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Cross-Talk Between Nitric Oxide and HSP70 in the Antihypotensive Effect of Adaptation to Heat

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Summary

In this work, we evaluated the effect of adaptation to heat on the fall of blood pressure (BP) induced by heat shock (HS) and the interrelation between nitric oxide (NO) and heat shock protein, HSP70. Experiments were carried out on Wistar rats. It was shown that HS resulted in a generalized and transient increase in NO production (the electron paramagnetic resonance method) and a fall of BP from 113±3 to 88±1 mm Hg ($p<0.05$). Adaptation to heat itself did not affect BP, but completely prevented the NO overproduction and hypotension induced by HS. The adaptation simultaneously increased the brain NO-synthase content and induced HSP70 synthesis (the Western blot analysis) in various organs. Both the antihypotensive effects of adaptation and HSP70 accumulation were completely prevented by L-NNA, an inhibitor of NO synthesis, or quercetin, an inhibitor of HSP70 synthesis. The data suggest that adaptation to heat stimulates NO synthesis and NO activates synthesis of HSP70. HSP70, which hampers NO overproduction, thus restricts the BP fall induced by heat shock.

Key words

Nitric oxide • Heat shock protein • Heat shock • Adaptation • Hypotension

Introduction

Acute fall of blood pressure (Manukhina *et al.* 1996), ischemic injury of cerebral cells, and brain edema (Le Greves *et al.* 1997) are important causes of lethality in heat shock. It is well known that an effective means of enhancing the organism's resistance to the detrimental action of an environmental factor is a prior adaptation to intermittent exposure to the same factor but of lesser intensity (Meerson 1984, 1991, Meerson and Malyshev 1993). However, the question concerning the molecular mechanisms of adaptive defense against hypotension is still open. In the present study, we based our premises on the following established facts 1) the fall of blood pressure induced by heat shock is related to nitric oxide (NO) overproduction (Hall *et al.* 1987, Malyshev *et al.* 1995), 2) overactivation of brain NO-synthase (bNOS) plays an important role in severe brain injury (Sharma *et al.* 1997) induced by heat shock, 3) central NO production is involved in regulation of systemic blood pressure (Gerová *et al.* 1995, Cabrera *et al.* 1996, Nurminen *et al.* 1997), 4) NO takes part in the activation of heat shock protein 70 (HSP70) synthesis (Malyshev *et al.* 1996, Kim *et al.* 1997), and 5) HSP70 limits the

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activation of NO synthase (Hauser *et al.* 1996). Taken together, these data suggested an important role of the interrelation between NO and HSP70 in the antihypotensive effect of adaptation to heat.

In the range of physiological concentrations, NO ensures appropriate regulation of the immunological, cardiovascular and nervous systems, platelet aggregation, etc. (Cooke and Tsao 1993, Bredt and Snyder 1994, Moncada 1994). However, at increased concentrations, NO is transformed into a detrimental factor which sharply decreases blood pressure, interferes with protein synthesis and DNA structure, and causes mitochondrial dysfunction and apoptosis (Marin and Rodriguez-Martinez 1997, Brune *et al.* 1998, Stoclet *et al.* 1998).

The HSP70 is the universal basis of intracellular defense. Typical manifestations of cellular stress include aggregation of denaturated proteins, protein degradation, free-radical oxidation, calcium overload and, in some cases, the development of apoptosis. Heat shock protein can restrict these disorders 1) at the expense of disaggregation of denaturated proteins (Pelham 1986), 2) by the utilization of damaged protein (Hershko 1988), 3) by induction of antioxidant enzymes (Privalle and Fridovich 1987), 4) by restriction of calcium-induced damage (Stevenson and Calderwood 1990), and finally 5) by preventing the activation of specific protein kinases (Gabai *et al.* 1998) that participate in apoptosis.

Accordingly, the interrelation between NO and HSP70 can serve as an example of coupling between regulatory molecules and endogenous defense systems.

In analyzing the interrelation between NO and HSP70 in adaptive defense, we studied 1) changes in NO production, the content of brain NOS and HSP70 during adaptation, 2) the effect of adaptation to heat on the NO overproduction induced by heat shock, and 3) the effect of HSP70 transcription inhibitor on the ability of adaptation to restrict the NO overproduction and, *vice versa*, the effect of NO-synthase inhibitor on the ability of adaptation to induce HSP70accumulation.

Methods

Experiments were carried out on Wistar male rats weighing 250-300 g.

Heat shock was inflicted by heating of conscious animals in a thermostat until the core temperature of 42 °C. The heating was then continued for additional 15 min (Currie *et al.* 1988). The total duration of heating did not exceed 30 min. Blood pressure was measured in conscious rats by the tail-cuff method.

Adaptation to a moderate heat was performed by repeated brief heat exposures of rats at the core temperature of 41 °C daily for 6 days. The duration of heat exposures was increased gradually from 5 to 10 min.

To measure NO production in rat tissues we used the capacity of NO to react with ferrous diethyldithiocarbamate (DETC) (Sigma, USA) resulting in formation of paramagnetic mononitrosyl iron complexes. This method has been described in detail elsewhere (Vanin *et al.* 1984, Mulsch *et al.* 1992). The electron paramagnetic resonance (EPR) signal from the samples was recorded on a EPR-radiospectrometer Radiopan (Poland) at 77 °K, with field modulation amplitude 0.5 mT and wave power 10 mW.

HSP70 was measured in the cytosolic fraction. The heart, brain or liver tissue was ground and placed into a hypotonic buffer (10 mM Tris, 10 mM KCl, pH 7.4) for 10 min at 4 °C. Then the tissue was homogenized in the same solution at the buffer:tissue ratio 5:1 (w/w). The obtained homogenate was filtered through eight gauze layers and centrifuged at 12 000 x *g* and 4 °C for 10 min. The supernatant containing cytosolic proteins was taken for electrophoresis and blotting. Electrophoresis was performed according to Laemmli (1970). Proteins were separated in 7 % PAAG. Proteins were transferred from PAAG onto a nitrocellulose membrane according to Towbin (1979). Western blots were incubated in the presence of monoclonal antibodies against HSP70 (Amersham, United Kingdom). After washing, the blots were incubated in the presence of horseradish peroxidase-conjugated anti-mouse Ig (Amersham, United Kingdom). Finally, bands of labeled antigen were detected by diaminobenzidine staining.

Brain NOS was measured in the brain tissue which was ground and placed into a buffer (0.5 M Tris, pH 6.8; 4 % SDS; 15 mM dithiotreitol; 0.2 mM PMSF; 10 μg/ml leupeptin; 10 μg/ml pepstatin; 10 μg/ml aprotinin) at 4 °C. Further treatment and electrophoresis were performed as described above. Western blots were incubated in the presence of polyclonal antibodies against bNOS (Biomol Research Laboratories, Inc.). After washing, the blots were incubated in the presence of alkaline phosphate-conjugated goat anti-rabbit IgG (Bio-Rad, USA). Finally, bands of labeled antigen were detected using a Bio-Rad Immuno-Blot Assay kit.

Quercetin (5 mg/kg body weight, i.p.) was used as a blocker of HSP70 transcription (Kukreja *et al.* 1995).

N^{ω}-nitro-L-arginine (L-NNA) (Merck, Germany) (15 mg/kg body weight, i.p.) was used as a NO synthase inhibitor. Both quercetin and L-NNA were injected one hour before each adaptive heat exposure.

The results were statistically evaluated with Student's t-test and presented as mean ± S.E.M.

Results

Adaptation to heat effectively protected the organism against acute hypotension in heat shock (Fig. 1). Heat shock induced a fall of blood pressure from 113±3 to 88±1 mm Hg ($p<0.05$). Adaptation itself did not affect blood pressure but completely prevented the hypotension induced by heat shock. The protective effect did not develop until day 6 of adaptation.

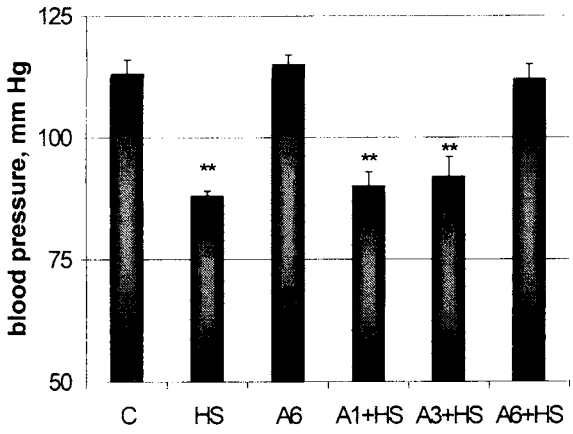

Fig. 1. *Effect of adaptation to heat on the fall of blood pressure induced by heat shock. Ordinate: blood pressure in mm Hg. C – control; HS – heat shock; A1, A3, A6: the 1st, 3rd and 6th days of adaptation to heat, respectively. Significant differences from the control, ** $p<0.01$*

Table 1. The effect of heat shock and adaptation to heat on the NO production.

Experimental groups	NO production (ng/g tissue) Liver	Brain	Heart
Control (n=10)	40.3±8.2	43.7±10.5	0
Heat shock (n=10)	233.2±16.1[a]	83.1±12.2[a]	7±2[a]
Adaptation to heat on the first day (n=10)	30.9±9.7	40.1±9.8	0
Adaptation to heat on the third days (n=10)	33.1±8.3	37.0±7.0	0
Adaptation to heat on the sixth day (n=10)	42.5±5.0	34.3±11.1	0
Adaptation to heat on the sixth day + heat shock (n=10)	29.7±10.1	45.5±7.4	0

[a] *Significant differences from the control ($p<0.05$)*

Table 1 presents quantitative data on tissue NO production in the controls, after heat shock, after adaptation to heat and after a heat shock against the background of prior adaptation. It can be seen that adaptation itself did not influence the NO production but completely prevented NO overproduction induced by heat shock. These data indicate that adaptation to heat induces some mechanism restricting the NO overproduction and that the mechanism may underlie the antihypotensive effect of adaptation.

The idea about the nature of this mechanism began to develop when changes in HSP70 and bNOS contents were compared to the development of the protective effects of adaptation. Figure 2 demonstrates that in the course of adaptation, the accumulation of HSP70 did not occur immediately but after a lag period of six days. Unlike HSP70, a small increase in bNOS content was observed as early as after one and three days of adaptation (Fig. 2). On day 6 of adaptation, the increase in bNOS content was substantial. A comparison of the time course of HSP70 and bNOS in adaptation shows that the increase in bNOS content precedes HSP70 accumulation. Furthermore it is important that the antihypotensive effect of adaptation (Fig. 1) developed concomitantly with the accumulation of HSP70 and a substantial increase in the bNOS content (Fig. 2). These

data suggest that HSP70 and NO contributed to the restriction of NO overproduction and thereby to the antihypotensive effect of adaptation to heat.

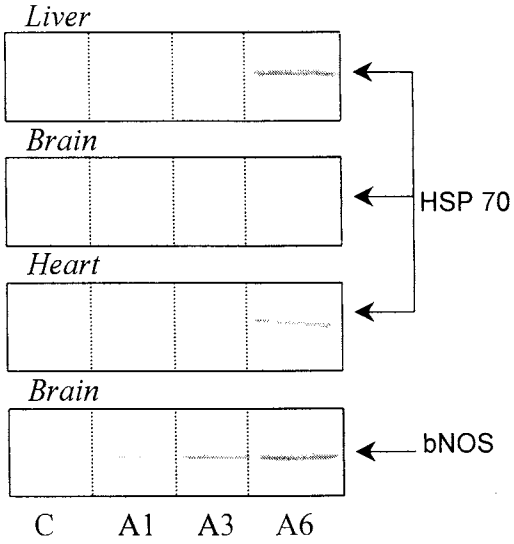

Fig. 2. *Effect of adaptation to heat on the HSP70 and brain NOS content in organs. Results of Western blot analysis are presented. The width and intensity of dark bands reflect accumulation of HSP70 and bNOS. C – control; A1, A3, A6: the 1st[h], 3r[d] and 6[th] days of adaptation to heat, respectively.*

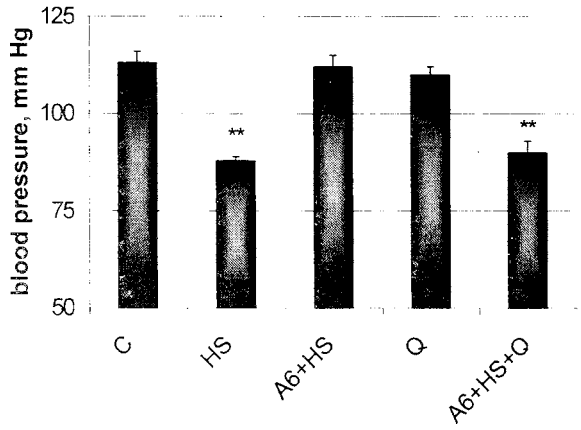

Fig. 3. *Effect of quercetin an inhibitor of HSP70 synthesis, on the antihypotensive effect of adaptation to heat. Ordinate: blood pressure in mm Hg. C – control; HS – heat shock; A6 – on the 6[th] day of adaptation to heat; Q - quercetin. Significant differences from the control, ** p<0.01.*

A confirmation was obtained in the next experimental series. It appeared that the inhibitor of HSP70 transcription quercetin did not influence blood pressure, but abolished the antihypotensive effect of adaptation (Fig. 3). Therefore, the HSP70 accumulation is a real molecular link in the adaptive mechanism, which restricts the NO overproduction and the fall of blood pressure induced by heat shock.

Furthermore, it was important to elucidate the mechanism for activation of HSP70 synthesis in adaptation to heat. Based on the idea about the important role of NO in the activation of HSP70 synthesis (Malyshev *et al.* 1996, Kim *et al.* 1997), we evaluated the effect of NO synthase inhibitor L-NNA on the HSP70 accumulation and the antihypotensive effect of adaptation to heat (Fig. 4). It appeared that L-NNA abolished both the HSP70 accumulation and antihypotensive effect. The data indicate that the increased NO synthesis is a stimulus for HSP70 accumulation in adaptation to heat.

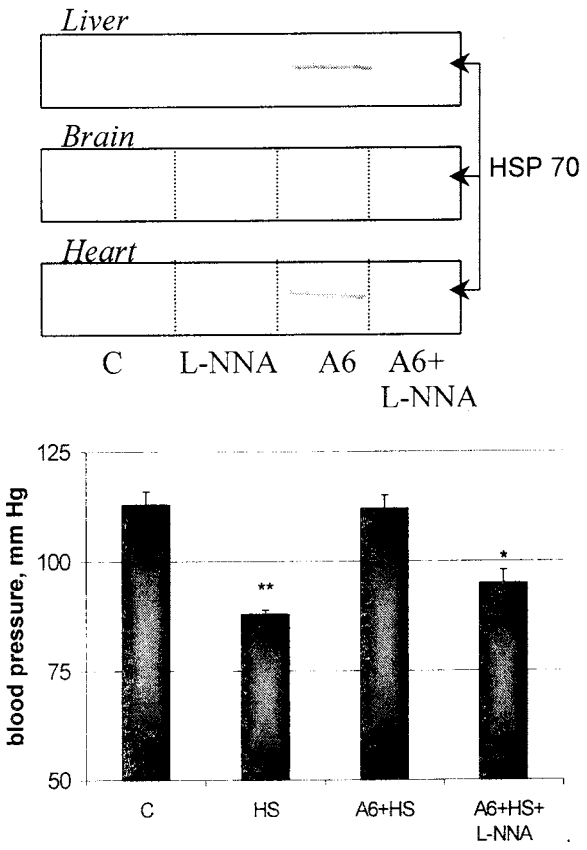

Fig. 4. *Effect of the NO-synthase inhibitor L-NNA on the HSP70 content in and the antihypotensive effect of adaptation to heat. C – control; HS – heat shock; A6 – on the 6[th] day of adaptation to heat. Upper panel: results of Western blot analysis. Width and intensity of dark bands reflect accumulation of HSP70. Lower panel: ordinate: blood pressure in mm Hg.*

Discussion

Increased resistance of the organism to an environmental factor can be achieved by a single prior exposure to the same factor of sufficient intensity. This effect is known as preconditioning. In our experiments, neither one nor three mild heat exposures provided protection against heat shock. The antihypotensive effect only developed after a series of six mild heat exposures. This protection was thus based on adaptation rather than on preconditioning.

The experimental data obtained in the present work expand the knowledge on the molecular mechanisms of adaptation to heat and suggest that the interrelation between NO and HSP70 plays an important role in the adaptive enhancement of resistance to the hypotensive effect of heat shock. However, a seemingly paradoxical situation occurs when evaluating the role of NO in the antihypotensive effect of adaptation to heat. According to the data of EPR-assay, such adaptation was not accompanied by any change in NO production. At the same time, we observed both an increase in the brain NOS content during adaptation and a preventive effect of L-NNA on the antihypotensive effect of adaptation to heat (Figs 2 and 4). These data suggest that the antihypotensive effect of such adaptation may be related to increased NO production.

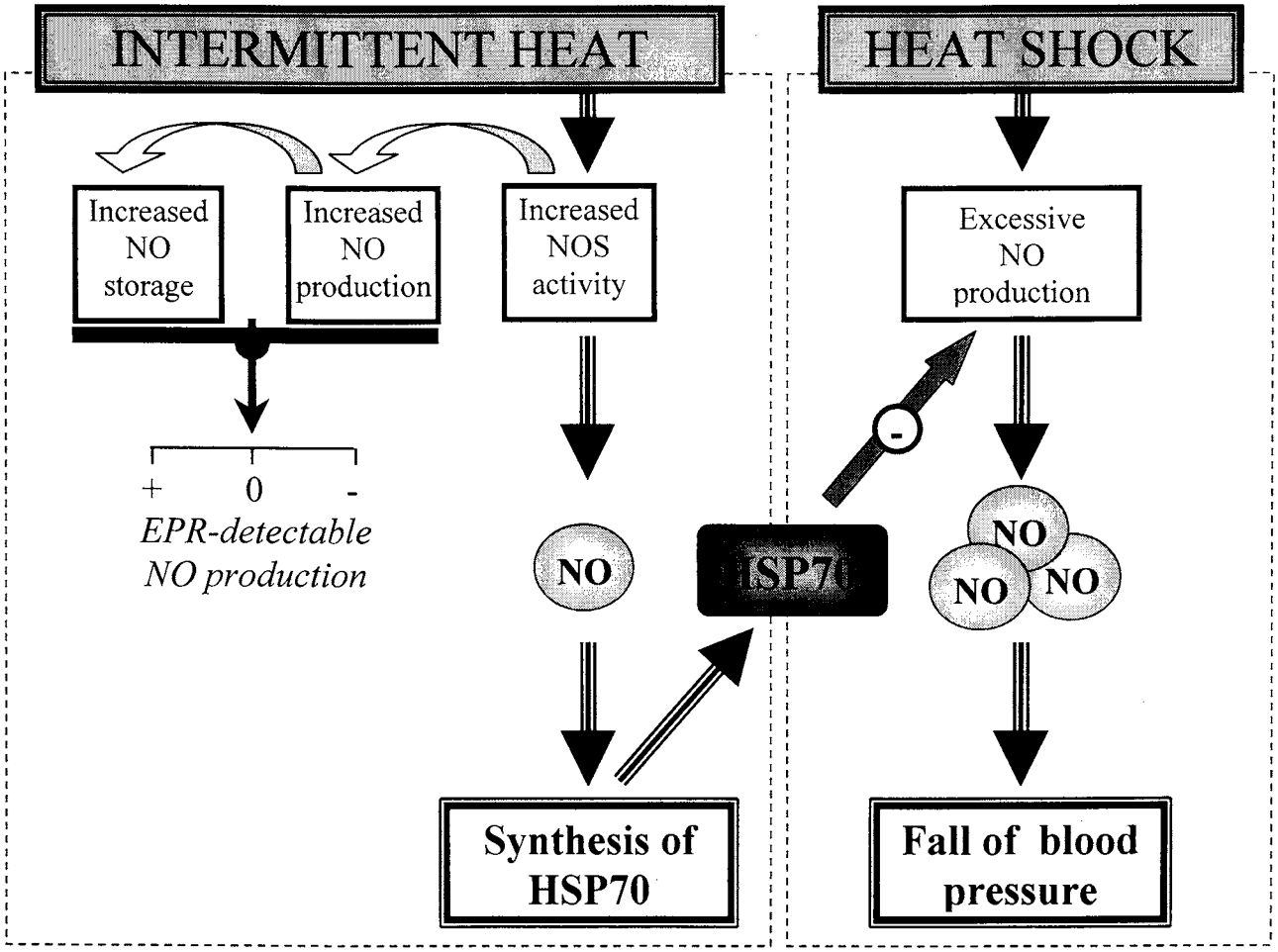

Fig. 5. *Cross-talk between NO and HSP70 in the antihypotensive effect of adaptation to heat (for further explanations see text).*

The paradox may have two hypothetical explanations. The first explanation is based on the assumption that the NOS activity in adaptation to heat is decreased and compensates for the increased NOS expression. However, this is unlikely because L-NNA, the inhibitor of NOS activity, interfered with the development of adaptation. The second explanation is based on the assumption that in adaptation, the bNOS activity is unchanged or increased. In this instance, the unchanged NO production detected in adaptation to heat by the EPR method may be the net result of two opposite processes, namely increased NO synthesis and increased

NO storage. Indeed, it has been shown that increases in NO synthesis induced by diverse factors, including heat exposure, resulted in progressive NO storage (Manukhina *et al.* 1999). In the same study, it was demonstrated on an example of adaptation to hypoxia that NO storage can conceal increased NO production. The diagram in Figure 5 summarizes our ideas about the cross-talk between NO and HSP70 in the antihypotensive effect of adaptation to heat.

In the present study, we have shown that the antihypotensive effect of adaptation to heat develops against the background of an increased bNOS and HSP70 content. The NO synthase inhibitor prevented both HSP70 accumulation and the development of the antihypotensive effect of adaptation to heat. This result led to the following idea. Adaptation to heat stimulates NO production, at least *via* expression of bNOS; NO activates HSP70 synthesis and this enhances the resistance of the organism to heat shock. This protection is apparently based on the well-known cytoprotective properties of HSP70 (Pelham 1986) and on its capacity to limit the factors inducing excessive activation of NO synthases and thereby NO overproduction (Privalle and Fridovich 1987, Stevenson and Calderwood 1990, Hauser *et al.* 1996). The prevention of NO overproduction restricts the fall of blood pressure induced by heat shock.

It can be concluded that the mechanism of coupling between the regulatory molecule NO and the endogenous defense system of heat shock proteins is apparently based on the principle of a negative feedback mechanism. Such mechanism not only provides activation of protective systems but also maintains the concentrations of regulatory molecules within physiological limits and thereby prevents the detrimental effects of their excessive production.

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