Nitric oxide is involved in heat-induced HSP70 accumulation

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Abstract Heat shock potentiated the nitric oxide production (EPR assay) in the liver, kidney, heart, spleen, intestine, and brain. The heat shock-induced sharp transient increase in the rate of nitric oxide production preceded the accumulation of heat shock proteins (HSP70) (Western blot analysis) as measured in the heart and liver. In all organs the nitric oxide formation was completely blocked by the NO-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA). L-NNA also markedly attenuated the heat shock-induced accumulation of HSP70. The results suggests that nitric oxide is involved in the heat shock-induced activation of HSP70 synthesis.

Key words: Heat shock; EPR; Western blot analysis; Nitric oxide; Stress protein

1. Introduction

All known organisms from primitive prokaryotes to higher eukaryotes respond to stresses of the environment by strikingly various ways. At the same time rapid synthesis of stress proteins of the heat shock protein (HSP70) family is a characteristic common feature of cell response to actions of the environment. It has been shown that HSP70 synthesis increases under most various actions: heat shock (HS); treatment of cells with sodium arsenate or a proline analogue; acute myocardial ischemia; acute hypoxia; cooling; etc. [1,2]. These proteins accumulate in cells of atherosclerotic blood vessels [3], in a culture of nerve cells contaminated with herpes virus [4], in hepatocytes in oncologic diseases [5], etc. HSP70 play an important role in the development of adaption to stress [6] and in such adaptive responses as thermal tolerance and early stages of cardiac compensatory hypertrophy [2,7,8]. At the same time the question as what cell mechanisms are involved in the activation of HSP70 synthesis remains open in many respects.

When analyzing this problem, the experimental facts have drawn our attention that the same agents can activate both HSP70 and nitric oxide (NO) synthesis. For instance, lipopolysaccharide, a classic inductor of NO synthesis [9], activate also HSP70 synthesis [10]. On the other hand, HS, which is a conventional way of inducing HSP synthesis [1,2,7], is attended by an increase in blood NO-heme [11]. Besides HS is known to result in a profound fall of blood pressure (BP) [22] which may also indirectly indicate an increase in generation of NO, a potent vasodilator. Our own experiments have demonstrated that, in adaptation of the organism to stress, a considerable activation of HSP70 synthesis in organs [6] occurs against the background of increased NO content in the same organs [12]. A similar situation was observed in inflammation: the NO content grew as the HSP70 content increased in the inflammation focus [13].

Taken together the data suggest that the sytem of NO generation is involved in the activation of HSP70 synthesis.

To verify this hypothesis we studied, first, the effect of HS on the NO content in rat organs, second, the effect of a NO-synthase inhibitor on the HS-induced accumulation of HSP70.

2. Materials and methods

Experiments were caried out on Wistar male rats weighing 250–300 g. The study was conducted in conformity with the policies and procedures detailed in the 'Guide for the Care and Use of Laboratory Animals'.

Heat shock was produced by heating of conscious animals in a thermostat to the core temperature of 41°C. After that the heating was continued for an additional 15 min. The total duration of heating did not exceed 30 min.

To estimate the amount of NO produced in rat tissues we used the capacity of NO to incorporate into ferrous diethyldithiocarbamate [Fe^{2+}-DETC, $(C_5H_{10}NS_2)_2Fe$] Sigma, USA) complexes to form paramagnetic mononitrosyl iron complexes (MNIC) with DETC. These complexes are characterized by electron paramagnetic resonance (EPR) signal with g-factor values $g_{\perp} = 2.035$ and $g_{||} = 2.012$ and a triplet hyperfine structure at $g_{\perp}$ (Fig. 2). The amount of MNIC–DETC complexes in the sample and, thereby, the amount of NO included into the complex was estimated by the signal intensity. The assay of NO by this method is provided by a high stability both of the used NO-trap, the Fe^{2+}–DETC complex and of MNIC–DETC, the product of NO binding to the trap. In fact, this method demonstrates the generation of tissue NO for a definite time interval (30 min) by the accumulation of MNIC with DETC, i.e. the rate of NO generation. This method has been described in detail elsewhere [14–17]. To form MNIC in the organism, we injected Na-DETC ($C_5H_{10}NS_2Na$) (50 mg/100 g body weight, i.p.) and $FeSO_4$ + Na-citrate (Sigma, USA) (20 mg + 95 mg/100 g body weight, s.c.) into the animal 30 min prior decapitation. The rats were decapitated 1, 4 and 24 h following HS. The heart, liver, kidneys, spleen, brain and small intestine were isolated, ground, frozen in a press-form and stored in liquid nitrogen. The EPR signal from the samples was recorded on an EPR-radiospectrometer Radiopam (Poland) at 77°K, with field modulation amplitude 0.5 mT and wave power 10 mW.

Another group of rats was additionally injected with a NO-synthase inhibitor N^{ω}-nitro-L-arginine (Merck, Germany) (20 mg/100 g body weight, i.p.) immediately after the HS termination. The rats were decapitated 1, 4 and 24 h following HS.

Since NO is a potent vasodilator, we used changes in BP, in addition to the direct EPR-assay of NO, as an indirect index of NO production. BP was measured by a bloodless method on the tail artery using a Physiograph DMP-4F (Narco Bio-Systems, USA). Electrophoresis and immunoblotting. Reagents and instruments of the firm Bio-Rad (USA) were used. The HSP70 content was measured in the cytosolic fraction. To this aim the hepatic tissue was ground and placed into a hypotonic buffer (10 mM Tris, 10 mM KCl, 10 mcM phenylmethylsulfonide fluoride (Cal-biochem, USA, 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 homogenate obtained was filtrated through eight gauze

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Abbreviations: BP, blood pressure; DETC, Fe^{2+}-diethyldithiocarbamate; EPR, electron paramagnetic resonance; HS, heat shock; HSP70 heat shock proteins with molecular weight 70 kDa; L-NNA, N^{ω}-nitro-L-arginine; MNIC, mononitrosyl-iron complex; NO, nitric oxide; cNOS, constituitive NO-synthase; iNOS, inducible NO-synthase

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layers and centrifuged at 12,000 × g and 4°C for 10 min. The supernatant containing cytosolic proteins was taken for analysis using electrophoresis and blotting. Electrophoresis was performed according to Laemmli [18]. Proteins were separated on 7% PAGE in the presence of SDS. Protein were transferred from polyacrylamide gels to a nitrocellulose membrane by electroelution according to Towbin [19]. The Western blots were preincubated in a buffer 50 Tris-HCl, 150 mM NaCl, pH 7.4 with low-fat milk (m/V) for 1 h. Then the Western blots were successively incubated in the presence of monoclonal antibodies against HSP70 (Amersham, London, UK) at a 1:500 dilution for 1 h. After washing the blots were incubated in the presence of ^{125}I-labeled antimurine IgG (Sigma) for 1 h. The antibodies were labeled using iodogen [20]. After the incubation with ^{125}I-labeled antimurine IgG, the blots were washed, dried and placed in a casette with X-ray film. HSP70 were detected autoradiographically. The site of the nitrocellulose membrane corresponding to HSP70 was excised and counted with a gamma-counter. To determine the nonspecific antibody binding, two sites were excised from the nitrocellulose band and the radioactivity was counted with a gamma-counter. The specific binding was determined as the difference between the value of radioactivity associated with HSP70 and the average value of nonspecific binding. HSP70 content was expressed in percent of the maximum HSP70 content in the liver 24 h following HS.

Results were statistically treated by the Student's t-test and were presented as mean ± S.E.M.

3. Results and discussion

3.1. HS sharply increases the NO production in rat organs

Fig. 1. demonstrates changes in the rate of NO generation in various rat organs after HS. It is seen that hyperthermia sharply increased the concentration of the MNIC–DETC complex, which reflected a potentiation of NO production in all organs studied. The rate of complex accumulation was at a maximum 1 h after the exposure termination. After 4 h, the NO generation began to decline. In 24 h, it almost did not significantly differ from the initial level. The EPR spectra from the MNIC–DETC complex given in Fig. 2 visualize the phenomenon with respect to hepatic tissue.

In animals treated with L-NNA, the inhibitor of NO synthesis, the MNIC–DETC signal was detected in none of the organs studied both in control and 1, 4 or 24 h after the thermal exposure.

The HS-induced increase in NO generation and the prevention of this phenomenon by L-NNA were reflected by corresponding changes in BP: in the control group, HS decreased BP from 110 ± 3 to 90 ± 2 mmHg (n = 5) (P<0.05) while in the rats injected with L-NNA following HS, BP only slightly decreased from 111 ± 4 to 104 ± 2 mmHg (n = 5).

Thus HS induces a sharp transient increase in NO production. Since the increase in accumulation of NO-containing MNIC–DETC complex was revealed in all organs studied, it is valid to say that this phenomenon is of a generalized nature. The fact that the NO production was completely abolished by L-NNA is evidence that NO synthesized following HS is formed in the NO-synthase reaction.

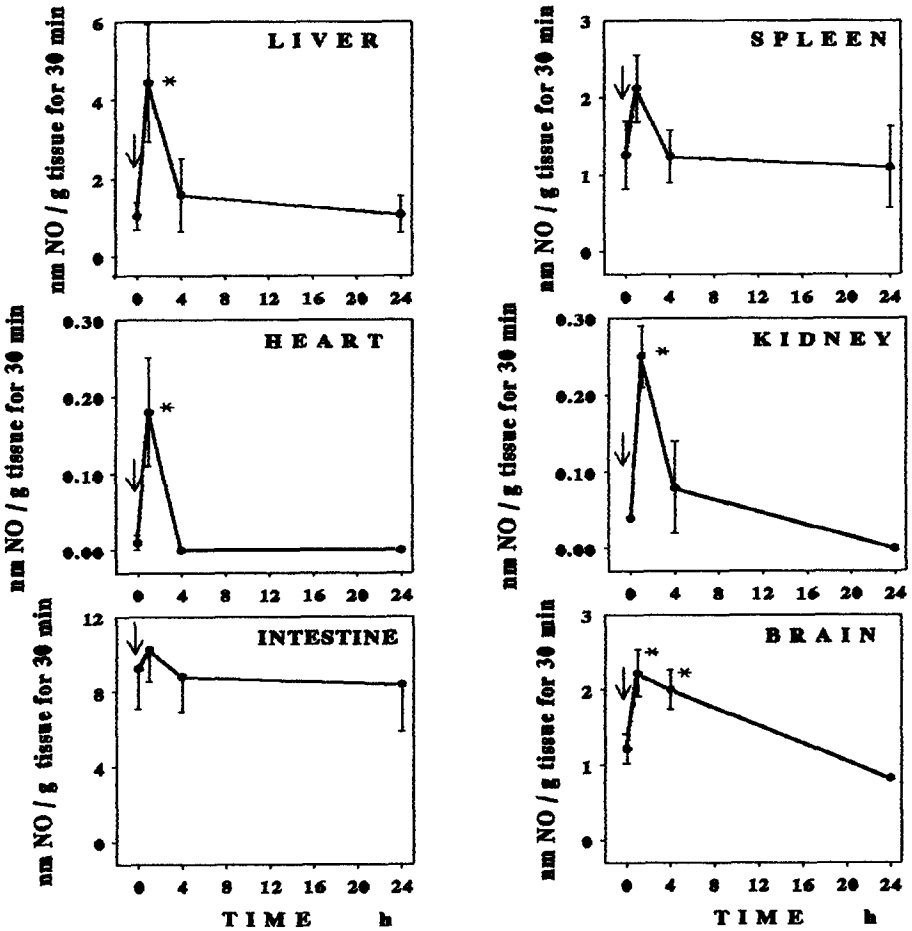

Fig. 1. Time course of NO production in rat organs after heat shock. Abscissa: time after heat shock. Ordinate: the rate of NO generation (nM NO/g wet tissue for 30 min). *Significant differences from the initial value, P < 0.05. The arrow indicates induction of HS that lasted 15 min. Five animals were studied in each series.

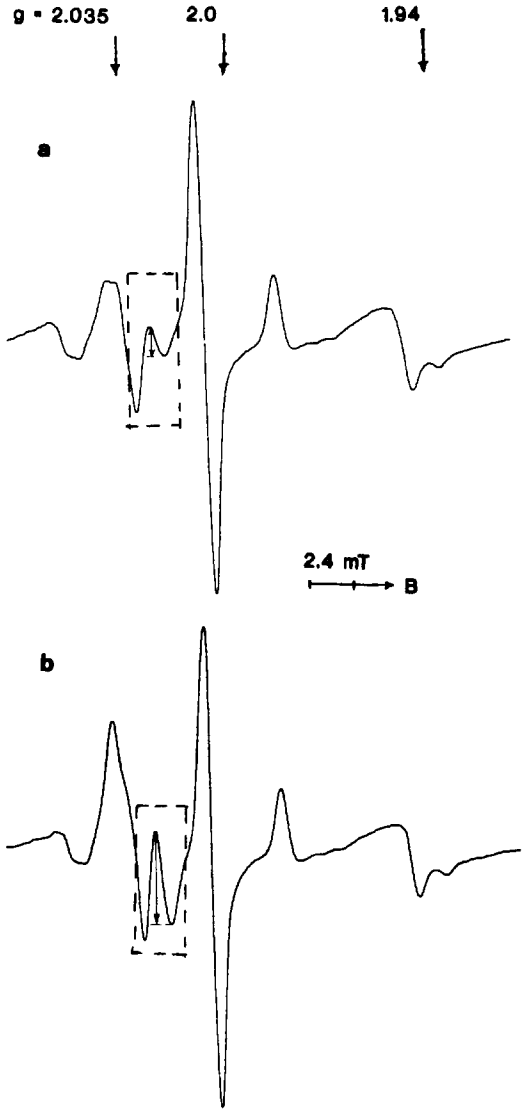

Fig. 2. The EPR signal from MNIC–DETC in the liver of control (a) and HS-exposed (b) rats. Dotted frame indicates the component of triplet hyperfine structure of the EPR signal, by the amplitude of which (as indicated by double arrow) the complex content was estimated.

3.2. *Inhibition of NO-synthase reduces HSP70 synthesis after HS*

The effect of L-NNA on HSP70 accumulation after HS we have estimated using the liver and heart as an example. The organs were chosen because after HS the NO increase was minimum in the heart and maximum in the liver in comparison with other organs (Fig. 1). One hour after HS, when the increase in NO content was maximum, there was no HSP70 accumulation in th eliver or the heart. Twenty-four hours after HS we observed the HSP70 accumulation in both the heart and the liver (Fig. 3, Table 1). Such time course of HSP70 accumulation with a maximum at 24 h is common and corresponds to data of many other investigators [21]. It is important to note that the HSP70 increase following HS was more pronounced in the liver than in the heart (Fig. 3, Table 1). This is in consistence with the greater NO production in the liver than in the heart. The main results of the work presented on Fig. 3 and Table 1 is the following: L-NNA decreased the HS-induced HSP70 accumulation by 75% in the liver and by 45% in the heart.

Table 1
The effect of NO-synthase inhibitor (L-NNA) on HSP70 accumulation in the rat liver and heart after heat shock

Groups of animals	Content of HSP70 (%)	
	Heart	Liver
Control, $n = 4$	0	0
Heat shock, $n = 4$	29 ± 4	100 ± 23
Heat shock + L-NNA, $n = 4$	16 ± 5	24 ± 5*

*Significant differences between the content of HSP70 with and without L-NNA, $P < 0.05$.

Taken together the results are as follows: (i) HS increases the NO content in organs. In this process, the NO increase shows a transient nature characteristic of trigger signals; (ii) After HS, the NO increase precedes the increase in HSP70 accumulation; (iii) the blockade of NO synthesis considerably altered the HS-induced HSP70 accumulation which was evident as a decrease in HSP70 level 24 h after HS; (iv) The greater the NO increase in an organ the greater the HSP70 accumulation in this organ.

On the whole the results support our hypothesis that one mechanism of the HS-induced increase in HSP70 synthesis is related with NO-synthase and increase NO production. It is obvious at the same time that NO is not the only factor of HS-induced activation of HSP70 synthesis. This follows from the fact that the inhibitor of NO synthesis completely abolished the NO generation but only partially reversed the HSP70 accumulation.

This raises two questions: first, what are the mechanisms mediating the HS-induced activation of NO-synthase, and second, what are the mechanisms of stress-protein synthesis activation by NO-synthase through NO generation? Tentative diagram on Fig. 4 reflects an attempt of answering these questions.

HS potentiates generation of free radicals in the body [22]. One intracellular target for free radicals is NFkB, a factor of transcription of many genes including that coding inducible NO-synthase (iNOS) [9]. Therefore the increased NO-synthase activity following HS may be connected with the enhanced concentration of free radicals.

It has been also shown that HS results in a multiple elevation of catecholamine and hormone concentration [23] and, as a result, in an increase of intracellular Ca^{2+}. Therefore another mechanism of potentiated NO synthesis in HS may be related with activation of Ca^{2+}-dependent constitutive NO-synthase (cNOS) isoforms [9] not associated with expression of corresponding genes.

At the present time there are few data to substantiate the mechanism by which NO could activate stress-protein synthe-

Fig. 3. The effect of NO-synthase inhibitor (L-NNA) on HSP70 accumulation in the rat liver and heart after heat shock. 1 – control, heart; 2 – heat shock, heart; 3 – heat shock + L-NNA, heart; 4 – control, liver; 5 – heat shock, liver; 6 – heat shock + L-NNA, liver.

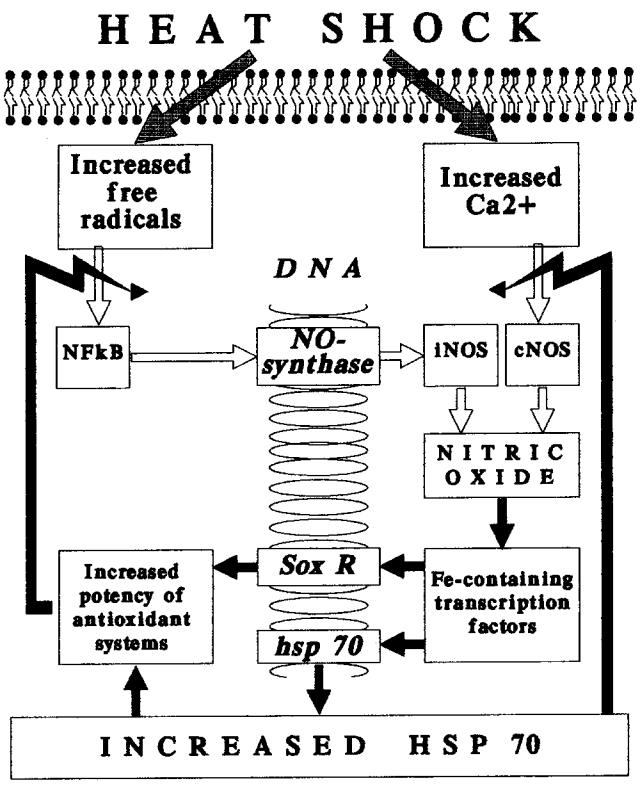

Fig. 4. Tentative interrelations between systems of NO and HSP70 synthesis. For explanation see text.

sis. Moreover, Curran et al. [24] have shown that NO reversibly and dose-dependently suppresses protein synthesis in hepatocytes an inhibits expression of specific hepatocyte proteins albumin and fibrinogen. In our experiments we have established that the HS-induced increase in NO generation is attended by HSP70 accumulation. This suggests that NO selectively influences synthesis of these stress proteins. A principle possibility of NO-activated expression of selected genes may be exemplified by expression of SoxR gene [25]. The latter is presumably provided by NO interaction with non-heme iron in the gene transcription protein. Since it is well known that expression of both soxR and HSP70 genes is induced by oxidative stress, it cannot be excluded that activation of both genes is mediated by the interaction of NO with an iron-containing transcription protein.

It is also possible that NO activates HSP70 synthesis indirectly, through changes in BP. For instance, HS is known to be attended by a fall of BP [26], which may be due to a considerable extent to the increase NO generation [11]. On the other hand, there is increasing evidence that changed blood flow to organs can activate synthesis of stress proteins [27].

Thus the precise mechanism(s) of interrelation between NO and activation of HSP70 synthesis remains unclear. However, irrespective of the nature of this mechanism, the facts established in the work demonstrate for the first time that NO is really involved in the activation of stress protein synthesis.

The applied significance of the work is that the system of NO generation and the system of heat shock proteins play an important role in both stress and adaptive responses of the organism. This is why understanding of the interrelation between these intracellular systems will allow to perform more targeted correction of stress damage and to control adaptive processes in the organism.

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