# Nitric oxide donor induces HSP70 accumulation in the heart and in cultured cells

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**Abstract As our group has shown, the NO-synthase inhibitor L-NNA decreased 2–3 times heat shock-induced synthesis of the heat shock protein HSP70 (FEBS Lett. 370 (1995) 159–162). It was suggested that NO is involved in such induction. In the present study, it was found that (1) injection of the NO donor dinitrosyl iron complex (DNIC) into rats results in accumulation of HSP70 in the heart; (2) heat shock is accompanied by increased generation of NO (EPR assay) and HSP70 accumulation in cultured cells; (3) DNIC induces HSP70 accumulation in cultured cells not exposed to heat shock.**

*Key words:* Nitric oxide; Heat shock protein; Cell culture; Electron paramagnetic resonance; Western blot analysis

## 1. Introduction

It has been shown that heat shock protein (HSP70) synthesis can be increased by quite different factors, i.e. heat shock (HS), acute myocardial ischemia, acute hypoxia, cooling, etc. [1,2]. HSP70 plays an important role in the restriction of stress damage [3] and in enhancing the resistance of the heart to ischemia and reperfusion after heat shock [4,5], ischemic preconditioning [6], and adaptation to stress [7]. At the same time, the question of what cell mechanisms are involved in the activation of HSP70 synthesis remains open in many respects. When analyzing this problem, the experimental fact has drawn our attention that the same agents can activate both HSP70 and nitric oxide (NO) synthesis. For instance, lipopolysaccharides, classic inducers of NO synthesis [8], also activate HSP70 synthesis [9]. On the other hand, HS, which is a conventional factor inducing HSP synthesis [1,2,10], is attended by an increase in nitrosyl heme content in blood [11]. Our own experiments have demonstrated that adaptation of the organism to stress both activates HSP70 synthesis [7] and potentiates NO generation in the same organs [12]. We have recently shown that HS-induced tissue synthesis of HSP70 is preceded by a transient increase in NO production in the same tissue. Furthermore the NO-synthase inhibitor $N^{w}$-nitro-L-arginine (L-NNA) completely abolished the HS-induced NO generation and decreased 2–3 times the HS-induced HSP70 synthesis [13].

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*Abbreviations:* DNIC, dinitrosyl iron complex; EPR, electron paramagnetic resonance; HS, heat shock; HSP70, heat shock protein with molecular weight 70 kDa; L-NNA, $N^{w}$-nitro-L-arginine; NO, nitric oxide

Taken together the data suggest that NO may contribute to the activation of HSP70 synthesis. The goal of the present study was to verify this hypothesis. We studied: (1) the effect of NO donor injection into rats on HSP70 accumulation in the heart; (2) the effect of HS on NO production in cultured cells, and (3) the effect of the NO donor on HSP70 accumulation in cultured cells.

## 2. Materials and methods

### 2.1. DNIC synthesis

Dinitrosyl iron complex (DNIC 1:2) with glutathione $[(GSH)_2\text{-}Fe^{+}\text{-}(NO^{+})_2]_2$ was used as the NO donor [14–16]. This complex was synthesized in phosphate buffered saline (PBS) according to the method described in [14]. It exists in two forms: DNIC 1:2 and DNIC 1:20. DNIC 1:2 is a dimeric diamagnetic complex synthesized by the treatment with NO of a $Fe^{2+}$-thiol (1:2) solution. DNIC 1:20 is a monomeric paramagnetic complex $(GSH)_2\text{-}Fe^{+}\text{-}(NO^{+})_2$ synthesized by the treatment with NO of a $Fe^{2+}$-thiol (1:20) solution. DNIC 1:20 is characterized by an EPR signal at $g_{\perp} = 2.041$ and $g_{\parallel} = 2.014$. Parameters of the EPR signal are independent of the nature of the thiol ligand (glutathione, cysteine or thiol groups of proteins). DNIC 1:2 turns into DNIC 1:20 on the addition of thiol to the medium in a concentration 20 or more times exceeding that of iron [14].

### 2.2. Animal experiments

Experiments were carried out on male Wistar 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'.

*2.2.1. DNIC treatment.* DNIC 1:2 was used as the NO donor [14]. It was injected into the caudal vein in the dose of 0.3 mmol/kg body weight. 30 and 90 min after the DNIC injection, blood samples (0.2 ml) were taken from the subclavian vein for measurement of DNIC content in blood. Animals were decapitated 24 h following the DNIC injection and taken for determination of DNIC content in the blood and heart and of HSP70 accumulation in the heart.

*2.2.2. Measurement of DNIC content in the blood and heart.* After injection, diamagnetic ('EPR-silent') DNIC 1:2 quantitatively transformed into paramagnetic DNIC 1:20 (see Section 3.1). This allowed us to use the EPR assay for determination of the DNIC content by a characteristic EPR signal according to a method described elsewhere [14]. Samples of blood and tissue were placed in glass ampoules and frozen in liquid nitrogen. EPR spectra were recorded on the EPR radiospectrometer Radiopan (Poland) in the X-diapason at 77 K, amplitude of hf modulation 0.2 mT, and microwave power 5 mW.

*2.2.3. Measurement of blood pressure.* Since DNIC releasing NO is a potent vasodilator [14], we used blood pressure (BP) as a physiological indicator of the NO release from DNIC. BP was measured by an indirect bloodless method on the tail artery using Physiograph DMP-4F (Narco Bio-Systems, USA).

*2.2.4. Electrophoresis and immunoblotting.* Reagents and instruments from Bio-Rad (USA) were used. The heart tissue was ground and placed in 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 a buffer:tissue ratio of 5:1 (w/w). The homogenate obtained was filtered through eight gauze layers and centrifuged at


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12000×*g* and 4°C for 10 min. The supernatant containing proteins was taken for electrophoresis and blotting. Electrophoresis was performed according to Laemmli [17] in 12% PAAG. They were transferred from PAAG to a nitrocellulose membrane according to Towbin [18]. Then blots were successively incubated in the presence of monoclonal antibodies (C92) against HSP70 (C92 was a gift from Dr. W.J. Welch, San Francisco, CA, USA) at a dilution of 1:500 for 1 h. The resulting immune complexes were reacted with horseradish peroxidase-conjugated anti-mouse Ig (Amersham, UK) and incubated for 1 h. Finally labeled antigen bands were detected by diaminobenzidine staining. For more precise identification of the proteins induced by DNIC, they were compared with HSP70 fractions induced by heat shock. Heat shock was produced by heating conscious animals in a thermostat to a core temperature of 41.5±0.5°C. After that the heating was continued for an additional 15 min. The total duration of heating did not exceed 30 min.

*2.3. Cell culture experiments*

Experiments were carried out using a human hepatoblastoma cell line, Hep G2. Hep G2 cells were grown on 35 mm plastic dishes. The cells were cultured in Eagle's modified minimum essential medium (EMEM) supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, containing 10% fetal bovine serum (FBS) and incubated in temperature-controlled, humidified incubators (5% $CO_2$).

*2.3.1. Heat shock.* The cells at near confluence ($1.0\times10^6$ cells/dish) were exposed to HS by the addition of fresh medium prewarmed to 42°C and subsequent incubation at this temperature. After 2 h the culture medium was removed, the cells were washed twice with EMEM and incubated for 1 or 24 h at 37°C. At the end of incubation cells were washed with 1 mM cold PBS, and prepared for the EPR assay and Western blotting.

*2.3.2. DNIC treatment.* The DNIC solution was added to the culture medium at a final concentration of 20 or 100 μM. The cells were incubated with DNIC. After 15 min, 4 or 24 h the cells and medium were EPR-assayed. After 4, 8, 16 or 24 h the cells were assayed by Western blot.

*2.3.3. EPR assay.* The cells were sedimented by centrifugation at 10000×*g* for 10 min at 4°C and the pellet was diluted with 0.3 ml of the supernatant. The suspension obtained or the medium was placed in glass ampoules and frozen in liquid nitrogen. The EPR assay was carried out as described above (see Section 2.2.2).

*2.3.4. Electrophoresis and immunoblotting.* The cells were washed three times with PBS. Cell pellets were suspended at the concentration of $2\times10^7$ ml in 100 μl lysis buffer containing 1.0% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl at pH 8.0 for 30 min at 4°C. The insoluble debris was removed by centrifugation at 18000×*g* for 10 min at 4°C. Subsequent electrophoresis and immunoblotting were performed as described above (see Section 2.2.4).

*2.4. Statistical analysis*

Results were statistically treated with Student's *t*-test and are presented as means±S.E.M.

## 3. Results and discussion

*3.1. Injection of DINC 1:2 results in appearance of DNIC 1:20 in blood*

Thirty minutes after the DNIC 1:2 (0.3 μmol/kg) injection, paramagnetic DNIC 1:20 appeared in blood in a concentration of 20 μM. This corresponds to 50% of the DNIC 1:2 injected. After 90 min and 24 h, DNIC 1:20 was not detected in the blood. In the heart DNIC 1:20 was undetectable.

The appearance of DNIC in the blood was accompanied by a transient decrease in BP from 110±3 to 87±9 ($P<0.05$). One hour after DNIC injection, the BP returned to the initial level, which corresponded to the disappearance of DNIC from the blood.

*3.2. DNIC induces HSP70 accumulation in the rat heart*

It is seen from Fig. 1A that DNIC (0.3 μmol/kg body weight) induced accumulation of HSP70 in the myocardium 24 h after the injection. This result demonstrates for the first time that an NO donor causes accumulation of HSP70. However it remains unclear whether this effect was due to a direct intracellular action of NO or was mediated by an NO-induced change in neuro-humoral regulation. This is why subsequent experiments were performed on cultured cells.

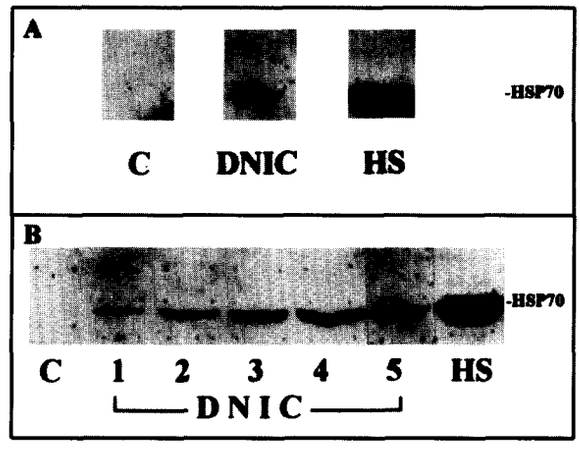


Fig. 1. The effect of DNIC 1:2 on HSP70 accumulation in the heart (A) and in Hep G2 cells (B). (A) C: control. DNIC: 0.3 μmol/kg. (B) C: control. Lanes 1, 2, 3, 4: 4, 8, 16 and 24 h after DNIC (100 μM) addition. Lane 5: 24 h after DNIC (20 μM) addition.

*3.3. HS increases NO generation in cell culture*

Heating of cultured cells for 2 h at 42°C resulted in the appearance of endogenous DNIC 1:20 in a concentration of 2 nmol/$10^6$ cells 1 h following HS (Fig. 2). Therefore HS potentiated the generation of NO and then the latter formed DNIC with endogenous iron and thiol-containing ligands.

*3.4. DNIC induces synthesis of HSP70 in cell culture*

Incubation of hepatocytes with DNIC 1:2 in the concentration of 100 μM resulted in the appearance of DNIC 1:20 derived from DNIC 1:2 in the cells. From the intensity of the EPR signal (peak at $g_1=2.041$), the intracellular concentration of DNIC 1:20 was calculated as 5 nmol/$10^6$ cells after 15 min of incubation (Fig. 2). Detection of the EPR signal from DNIC 1:20 in cells after contact of the cells with exogenous DNIC 1:2 ensures that this NO donor has really entered the cell. The major part of DNIC 1:2 remained in the extracellular medium. To evaluate the size of this portion, the medium was supplemented with 5 mM glutathione. This induces the transformation of diamagnetic DNIC 1:2 into paramagnetic DNIC 1:20 and makes it possible to measure extracellular DNIC 1:2 using the EPR method. This concentration comprised 75 μM which was close to the amount of DNIC 1:2 added. If the extracellular medium was not treated with glutathione, no signal of DNIC was observed. Within 4 h, both extra- and intracellular DNIC concentrations fell to below the limit of detection (0.3 nmol per sample).

Fig. 1B shows that both HS and exogenous DNIC 1:2 administration induced synthesis of HSP70. The DNIC-induced HSP70 synthesis was time- and dose-dependent. So, for the first time we were able to show that NO itself can activate synthesis of HSP70 in cells. It seems reasonable to discuss the mechanism of NO-induced activation of HSP70

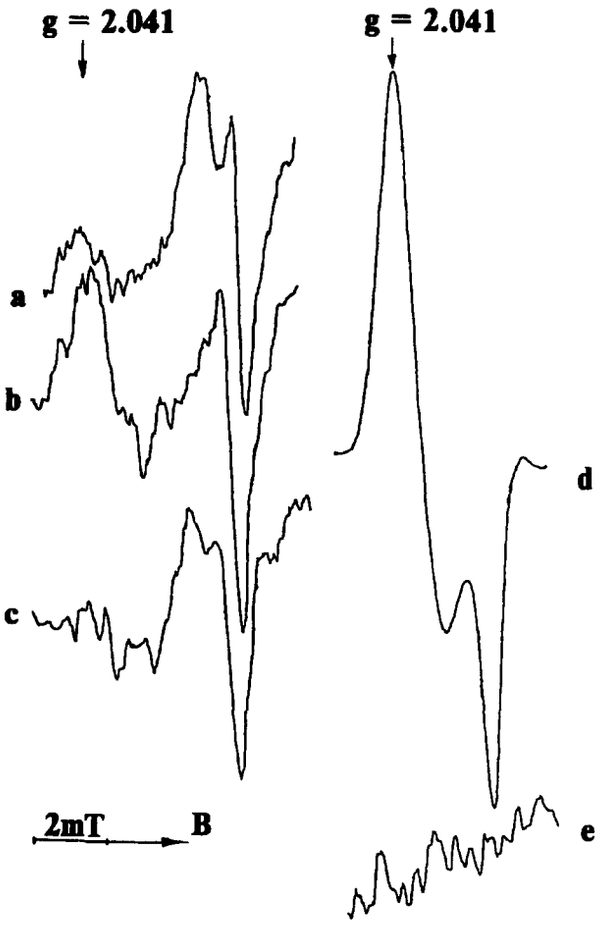


Fig. 2. EPR spectra from hepatocytes and culture medium. a: Hepatocytes exposed to heat shock. b and c: Hepatocytes 15 min and 4 h after DNIC addition, respectively. d and e: Culture medium 15 min and 4 h after DNIC addition, respectively. Recorded at 77 K; amplitude of hf modulation 0.2 mT; microwave power 5 mW. Relative magnification of the spectrometer in recording spectra a, b, c, e: ×10 and d: ×1.

synthesis. We suggest that the mechanism may be as follows. It is known that, in basal conditions, the HSP70 transcription factor (HSF) is inactive. Stress factors induce trimerization of HSF [19]. As a result, the trimeric HSF enters the nucleus and activates transcription of *hsp70* genes. It is quite possible that NO, specifically in its ionized form, nitrazonium ion $NO^+$, catalyzes the trimerization process and accelerates the formation of disulfide bonds between HSF molecules. DNIC releases NO in the form of $NO^+$ [20]. Based on current notions [21], the chemical reaction appears as follows: $NO^+$ interacts with SH-groups of HSF to form the intermediate S-nitrosothiol. Degradation of the latter with formation of highly reactive thiol radicals results in the formation of disulfide links between HSF molecules, which leads to trimerization and activation of HSF.

It is commonly accepted that NO is a key molecule in the regulation of various physiological processes including the immune, nervous and cardiovascular systems [22]. Our results supplement this picture: NO can operate as a factor initiating the synthesis of the stress protein HSP70. Taking into account that HSP70 plays an important role in stress and adaptive responses of the organism [1–7], the present results allow us to classify the system of NO generation as one that determines the resistance of the organism to stress as well as the adaptive capacity of the organism.

## References

[1] Schlesinger, M.J., Ashburner, M. and Tissieres, A. (1982) Heat Shock: From Bacteria to Man. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[2] Welch, W.J. and Suhan, J.P. (1986) J. Cell. Biol. 103, 2035–2052.
[3] Pelham, H.R.B. (1986) Cell 46, 517–528.
[4] Currie, R.M., Karmazyn, M., Kloc, M. and Mailer, K. (1988) Circ. Res. 63, 543–549.
[5] Karmazyn, M., Mailer, K. and Currie, R.M. (1990) Am. J. Physiol. 259, 424–431.
[6] Marber, M.S., Latchman, D.S., Walker, J.M. and Yellon, D.M. (1993) Circulation 88, 2023–2032.
[7] Meerson, F.Z. and Malyshev, I.Yu. (1993) Phenomenon of Adaptive Stabilization of Structures and Protection of the Heart, Nauka, Moscow (in Russian).
[8] Bredt, D.S. and Snyder, S.H. (1994) Annu. Rev. Biochem. 63, 175–195.
[9] Zhang, Y., Takahashi, K., Jiang, G.-Z., Zhang, X.-M., Kawai,M., Fukada, M. and Yokochi, T. (1994) Infect. Immun. 62, 4140–4144.
[10] Tomasovic, S.P. (1989) Life Chem. Rep. 1, 33–63.
[11] Hall, D.M., Buettner, G.R., Matthes, R.D. and Gisolfi, C.V. (1994) J. Appl. Physiol. 77, 548–553.
[12] Meerson, F.Z., Lapshin, A.V., Mordvincev, P.I., Mikoyan, V.D., Manukhina, E.B., Kubrina, L.N. and Vanin, A.F. (1994) Byull. Eksp. Biol. Med. 117, 242–244 (in Russian).
[13] Malyshev, I.Yu., Manukhina, E.B., Mikoyan, V.D., Kubrina L.N. and Vanin, A.F. (1995) FEBS Lett. 370, 159–162.
[14] Vedernikov, Y.P., Mordvintcev, P.I., Malenkova, I.V. and Vanin, A.F. (1992) Eur. J. Pharmacol. 211, 313–317.
[15] Mulsch, A., Mordvintcev, P., Vanin, A.F. and Busse, R. (1991) FEBS Lett. 294, 252–256.
[16] Feelisch, M., te Poel, M., Zamora, R., Deussen, A. and Moncada, S. (1994) Nature 368, 62–65.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Rabindran, S.K. (1994) Mol. Cell. Biol. 14, 6552–6560.
[20] Vanin, A.F. (1995) Biochemistry (Moscow) 60, 441–447.
[21] Stamler J.S. (1994) Cell 78, 931–936.
[22] Moncada, S.J. (1994) Hypertension 12 (Suppl. 10), S35–S39.