 1 µM) followed by cumulative concentrations of acetylcholine (ACh, 1 nM -10 µM). After washout and recovery, rings were exposed to N(G)-nitro-L-arginine methyl ester (L-NAME; 100 µM) and 30 min later to cumulative concentration of PE followed by cumulative concentration of sodium nitroprusside (SNP, 0.1 nM - 1 µM). In some instances, rings were collected, opened and immersed in SDS-loading buffer for Western blot analysis.

Removal of Endothelium

To remove endothelium, anesthetized mice were perfused intracardially with 3 ml phosphate buffered saline (PBS; pH 7.4) followed by 1.5 ml of 0.03% Triton-X100 in water. Aortas were then removed and rings prepared. This treatment did not affect the maximal contractile response to PE, but abolished the relaxing response to 3 µM ACh.

HUVEC Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Manassas, VA, USA) and cultured in M199 supplemented with 20% fetal bovine serum, 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement (ECGS) on collagen-coated dishes (BD Biosciences, San Jose, Ca, USA). Heat shock was performed on exponentially growing cells by placing the culture dish in a 43.5°C water bath for 15 min. After heat shock, cultured cells returned to their incubator for an additional 18 h before being harvested in

RIPA buffer (10 mM HEPES, pH 7.4, 0.1% CHAPS, 2 mM EDTA, 1 mM EGTA, 5 mM DTT, 1 mM PMSF, 10 µg/ml proteinase inhibitor cocktail).

Western Blot Analysis

Western blot analysis was performed using whole aortas lysed in RIPA buffer or single aortic rings collected in denaturing buffer. Lysates were centrifuged at 11,000 x g for 15 min at 4°C. Protein concentration in supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were denatured in SDS-denaturing buffer (5M Tris, pH 6.8), 20% SDS, 20% glycerol, 1M β-mercaptoethanol, boiled for 2 min, separated using SDS-PAGE on 4-20% Tris-glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred onto Immobilon™-P membranes (Millipore Corporation, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline (TBS; 50 mM Tris·HCl, 150 mM NaCl) with 0.05% Tween 20 (Sigma), the membranes were incubated with primary antibody (see reagents). After incubation, membranes were washed in TBS-Tween and incubated with secondary antibodies (1:1,000; Amersham) for 1 h at room temperature. Detection was achieved using a chemiluminescence system (ECL system; Amersham, Buckinghamshire, UK) and exposing to Hyperfilm (Amersham). Equal loading of protein was confirmed by re-probing membranes using an anti-β-Actin antibody (1:5,000; Amersham).

Immunoprecipitation

Immunoprecipitation was performed according to Garcia-Gardena et al. [29]. The samples were placed in immunoprecipitation (IP) buffer (100mM Tris-HCl, pH 7.4, 1% v/v nonidet P-40; 10µl/ml proteinase inhibitor cocktail), homogenized on ice and centrifuged at 12,000 × g for 10 min at 4°C. Equal amounts of proteins were pre-cleaned by incubation with protein G-agarose and normal rabbit IgG for 2 h at 4°C, and then incubated with 2µg of anti-eNOS polyclonal antibody (N30030; Transduction Laboratories) overnight. Protein complexes were then precipitated with protein G-agarose for 2 h at 4°C. The immunoprecipitants were washed 5 times in IP buffer, boiled 2 min in denaturing buffer, separated by 4-20% Tris-glycine SDS-PAGE and transferred to Immobilon™-P membrane. Membranes were then incubated with primary antibodies and processed as described above for Western blot analysis.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). Concentration-response curves were analyzed using two-way ANOVA. Difference between maximal contractions was analyzed using unpaired Student *t*-test or one-way ANOVA, followed by Tukey test. Statistical significance was set at P<0.05.

Results

Induction of Stress Response in Isolated Mouse Aortas by Heat Shock

In order to provide a better control for individual variability, we first established *in vitro* incubation of isolated mouse aortas; this allowed us to compare untreated vascular segments (control) versus segments in which the stress response (after heat shock) was induced; in this protocol the stress response was defined as a condition where heat shock induced a robust HSP70 expression, which was monitored in each experiment by western blot. In control (not subjected to heat shock) aortic rings, contractile responses to KCl-induced depolarization, phenylephrine, or acetylcholine were not changed by 18 h-incubation in culture medium (data not shown). Since incubation did not change physiological responses of isolated aortic rings, we used this *in vitro* model to examine the effect of heat shock on vasomotor function. Our *in vitro* approach offered the additional advantage of excluding possible confounding effects, observed after heat shock *in vivo*, such as heat stress-mediated activation of neuronal and/or hormonal axis. In a first series of experiments, aortic rings were subjected for 15 min to either 42°C or 43.5°C. Eighteen hours later, the stress response induction and vasomotor function were examined (Figure 1). A 15-min exposure to 42°C barely affected HSP70 expression in aortic rings, as revealed by Western blot analysis; in contrast, a robust induction of HSP70 expression in isolated mouse aortas was obtained after treatment to 43.5°C for 15 min (Figure 1). Vasoconstriction induced by 1 μ M phenylephrine (PE) was not different in 42°C-heat shocked aortic rings and controls.

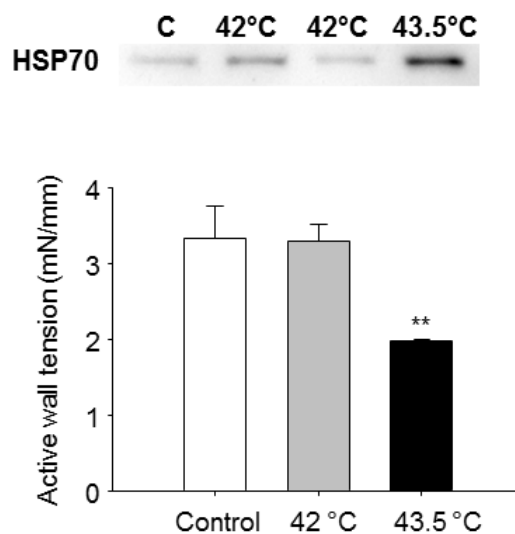


Figure 1. Induction of a stress response as detected by HSP70 expression was accompanied by changes in vasomotor functions. Western blot analysis of HSP70 expression in 4 aortic rings incubated at 37°C (Control, C), 42°C (2 samples in middle lanes) or 43.5°C. The bar graph shows comparison of 1 μ M phenylephrine (PE)-induced contractions between aortic rings incubated at 37°C, 42°C or 43.5°C for 15 min, and then maintained in DMEM (with 10% FBS) for 18 h before physiological analysis. Each column represents the mean (\pm SEM, vertical bars) from 6-8 different rings. Notice the lack of change in PE-induced vasoconstriction following incubation at 42 °C, paralleled by lack of HSP70 induction as detected by western blot. **P<0.01 versus Control; one-way ANOVA and Tukey test.

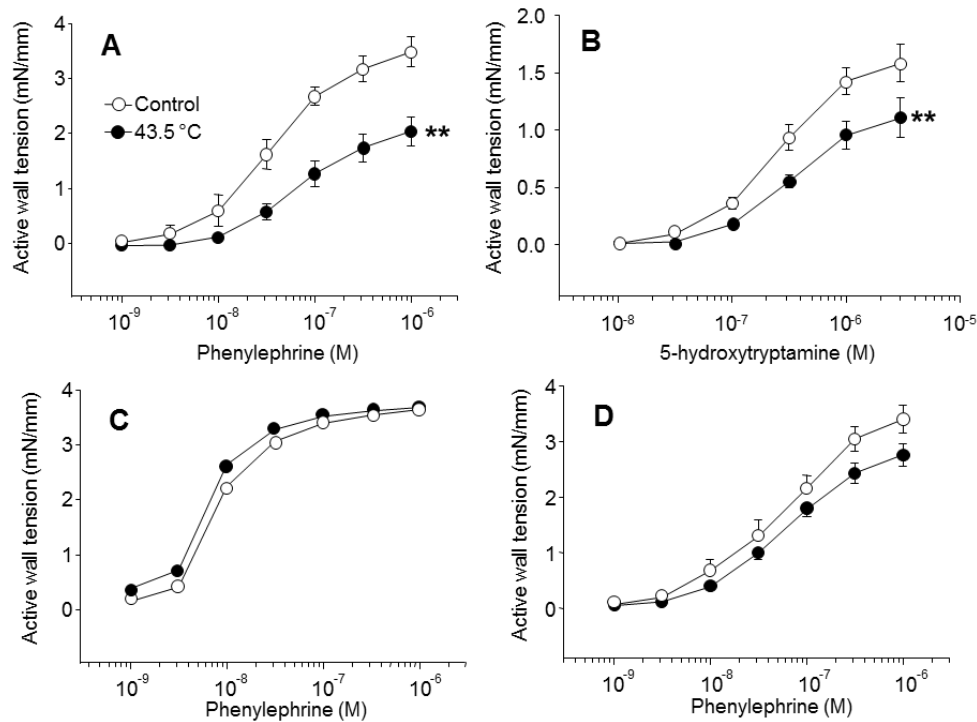


Figure 2. Heat shock effect on vascular tone was mediated by endothelial nitric oxide synthase (eNOS). Active arterial wall tension, induced *in vitro* by cumulative concentrations of phenylephrine (A) or 5-hydroxytryptamine (B), was reduced in heat-shocked (43.5 °C) mouse aortic rings compared to controls. In endothelium-denuded rings (C) and in rings from eNOS deficient mice (D) the effect of heat shock on phenylephrine-induced tone was not significant. Each curve represents the mean (\pm SEM, vertical bars) from 8-12 different rings. ** $P < 0.01$ versus Control; two-way ANOVA.

Conversely, exposure to 43.5°C, which induced a stress response as detected by HSP70 expression, was accompanied by significant decrease in vasoconstriction to either PE or 5-hydroxytryptamine (5-HT, Figure 1 and 2 A, B). Therefore, 43.5°C temperature was used as heat shock treatment throughout the experiments described. Contraction to 100 mM KCl was also reduced in heat shocked aortas compared to controls (2.54 ± 0.12 mN/mm vs. 3.30 ± 0.34 mN/mm, respectively; $p < 0.05$).

Heat Shock Effect on Vascular Tone Was Mediated by Endothelial Nitric Oxide Synthase (eNOS)

Basal (unstimulated) release of NO from endothelium modulates the responses of isolated vessels to vasoconstrictors, such as PE and 5-HT [30]. To assess if the decrease of contraction to PE after heat shock involved endothelial mechanisms, we tested some endothelium-denuded isolated aortas (see also methods) in the same experimental paradigm.

Control and heat shocked aortic rings without endothelium showed similar contraction to cumulative concentrations of PE (Figure 2 C), indicating that heat shock exerted its effect through endothelium. More detailed evidence was obtained using eNOS deficient mice, were

heat shock did not change PE-induced contraction (Figure 2 D). Furthermore, the treatment with L-NAME, a NOS inhibitor, abolished the difference in PE-induced contraction between heat shocked and control aortas (Figure 3 A), suggesting that heat shock did not affect contractile function *per se* but rather increased basal NO production. In order to exclude the potential involvement of inducible NOS (iNOS or NOS II), we tested also aortas from iNOS null mice. These preparations responded to heat shock in a manner similar to aortas from wild type (Figure 3 C), ruling out iNOS as potential mediator of increased basal NO production in this system.

Finally, because heat shocked and control aortas showed similar relaxation to the NO donor, sodium nitroprusside (Figure 3 D), we could exclude an effect of heat shock on signaling downstream to NO and/or in the ability of smooth muscle cells to relax in response to NO. Thus, these results indicated that heat shock treatment increased NO production in isolated aortas, through an increase of eNOS activity under basal conditions.

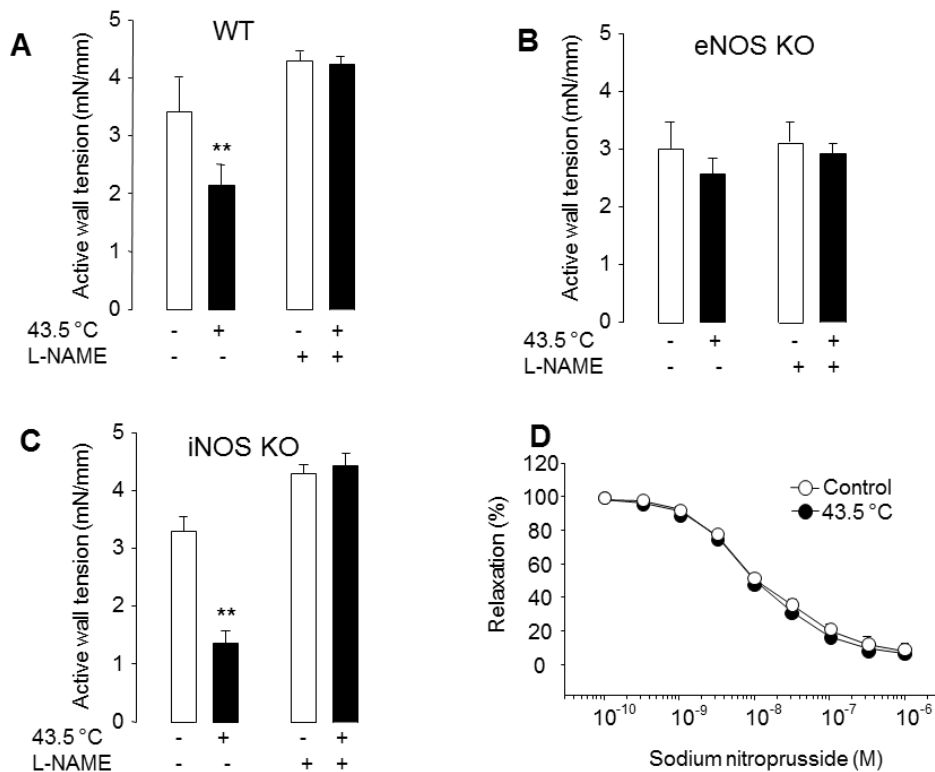


Figure 3. Heat shock effect on vascular tone increased basal NO (was abolished by L-NAME), without changing sensitivity to exogenously added NO. Active arterial wall tension to 1 μ M phenylephrine, reduced in heat-shocked (43.5 °C) aortas from wild type mice (WT, A) or iNOS deficient mice (iNOS KO, C), was restored to control level by incubation with the nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME; 100 μ M). Notice, in (B), the absence of effect of heat shock and/or L-NAME in rings from eNOS deficient mice. In (D), concentration-dilatation curves to cumulative concentrations of the NO-donor sodium nitroprusside, in control and heat-shocked rings from control wild type mice. Each column or curve represents the mean (\pm SEM, vertical bars) from 8-12 different rings. **P<0.01 versus Control; one-way ANOVA and Tukey test.

Stress Response Induction Increased the Expression of Heat Shock Proteins but Not of Nitric Oxide Synthase Isoforms

Both HSP90 and HSP70 were constitutively expressed in aortas as shown by Western blot analysis. After heat shock, HSP90 was slightly increased (Figure 4) while HSP70 expression was markedly increased (Figure 1). We also examined whether heat shock altered the expression of caveolin-1, a protein exhibiting an inhibitory interaction with endothelial NOS [25, 29]. Western blot analysis revealed that caveolin-1 was unchanged after heat shock in isolated aortas (not shown). The effect of heat shock on expression of NOS isoforms was also examined by Western blot analysis (Figure 4). Neuronal NOS (nNOS, NOS type I) or inducible NOS (iNOS, NOS type II) were barely detected in control aortas and did not increase in heat shocked aortas. Finally and more importantly, eNOS expression was not increased after heat shock (Figure 4). These data therefore showed that increased basal NO production after heat shock could not be explained by an increased expression of any NOS isoform (including non-endothelial isoforms).

Inhibition of HSP90 Did Not Inhibit the Effect of Heat Shock on Phenylephrine-Induced Vasoconstriction

Under stimulated conditions, i.e. when challenging with endothelium-dependent vasodilators, HSP90 has an activator effect on eNOS activity, which can be blocked by geldanamycin, an inhibitor of HSP90 [27]. To determine whether or not the increase in HSP90 observed after heat shock (Figure 4) was related to endothelium-dependent inhibition of vasoconstriction to PE, we challenged some aortic rings with geldanamycin (20 μ M).

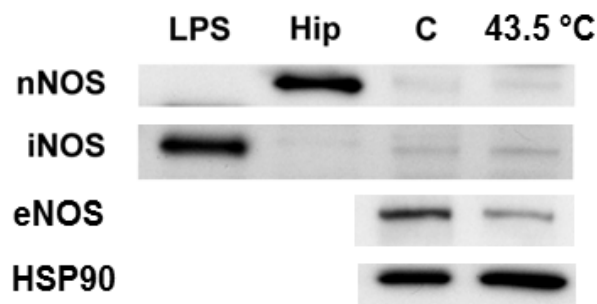


Figure 4. Induction of a stress response increased expression of heat shock proteins (HSP) without changing the expression of any NOS isoform. Western blot analysis for nitric oxide synthase isoforms was carried out in control (C) and heat-shocked (43.5 °C) aortic rings. Expression of neuronal NOS or NOS I (nNOS), inducible NOS or NOS II (iNOS) and endothelial NOS or NOS III (eNOS) were not increased by heat shock. Because nNOS and iNOS were barely detectable in mouse aorta, mouse hippocampus (Hip) and LPS-treated mouse microglial N11 cells (LPS) were run as positive controls for nNOS and iNOS, respectively. Expression of 90 kD heat shock protein (HSP90) was increased by heat shock, as expected.

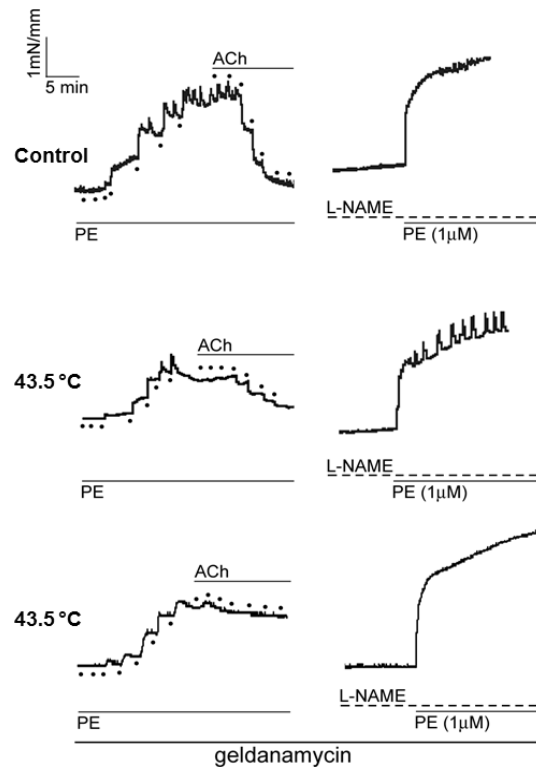


Figure 5. Inhibition of HSP90 did not inhibit the effect of heat shock on phenylephrine-induced vasoconstriction. Typical experiment repeated three times with similar results. Cumulative concentrations (1 nM -1 μ M) of phenylephrine (PE) followed by ACh, were added to the organ chamber by half log increase, as indicated by black dots on the tracing. After a first run, preparations were washed and allowed to recover for 30 min, incubated with 100 μ M L-NAME, and challenged again with 1 μ M PE. Upper trace, Control; middle trace heat-shock; lower trace heat-shock + 20 μ M geldanamycin (HSP90 inhibitor). Notice that the contractile response evoked by PE in the absence of L-NAME is depressed by heat shock, regardless of geldanamycin-treatment (as further demonstrated by the increase in contraction with L-NAME, in both middle and lower traces). In contrast, relaxation to ACh is abolished by geldanamycin but is unaffected by heat shock.

As expected [27], geldanamycin blocked acetylcholine(ACh)-induced relaxation, indicating that inhibition of HSP90 and thereby of ACh-stimulated eNOS activity, effectively occurred in our system (Figure 5); however, geldanamycin failed to restore the contractile response to PE in heat shocked aortic rings, indicating that increased basal NO production was unrelated to HSP90, while L-NAME was able to restore PE-induced maximal contraction in heat shocked aortas, in a similar manner, with or without geldanamycin (Figure 5).

HSP70 Interacts with eNOS after Heat Shock

Protein-protein interaction between eNOS and the inducible HSP70 was examined in isolated aortas after heat shock using immunoprecipitation with anti-eNOS polyclonal antibody (Figure 6). HSP70 was detected in eNOS- immunoprecipitants from heat shocked aortas but not from controls, although there was constitutive HSP70 expression in isolated

aortas without heat shock treatment (Figure 1). Similar results were also observed in eNOS-immunoprecipitants from heat shocked HUVEC using either monoclonal or polyclonal anti-eNOS antibodies (not shown). In addition, eNOS expression was similar in control and heat shocked aortas (Figure 6), while HSP90 was not co-immunoprecipitated with eNOS, either in control or heat shocked aortic rings (not shown).

HSP70-eNOS in vitro interaction was further examined by measuring eNOS enzymatic activity as conversion of L-arginine to L-citrulline. As shown in Figure 7, addition of recombinant human HSP70 to recombinant bovine eNOS significantly increased eNOS enzymatic activity, which was strongly inhibited when L-NAME had been included in the incubation medium.

Discussion

Induction of heat shock proteins, especially HSP70, in blood vessels is a relatively common phenomenon observed in various experimental models and in some pathophysiological conditions [31, 32]. Induction of HSP70 in vascular endothelium occurs following heat shock preconditioning, suggesting a central role of blood vessels in heat shock-mediated protection against myocardial ischemia [11]. In this report, we show that heat shock preconditioning modified blood vessel physiology and endothelial function. More specifically, heat shock reduced blood vessel vasoconstriction to various stimuli (high K^+ -induced depolarization, 5-hydroxytryptamine, phenylephrine) by increasing eNOS activity under basal conditions, via a mechanism independent of HSP90. The other potential candidate as modulator of eNOS activity was HSP70, because we found that it was strongly induced by heat shock in our system. In this respect, we showed, by immunoprecipitation, that eNOS and HSP70 interacted in heat shocked aortas or HUVEC, and confirmed, in a cell-free assay, that HSP70 can increase eNOS enzymatic activity. These observations indicate that HSP70 interacts with eNOS and increases its enzymatic activity, opening new therapeutic targets which could be relevant in pathological conditions such as myocardial infarction and stroke.

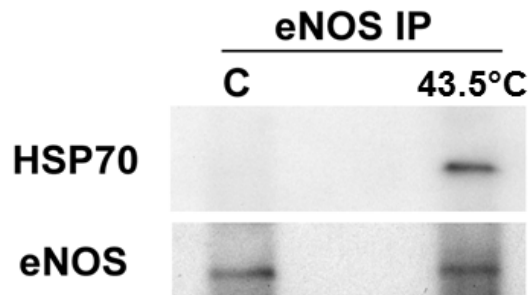


Figure 6. Interaction between HSP70 and eNOS in vascular endothelium. Typical experiment repeated three times with similar results. Protein lysates (120 μ g) from control (C) and heat shocked (43.5°C) mouse aorta were immunoprecipitated using eNOS polyclonal antibody and further analyzed by SDS-PAGE and Western blot, using HSP70 antibody. The same membrane was rinsed and probed again using a specific eNOS monoclonal antibody to show equal amount of eNOS in both lysates. Similar results were obtained by immunoprecipitation of lysates from heat shocked HUVEC.

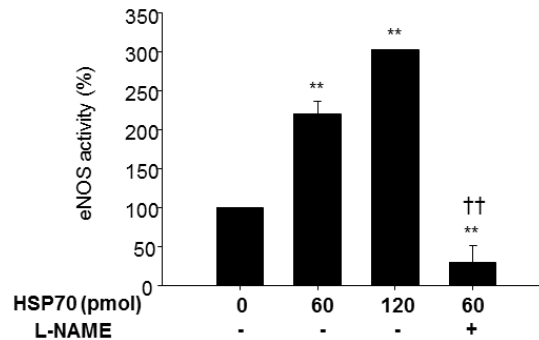


Figure 7. Interaction between HSP70 and eNOS in a cell free system. Recombinant bovine eNOS was incubated in Tris buffer with various amount of recombinant human HSP70 in absence or presence of L-NAME. After 15 min incubation, NOS activity was determined by measuring conversion of L-arginine to L-citrulline. Each column represent the mean (\pm SEM, vertical bars) from two different experiments, each performed in triplicate. ** $P < 0.01$ versus HSP70 0, †† $P < 0.01$ versus HSP70 60 pmol; one-way ANOVA and Tukey test.

The *in vitro* induction of the stress response in aortic segments, as defined by HSP70 expression, occurred after heat shock at 43.5 °C in our system, but not after heat shock at 42 °C. This is consistent with induction of a stress response in cultured endothelial cells where HSP70 expression and cell resistance to injury have been observed after exposure to 43°C for 1.5 h [33]. In contrast, *in vivo*, a single hyperthermic episode at 42°C for 15 min may be sufficient to induce an endothelial stress response measured by HSP70 expression [11, 34], suggesting that additional factors *in vivo* contribute to lower the threshold for induction of a stress response.

Our data show that the reduction of contractile responses induced by heat shock was caused by increased NO production, but was not due to higher expression of NOS isoforms, in particular of eNOS, as detected by western blotting. Therefore, heat shock preconditioning differs from other treatments, such as endotoxic shock and LPS-treatment, (that induce iNOS gene expression) [35], or statin administration (that stabilizes eNOS mRNA) [36], because it increases NO production in blood vessels without changing the abundance of eNOS or iNOS. The fact that heat shock-induced vasomotor changes required the presence of endothelium and were absent in eNOS deficient mice, indicates that heat shock acted through a modification of eNOS enzymatic activity.

Interestingly, in our system, heat shock increased basal eNOS activity without changing eNOS activity stimulated by ACh, at variance with the effect of the activator protein HSP90, which has been shown to interact with eNOS following agonist-stimulated endothelium-dependent vasodilation [27]. Although HSP90 was slightly increased by heat shock in our system, three arguments indicate that heat shock-increased basal eNOS activity did not involve HSP90. Firstly, agonist (ACh)-mediated relaxation in heat shocked aortas was not increased but, rather, it was reduced (not shown). This effect is opposite to that reported in HSP90-transfected endothelial cells [27]. Secondly, inhibition of HSP90 (using a specific HSP90 inhibitor, geldanamycin) did not restore contractility in heat shocked aortas while still blocking ACh-mediated relaxation, confirming that HSP90 regulates eNOS upon agonist stimulation but not basal eNOS activity [27]. Finally, HSP90 only barely co-immunoprecipitated with eNOS in control or heat shocked aortas (not shown), confirming other reports where HSP90-eNOS interaction, drastically increased after agonist stimulation,

was barely detectable under basal conditions [27, 37]. From these results, it appeared that heat shock enhanced eNOS activity via a mechanism independent of HSP90.

Another mechanism reported to increase basal eNOS activity involves downregulation of caveolin [38]. However, when probing for caveolin-1, we found that heat shock did not modify the total amount of caveolin-1 in isolated aortas (not shown). Since heat shock has been reported to acutely modify caveolin localization in cultured mouse NIH3T3 fibroblasts and to induce internalization of caveolin followed by a rapid (within 4 h) return to plasma membrane [39], it is also possible that long-term effects could take place after heat shock and account for increased eNOS basal activity. Further investigations are needed to clarify the role of caveolin and caveolin-eNOS interaction after heat shock preconditioning.

We showed that HSP70 co-immunoprecipitated with eNOS in mouse aortas and HUVEC. Although reverse immunoprecipitation experiment (detection of eNOS in immunoprecipitated proteins using HSP70 antibody) was not feasible due to high amount of proteins competing for HSP70 binding after heat shock preconditioning, we observed HSP70-eNOS interaction in various paradigms (cell culture or cell-free assay), in different species (mouse and human) and using monoclonal or polyclonal anti-eNOS antibodies, which strengthens the observation in isolated blood vessels. Furthermore, addition of recombinant HSP70 could increase eNOS activity in a cell-free assay, indicating that HSP70-eNOS interaction may have a functional outcome.

Obviously HSP70 is known to chaperone denatured proteins after treatment with elevated temperatures, which helps in guiding their refolding and/or triage to the proteasome [40]. In our system, however, eNOS did not appear to be denatured, as indicated by a maintained ACh-mediated relaxation in mouse aortas immediately or 5 h after heat shock (not shown). Moreover, one would not expect a recovered activity to be significantly increased compared to initial conditions. It seems more likely that HSP70 interacts with native eNOS. We propose that after heat shock, HSP70-binding domains are exposed due to post-translational modifications of eNOS or removal of masking protein(s). Indeed, HSP70 is able to interact with co-chaperones, such as CHIP (carboxyl terminus of Hsp70-interacting protein), which regulates eNOS activity and trafficking between subcellular compartments [41]. Further experiments are needed to determine the precise molecular mechanism through which HSP70 modulates eNOS activity following heat shock preconditioning.

Our data cannot rule out that additional mechanisms regulating eNOS activity, such as fatty acylation or phosphorylation of eNOS or substrate/co-factor availability, could also contribute to heat shock-mediated effects. Analyses of eNOS phosphorylation state, eNOS subcellular localization and *in vitro* NOS activity will help in clarifying these issues. Preliminary experiments revealed that heat shock did not increase the amount of Akt or phosphorylated isoforms of Akt (not shown), a kinases that phosphorylates eNOS and increases its activity [42].

Our findings are important for several reasons. Firstly, we provide a mechanistic basis to explain heat shock (and HSP70) protective effect *in vivo* against myocardial and cerebral ischemia. Indeed, increase in basal endothelial NO production enhances blood supply to ischemic tissue as shown after eNOS upregulation, and results in decreased infarct size [24]. Similarly, NO increase could also provide explanation for heat shock-mediated prevention of leukocyte adherence after ischemia/reperfusion injury [43].

Secondly, we showed that HSP70 interacts with eNOS under stressful conditions, but it is likely that similar interactions also occurs in pathological conditions known to induce HSP70

synthesis in endothelial cells and blood vessels. For example, endothelial cells overexpress HSP70 in response to exposure to oxidized LDL [16] and HSP70 has been observed in atherosclerotic human arteries [44] or aortas after physiological stress [45]. Our data provide evidence that HSP70 interacts with eNOS and could thereby modify eNOS function, giving a new perspective to previous observations.

Thirdly, regulation of NO production by endothelium is of paramount importance in pathological conditions such as stroke, atherosclerosis, hypertension to mention a few. For example, stroke prevention favors treatments with statins that increase basal eNOS activity. Here we observed that heat shock preconditioning also increase basal eNOS activity without eNOSupregulation, suggesting that alternative (and may be synergistic) mechanisms may be explored and provide new therapeutic targets in the treatment of vascular diseases.

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