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Adaptation to Heat of Cardiomyoblasts in Culture Protects Them against Heat Shock: Role of Nitric Oxide and Heat Shock Proteins

E. A. Monastyrskaya[1], L. V. Andreeva[2], M. R. Duchen[2], F. Wiegant[3], L. A. Bayda[1], E. B. Manukhina[1], and I. Yu. Malyshev[1]*

[1]Institute of General Pathology and Pathophysiology, ul. Baltiiskaya 8, Moscow 125315, Russia; fax: (095) 151-0421; E-mail: igor.malyshev@mtu-net.ru
[2]University College London, Department of Physiology, Gollower Street, London, WC1E 6BT, United Kingdom; E-mail: m.duchen@ucl.ac.uk
[3]University Utrecht, Department of Molecular Cell Biology, Padualaan 8, 3584CH, Utrecht, The Netherlands; fax: 31-30-2512655; E-mail: f.a.c.wiegant@bio.uu.n

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Abstract—Dosed adaptation to environmental factors is an efficient non-drug means for increasing the resistance of organs or the body as a whole. We demonstrated earlier that nitric oxide (NO) plays an important role in adaptive defense of the organism, in particular due to activation of heat shock protein (HSP) synthesis. A key question remained open—to what extent the formation of adaptive defense depends on central mechanisms and to what extent on the intracellular mechanisms immediately responding to the adapting factor, and whether the NO-dependent activation of HSP synthesis plays a role in adaptation of isolated cells. In the present study we looked into the possibility of producing a protective effect of adaptation to heat in cell culture. A 6-day adaptation to heat limited to 17% the decrease in metabolic activity induced by heat shock in H9c2 cardiomyoblasts. The development of adaptation was associated with increased NO production. Treatment of cells with the inhibitor of NO synthase L-NNA (100 μM) prevented the development of adaptive protection. Adaptation of cell culture enhanced synthesis of HSP70 but not HSP27. Blockade of HSP70 synthesis with quercetin (50 μM) left unchanged the protective effect of adaptation. Inhibition of NO synthesis restricted the adaptation-induced HSP70 synthesis. Therefore, the formation of adaptation at the cell level may result from a direct action of an environmental factor without participation of neurohumoral factors. Such adaptation involves NO-dependent mechanisms divorced from the activation of HSP70 synthesis.

Key words: cardiomyoblasts, adaptation, heat shock, nitric oxide, heat shock proteins

It is known that a single pre-exposure of a cell to a detrimental factor can enhance the cell resistance to subsequent more intense action of the same factor [1]. This phenomenon is termed ischemic preconditioning (if brief ischemia is used as a preconditioning factor [2]) or heat preconditioning (if a single thermal exposure is used as a preconditioning factor [3]). A prerequisite for enhancing the resistance of a cell in the process of preconditioning is that the prior stimulus should induce a slight injury, which would be in turn sufficient for activation of intracellular defense systems. In the instance of heat preconditioning, for example, the prior heat exposure induces moderate denaturation of cell proteins and attenuates total protein synthesis and ATP production. Thereby heat shock activates synthesis of heat shock proteins (HSPs) HSP27, HSP32, HSP70, and others [4] to provide protection of the cell from subsequent severe heat shock [5]. Protective properties of HSPs are related to their ability to utilize damaged proteins [6], prevent aggregation of abnormal proteins [7], restrict NO overproduction [8], and exhibit antiapoptotic effects [9].

The potential of preconditioning for increasing the resistance of cells was demonstrated for the first time on a model of myocardial ischemia–reperfusion [2]. This led to many attempts at clinical use of the phenomenon [10], such as the enhancement of heart resistance in myocardial infarction [1]. However, the necessity of inducing an initial injury in the organ affected by the disease limits broad application of preconditioning for protection purposes.

More efficient increase in resistance of organs or the whole body can be accomplished using non-drug methods of adaptive medicine. In this case, the increased resistance is provided by periodic mild exposures to the

* To whom correspondence should be addressed.

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environmental factor. This method differs from preconditioning in that adaptation causes virtually no damage to organs and cells [11]. Indeed, it has been demonstrated that adaptation of the organism to intermittent mild stress exposures enhances the resistance of heart to myocardial infarction [12] and disorders in post-infarction cardiosclerosis [13], whereas adaptation to heat restricts development of ischemic and reperfusion arrhythmias (Monastyrskaya et al., unpublished data). Clinical use of adaptation methods will be impossible without understanding the molecular and cell mechanisms of adaptation.

Experimental studies of long-term adaptation to environmental factors (hypoxia, heat, stress, exercise) have shown that protective effects of adaptation develop due to activation of both neuroendocrine and intracellular mechanisms [11]. Neuroendocrine mechanisms attenuate the intensity and duration of stress response at central and local levels. The concept on cell mechanisms of adaptation was substantiated by experiments on isolated heart, sarcoplasmic reticulum, and cardiomyocyte nuclei [14] isolated from adapted animals. Such organs and cell structures were noted for their higher resistance to damage as compared to controls.

Recent data have demonstrated that nitric oxide (NO), a universal regulator participating in the majority of physiological and biochemical processes, possesses antistress and adaptogenic functions which underlie its important role in the adaptation defense of the organism [15]. Mechanisms of the NO-related cell defense include antiapoptotic [16], antioxidant [17], and anti-inflammatory effects [18] as well as the effects mediated by cGMP production [19]. In adaptation of the organism, NO activates synthesis of protective HSPs and thereby enhances the organism's resistance to injury [20].

The existing concept of adaptation has left open an important question: to what extent the formation of adaptive defense depends on central mechanisms and to what extent it is predetermined by the intracellular mechanisms immediately responding to the adapting factor. To solve this question it was necessary to elucidate whether the formation of adaptive defense is possible in the conditions of cell culture, when the effect of neural and humoral factors on cells is absent.

If adaptation of cell culture is possible, another important question arises: what cell mechanisms can underlie adaptation, and whether the NO-dependent activation of HSP70 synthesis plays a no less important role in adaptation of isolated cells than in adaptation of the organism.

Accordingly, the aims of the present study were: 1) to elucidate the possibility of producing adaptive defense under the conditions of cell culture; 2) to evaluate changes in NO production and HSP70 synthesis during periodic heat exposures of cell culture; and 3) to study the effect of a HSP70 transcription inhibitor and a NO synthase inhibitor on the accumulation of HSP70 and protective effect of adaptation to heat.

MATERIALS AND METHODS

Cell culture. H9c2 cells (undifferentiated rat cardiomyoblasts) were obtained from the American Type Culture Collection (CRL-1446, ATCC, Rockville, MD, USA) and were cultured in full growth medium which was Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA), containing 4.5 g/liter glucose and supplemented with 10% fetal calf serum (Sigma) and 1% penicillin-streptomycin (Gibco, USA), in an atmosphere of 10% CO_2. For the experimental work, cells were grown in six-well plates, round cover slips for fluorescent examination, or flasks for Western blot analysis.

Heat adaptation of cells. For adaptation to heat, cells were exposed to heat for 6 days by placing sealed flasks on a water bath at 41.5°C for 1 h daily. Cells were taken for the experiment 24 h after the last session of adaptation.

Heat shock model. Heat shock was used as a model of cell stress. For this purpose sealed flasks with cells were placed on a water bath at 45°C for 1 h. Experiments were carried out 24 h after heat shock.

Inhibition of NO synthesis. Synthesis of NO was inhibited with the nonspecific NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA, Sigma) at 100 μM concentration [21]. Incubation with L-NNA was started 30 min prior to each session of adaptation; washout was performed 2 h post-session.

Inhibition of heat shock protein synthesis. Synthesis of HSPs was inhibited during adaptation using the inhibitor of HSP70 gene transcription factor quercetin (Sigma) [22] at 50 μM concentration [23]. Preliminary experiments demonstrated that higher quercetin concentrations are toxic for this cell line. The scheme of incubation with quercetin was similar to that with L-NNA.

Measurement of cell metabolic activity. Cell metabolic activity was measured using the MTT bioreduction method as described by Mosmann [24]. The MTT bioreduction method is based on reduction of (3,4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide to the formasan pigment. In cells this reaction occurs due to activity of mitochondrial enzymes, namely, due to catalytic reactions coupled with NADP- and NADPH-dependent redox systems. Since NADP/NADPH are the major mediators of oxidative phosphorylation in the cell and the process of oxidative phosphorylation is the key one in maintaining the cell energy conditions, the method of MTT bioreduction measurement is commonly used for evaluation of the overall "vital activity" in a cell population [25-28].

Cell culture plates were washed with phosphate buffer and incubated with 5 mg/ml solution of MTT reagent (Sigma) at 37°C for 60 min. The blue crystals that

appeared were dissolved in a solution containing isopropanol with 0.1 M HCl and 10% Triton X-100. The absorbance was read spectrophotometrically at 590 nm.

Measurement of NO production. NO production was evaluated by the total level of stable NO metabolites nitrite and nitrate. The level of nitrite and nitrate was measured in the cultural medium using chemiluminescence analysis by NO recovery [29] with a Sievers Instruments analyzer (USA) in accordance to the manufacturer's instructions.

Measurement of HSP content. The content of HSP70 and HSP27 was measured using Western blot analysis. Cells were washed with phosphate buffer, scraped off on ice, and centrifuged at 1000*g* for 10 min. The obtained sediment was dissolved in phosphate buffer by intense vortex mixing with addition of 0.1% Triton X-100 and protease inhibitor cocktail (2.5 μg/ml antipain, 2.5 μg/ml leupeptin, 1.75 μg/ml pepstatin A, and 0.95 μg/ml aprotinin; Sigma). Protein was measured using the Bradford reagent (Bioquant Protein, Merck, Germany). Ready samples were heated at 95°C with Laemmli buffer at 1 : 1 ratio. Proteins were separated by electrophoresis in 12% polyacrylamide gel with sodium dodecyl sulfate for 50 min. Then proteins were transferred onto nitrocellulose membrane (Hybond, Amersham, UK). Membranes were washed in 0.1% milk (Bio-Rad, USA) and 0.05% Tween-20 (Bio-Rad) in phosphate buffer (PBST). Membranes were blocked in the same buffer with 5% milk for 12 h and then exposed to primary antibodies (1 : 1000) to HSP70 (murine monoclonal, StressGen) and to HSP27 (rabbit polyclonal, Santa Cruz, USA); secondary antibodies (1 : 5000) (anti-rabbit immunoglobulin G (IgG) for polyclonal primary antibody to HSP27 and anti-mouse IgG for monoclonal primary antibody to HSP70; all conjugated with horseradish peroxidase, Bio-Rad). Membranes were developed using enhanced chemiluminescence system (ECL, Amersham, UK) and exposed to autoradiography film. Then films were scanned and densitometric analysis was performed.

Statistical treatment of data. Data were treated using Student's *t*-test. Differences were considered significant at two-way significance level of $p < 0.05$. Data are presented as means ± SEM. Analysis was performed using GraphPad Prizm 3.0 software (USA).

RESULTS

Adaptation to heat protects cells against injury in heat shock. Figure 1 shows that heat shock significantly decreased the H9c2 cell metabolic activity to 46 ± 3% of the control level. Adaptation itself did not induce changes in this parameter but significantly prevented its decrease in cells exposed to heat shock. Metabolic activity of adapted cells was reduced by heat shock only to 63 ± 3% of the control level.

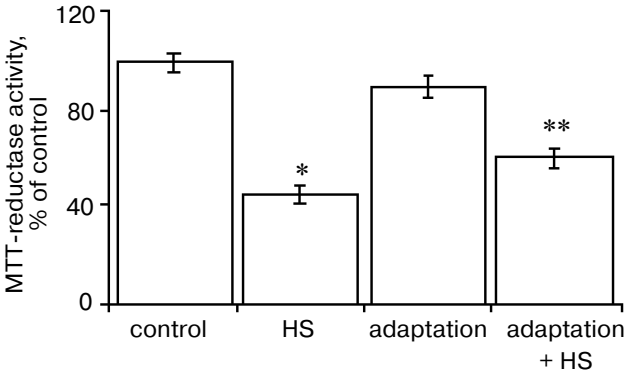

Fig. 1. Effect of adaptation to heat on the heat-shock (HS) induced decrease in metabolic activity of H9c2 cells. * Significant difference from control, $p < 0.05$. ** Significant difference from heat shock, $p < 0.05$.

NO participates in cell adaptive defense against injury in heat shock. Data in Fig. 2 show that periodic thermal exposure of cell culture for six days increased the total level of nitrite and nitrate in the culture medium from 15.1 ± 1.2 to 19.0 ± 1.1 μM ($p < 0.05$). Therefore, heat adaptation of cells increased NO production.

Synthesis of NO was inhibited with 100 μM L-NNA. This concentration of L-NNA neither exerted any toxic effect nor induced changes in metabolic activity of control cells (Fig. 3a). At the same time, L-NNA restricted the increased NO production in adapted cells (Fig. 2) and abolished the protective effect of adaptation against subsequent heat shock (Fig. 3b). These data support the participation of NO in the protective effect of adaptation in cell culture.

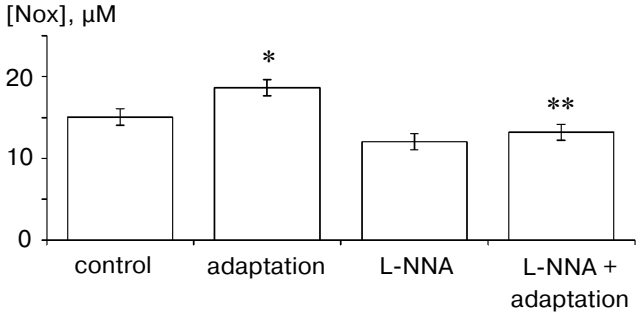

Fig. 2. Effect of adaptation to heat on total nitrite and nitrate level in the culture medium of H9c2 cells. * Significant difference from control, $p < 0.05$. ** Significant difference from adaptation, $p < 0.05$.

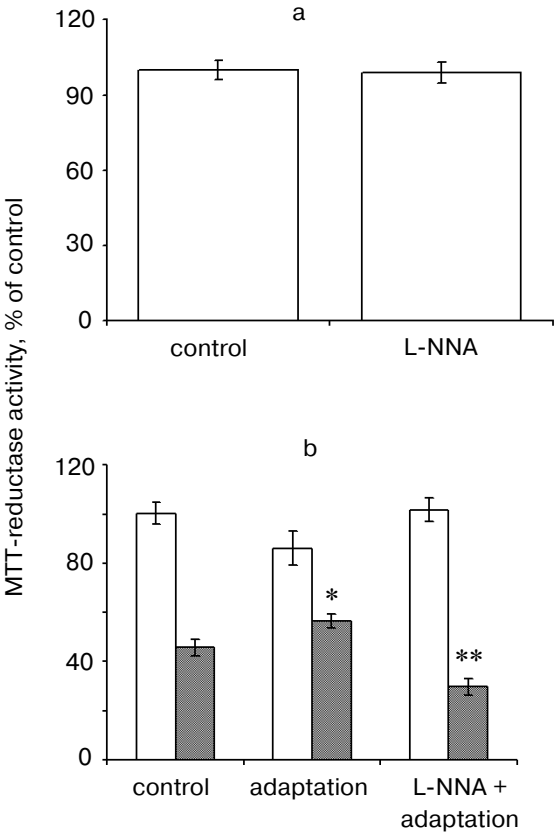

Fig. 3. Effect of the NO synthase inhibitor L-NNA on the metabolic activity of cells before (a) and after heat adaptation and heat shock (b). Empty bars, without damaging impact; full bars, heat shock. * Significant difference from control, $p < 0.05$. ** Significant difference from adaptation, $p < 0.05$.

Nitric oxide is involved in activation of HSP70 synthesis in cells during heat adaptation. Evaluation of HSPs content using Western blot analysis demonstrated the absence of HSP70 and HSP27 in control cells. The 6-day heat adaptation of cells stimulated the expression of HSP70 (Fig. 4a) and left unchanged synthesis of HSP27 (Fig. 4b).

To evaluate the possible role of NO in the adaptation-induced synthesis of HSPs, we reproduced the adaptation on the background of the NO synthase inhibitor L-NNA. It appeared that L-NNA alone did not influence the HSP70 synthesis, but it restricted the HSP70 accumulation in heat adaptation of cells (Fig. 4a). Therefore, the adaptive activation of HSP70 synthesis was related, at least partially, with NO-dependent mechanisms.

HSP70 apparently does not play a role in development of the protective effect of heat adaptation. In evaluating the role of HSP70 accumulation during adaptation to heat, we used the HSP70 synthesis inhibitor quercetin at a concentration of 50 μM. This concentration of quercetin was nontoxic for H9c2 and did not induce changes in cell metabolic activity (Fig. 5). As shown on the representative blot (Fig. 4a), quercetin completely inhibited the HSP70 induction in adaptation of cells but did not affect the development of adaptive protection (Fig. 5). This result suggests that HSP70 does not contribute significantly to the adaptive enhancement of cell resistance to heat.

Heat shock did not induce HSP70 in control cells (Fig. 4a) but notably increased synthesis of HSP27 (Fig. 4b). Heat shock of adapted cells did not result in additional induction of HSP70 as compared to that observed in the adapted cells before heat shock (Fig. 4a) and slightly increased synthesis of HSP27 as compared to heat shock of non-adapted cells (Fig. 4b).

Incubation of adapted cells with L-NNA potentiated HSP27 synthesis in adapted cells exposed to heat shock (Fig. 4, a and b).

DISCUSSION

The present study demonstrates the possibility of producing adaptive protection against heat shock under the conditions of cell culture. A key difference of such

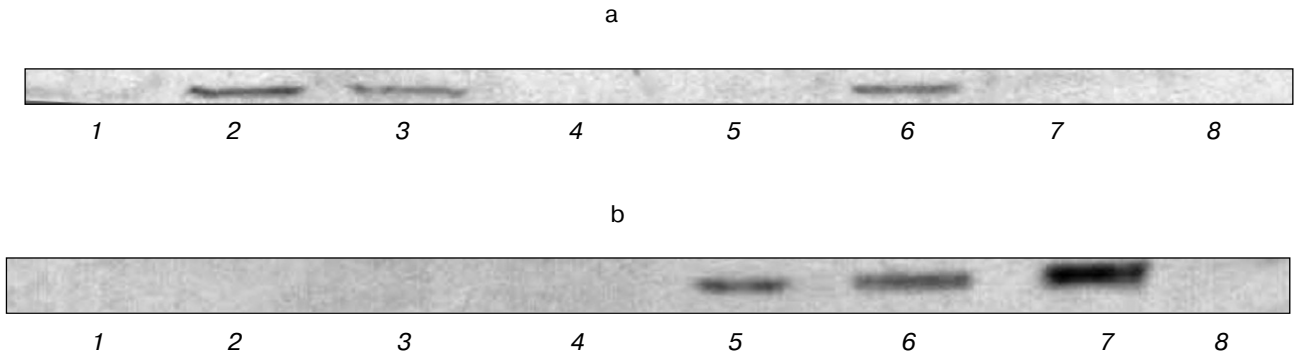

Fig. 4. Effects of heat adaptation and heat shock on synthesis of HSP70 (a) and HSP27 (b). Results of Western blot analysis: *1*) control; *2*) adaptation; *3*) L-NNA + adaptation; *4*) quercetin + adaptation; *5*) heat shock; *6*) adaptation + heat shock; *7*) L-NNA + adaptation + heat shock; *8*) quercetin + adaptation + heat shock.

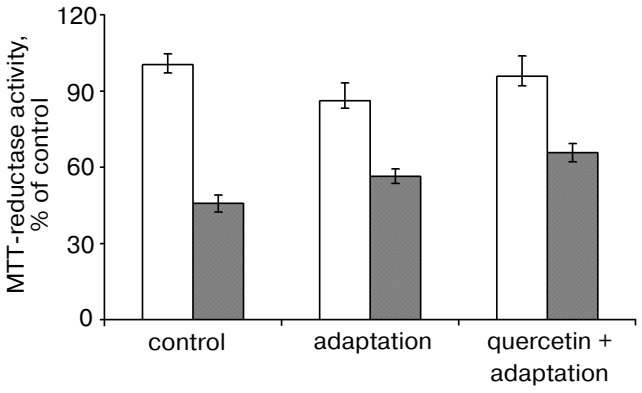

Fig. 5. Effect of the inhibitor of HSP synthesis quercetin on cell metabolic activity after heat adaptation and heat shock. Empty bars, without damaging impact; full bars, heat shock.

adaptive protection from the protective effect of preconditioning is that the former is achieved due to periodic heat exposures, which themselves do not induce cell injury. In our model, the protective effect developed only after 6 thermal exposures whereas a single or even three thermal exposures did not result in development of stable protection of cells against subsequent heat shock (data in press).

Studies of our laboratory have previously demonstrated that heat adaptation of the organism was associated with increased NOS expression [20] and NO production. Furthermore, the NO-dependent mechanisms were suggested to be involved in formation of adaptive defense by both restriction of neuro-humoral stress mechanisms and activation of intracellular protective systems, primarily heat shock proteins HSP70 [15].

In the present work, we showed that periodic heating of cell culture resulted in increased NO synthesis. This fact suggests that the increased NO production accompanying adaptation of the organism might be related not only with neuro-humoral mechanisms, but also with an immediate effect of the physical factor on the cell.

The fact that the NO synthesis inhibitor L-NNA prevented the formation of adaptive defense supports the role of NO in development of this effect under the conditions of cell culture. The protective effect of NO has been demonstrated also with other models of cell culture. For example, a NO donor protected cardiomyoblasts H9c2 against oxidative injury [30]. The protective effect of NO might be mediated by the NO-dependent activation of antiapoptotic and antioxidant mechanisms (for review see [31]) and activated synthesis of the protective proteins HSP70 [32].

In the present study, we observed that L-NNA restricted the HSP70 synthesis induced by heat adaptation of cell culture. This indicates that a mechanism for NO-dependent activation of HSP70 synthesis occurs in isolated cells as in the whole organism under heating. However, this mechanism plays apparently different roles in the formation of adaptive defense in the organism and in isolated cells. We have earlier shown that both the NOS inhibitor L-NNA and the HSP70 synthesis inhibitor quercetin attenuated the adaptive activation of HSP70 synthesis and hampered the formation of heat resistance in the organism [20]. Accordingly, we concluded that the NO-dependent activation of HSP70 synthesis plays an important role in protective effects of organism adaptation. The present results showed that quercetin completely blocked HSP70 synthesis in isolated cells but left unaffected the protective effect of adaptation. Therefore HSP70 and the NO-dependent activation of HSP70 synthesis apparently do not play a substantial role in the adaptive protection of cell culture from heat shock as evaluated by MTT bioreduction. Since HSPs are able to stabilize structural proteins of the cell [33], this stabilization could contribute to general resistance of the organism by maintaining endothelial and epithelial barrier function, which suffers in heat shock [34]. Nevertheless, the question of what factors occurring in the organism but lacking in cell culture play a role in the protective effect of HSP70 in heat shock remains open and needs further investigation.

In the course of adaptation we did not observe any change in HSP27 content. Probably HSP27, like HSP70, does not play a substantial role in the protective effect of adaptation in isolated cells. Noteworthy are the following differences in synthesis of HSP27 and HSP70: adaptation of cells to moderate thermal exposures induces synthesis of HSP70 but not HSP27 and, *vice versa*, severe heat shock induces synthesis of HSP27 but not HSP70. It is not yet clear whether this difference is due to a possible mutual influence of HSP27 and HSP70 on each others mechanisms of synthesis or this is merely related with different thermal sensitivity in the biosynthetic apparatus of different HSP family members. The latter hypothesis may be supported by the fact that synthesis of HSP27 in H9c2 cells is efficiently activated at a temperature of 42-42.5°C [35]; therefore, the temperature of 41.5°C, at which we performed adaptation, might be merely insufficient for induction of HSP27 in this cell line. On the other hand, A. Samali et al. [36] showed that induction of HSP70 was not observed at temperatures above 42°C (temperature of heat shock in our experiments) despite activation of the HSP70 transcription factor HSF-1 at 40-46°C. This absence of HSP70 induction was apparently due to disturbed process of protein translation.

Another difference in regulatory mechanisms of HSP70 and HSP27 synthesis was that L-NNA partially blocked synthesis of HSP70 but not HSP27. Therefore,

NO plays different roles in regulation of HSP70 and HSP27 synthesis.

On the whole, the data suggest that adaptation can develop at the cell level due to a direct influence of an environmental factor, without participation of neurohumoral factors. Formation of such adaptation involves NO-dependent mechanisms not related with activation of HSP70 synthesis.

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