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Role of Heat Shock Proteins HSP70 and HSP32 in the Protective Effect of Adaptation of Cultured HT22 Hippocampal Cells to Oxidative Stress

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Preadaptation of cultured HT22 mouse hippocampal neurons to oxidative stress prevented cell damage induced by severe oxidative stress. This protection manifested in a decrease in metabolic disturbances in neurons. Adaptation of neurons to oxidative stress was accompanied by accumulation of HSP32 and HSP70. HSP synthesis inhibitor quercetin abolished the protective effect of adaptation under conditions of oxidative stress. Activation of HSP70 synthesis in neurons is an important mechanism for adaptive protection of cells.

Key Words: *adaptation to oxidative stress; cell culture; heat shock proteins HSP32 and HSP70*

Preadaptation to hypoxia prevents cognitive disorders and neurodegenerative brain damage in rats with experimental Alzheimer's disease. *In vivo* experiments showed that the protective effect of adaptation to hypoxia on cognitive function in rats is mediated by inhibition of oxidative stress in the hippocampus [2,4].

Repeated activation of moderate oxidative stress contributes to adaptation to hypoxia [1]. Hence, adaptation to oxidative stress can be considered as an important factor of protective effect of adaptation to hypoxia. Previous studies showed that stimulation with low doses of H_2O_2 activates the antioxidant defense system and increases the resistance of pheochromocytoma cells to oxidative stress induced by H_2O_2 and related compounds [5]. An increase in the synthesis of stress proteins HSPs has a key role in adaptive protection. These proteins are the major components of the intracellular defense system [8].

Here we evaluated the possibility for adaptive protection of cultured HT22 mouse hippocampal neurons from oxidative stress. The role of heat shock proteins HSP32 and HSP70 in neuroprotective adaptation was studied.

MATERIALS AND METHODS

Experiments were performed on HT22 cells obtained from Dr. F. A. C. Wiegant (Department of Molecular and Cellular Biology, Biological Faculty, Utrecht University, Netherlands). This cell line is a subclone of genuine clone HT4 from mouse hippocampus [9]. HT22 cells are often used to study diseases associated with nerve cell death caused by oxidative stress [12]. HT22 cells were grown in

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Dulbecco's medium supplemented with 10% fetal bovine serum and 1% solution of penicillin and streptomycin at 37°C and 10% CO_2.

Adaptation to non-damaging effects of H_2O_2 (3 μM) was performed for 3 days (2 h per day). Cell damage was induced by addition of 600 μM or 1.5 mM H_2O_2 to the culture medium 24 h after the last adaptation session. MTT reductase activity in cells was measured after 2 h. The synthesis of HSP32 and HSP70 was studied 8 and 18 h after H_2O_2 treatment, respectively.

Study of MTT reductase [6] activity is based on evaluation of the function of NADH and NADPH dehydrogenases (respiratory complex I) and reflects total metabolic activity of this cell population. The cells were incubated with MTT in a concentration of 0.5 mg/ml at 37°C for 30 min. Violet crystals were dissolved in a solution of isopropanol with 0.1 M HCl and 10% Triton X-100. Optical density of the solution was proportional to metabolic activity of cells. It was measured on a KFK-3 spectrophotometer at 570 nm after 40 min.

HSPs content was measured by Western blot analysis in 12% polyacrylamide gel [7]. We used BioRad equipment and reagents. The content of these proteins was estimated by enzyme immunochemiluminescence using primary monoclonal antibodies against inducible HSP70 and HSP32 (StressGen Biotechnologies) and secondary antibodies conjugated with horseradish peroxidase (Amersham). The test antigen was visualized with ECL kit (Amersham) and exposed with Hyperfilm ECL film (Amersham). Protein content was estimated from the width and staining intensity of antibody-binding band with the corresponding protein molecular weight. A quantitative study of scanned immunoblots was performed by means of Photoshop software.

Quercetin in a concentration of 50 μM (Sigma) served as a HSP synthesis inhibitor. Preincubation with quercetin in a solution of dimethylsulfoxide (MP Biomedicals, Inc.) was performed 1.5 h before each adaptation session. Metabolic activity of cells was measured by the end of study.

The results were analyzed using Student's *t* test. The differences were significant at $p<0.05$. The data are presented as $M\pm m$.

RESULTS

When developing the model of adaptation of cultured HT22 mouse hippocampal neurons to a non-damaging effect of H_2O_2, two main criteria were used. First, each adaption exposure and total course of adaptation should not induce metabolic disturbances in cells (MTT test). And second, single treatment does not have a protective effect, while the course of adaptation protects cultured cells from oxidative stress induced by 600 μM and 1.5 mM H_2O_2.

We studied the influence of 3-day adaptation to non-damaging effects of H_2O_2 (3 μM) on the decrease in metabolic activity of HT22 cells induced by oxidative stress (Fig. 1). Adaptation did not affect cell survival (this parameter was similar to the control). Incubation with 600 μM H_2O_2 decreased metabolic activity of cells by 38.6±3.1%. Preadaptation to 3 μM H_2O_2 completely abolished this effect. Hence, adaptation protected HT22 cells from damage induced by oxidative stress.

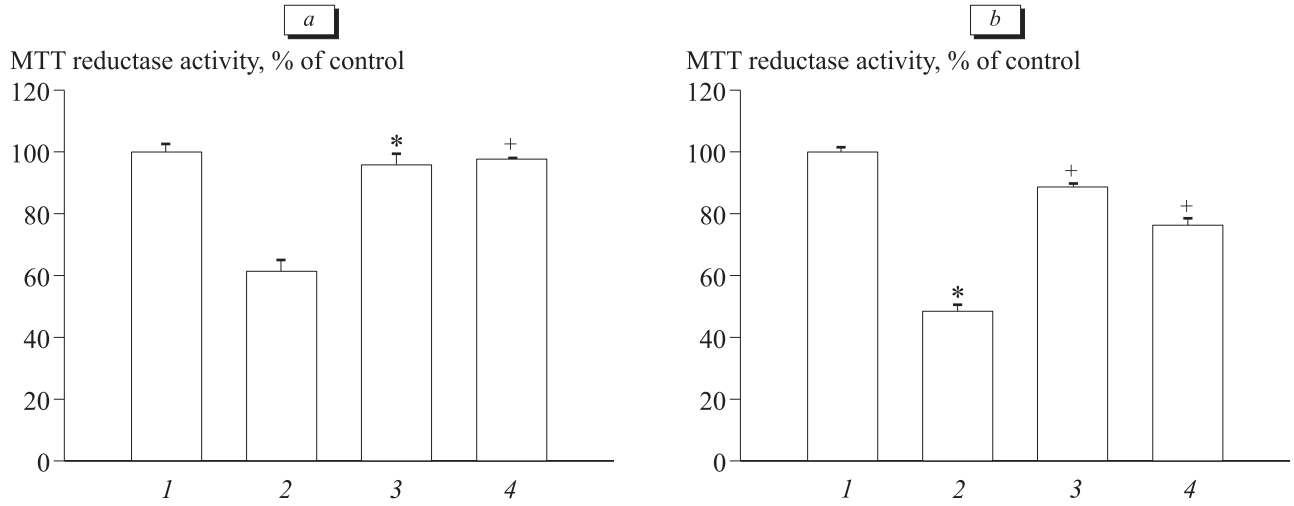

Fig. 1. Effect of 3-day adaptation to 3 μM H_2O_2 on the decrease in metabolic activity of HT22 cells induced by oxidative stress (600 μM H_2O_2, *a*) or 1.5 mM H_2O_2 (*b*). Control (*1*); damage (*2*); 3-day adaptation (*3*); and 3-day adaptation and damage (*4*). $p<0.001$: *compared to the control; +compared to damage (Student's *t* test).

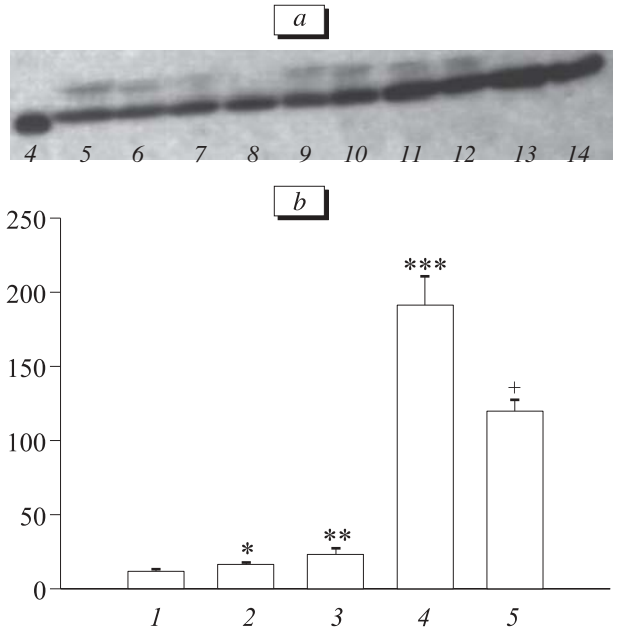

Fig. 2. Effect of adaptation to 3 μM H_2O_2 on HSP32 synthesis in HT22 cells. Representative electrophoretogram of HSP32 accumulation in HT22 cells (*a*): HSP32 marker (*4*); control (*5*, *6*); single treatment with 3 μM H_2O_2 (*7*, *8*); 3-day adaptation to 3 μM H_2O_2 (*9*, *10*); 3-day adaptation to 3 μM H_2O_2 and oxidative stress with 1.5 mM H_2O_2 (*11*, *12*); oxidative stress with 1.5 mM H_2O_2 (*13*, *14*). HSP32 content in HT22 cells (relative densitometric units, product of signal area and intensity; *b*): control (*1*); single treatment with 3 μM H_2O_2 (*2*); 3-day adaptation to 3 μM H_2O_2 (*3*); oxidative stress with 1.5 mM H_2O_2 (*4*); 3-day adaptation to 3 μM H_2O_2 and oxidative stress with 1.5 mM H_2O_2 (*5*). **p*<0.02, ***p*<0.05, and ****p*<0.001 compared to the control; ^+p<0.02 compared to oxidative stress (Student's *t* test).

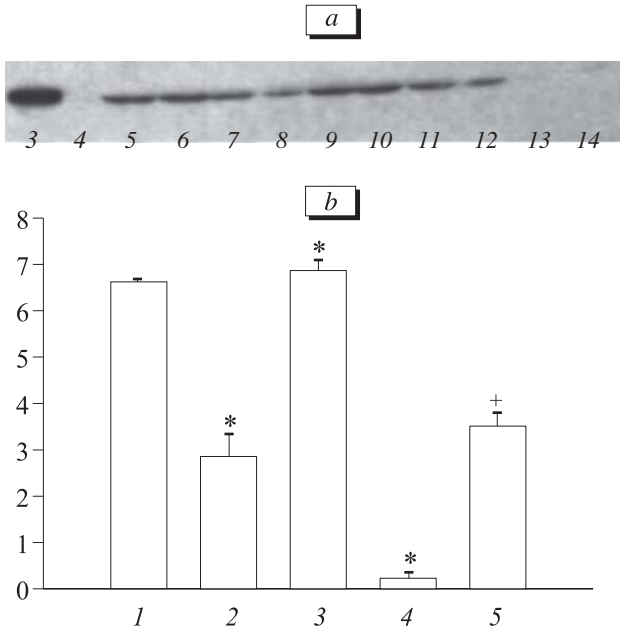

Fig. 3. Effect of adaptation to 3 μM H_2O_2 on HSP70 synthesis in HT22 cells. Representative electrophoretogram of HSP70 accumulation in HT22 cells (*a*): HSP70 marker (*3*); HSP32 marker (*4*); control (*5*, *6*); single treatment with 3 μM H_2O_2 (*7*, *8*); 3-day adaptation to 3 μM H_2O_2 (*9*, *10*); 3-day adaptation to 3 μM H_2O_2 and oxidative stress with 1.5 mM H_2O_2 (*11*, *12*); oxidative stress with 1.5 mM H_2O_2 (*13*, *14*). HSP70 content in HT22 cells (relative densitometric units, product of signal area and intensity; *b*): control (*1*); single treatment with 3 μM H_2O_2 (*2*); 3-day adaptation to 3 μM H_2O_2 (*3*); oxidative stress with 1.5 mM H_2O_2 (*4*); 3-day adaptation to 3 μM H_2O_2 and oxidative stress with 1.5 mM H_2O_2 (*5*). **p*<0.001 compared to the control; ^+p<0.001 compared to oxidative stress (Student's *t* test).

Figure 1, *b* presents the data on the effect of a more potent stress factor on survival rate of nerve cells and the protective effect of preadaptation. Incubation with 1.5 mM H_2O_2 decreased metabolic activity of cells by 51.5±1.7%. Despite massive cells death, preadaptation to 3 μM H_2O_2 reduced the decrease in this parameter by 27.8±2.2%. Therefore, preadaptation to H_2O_2 increased the resistance of nerve cells to severe oxidative stress.

TABLE 1. Influence of Quercetin on the Protective Effect of Adaptation to H_2O_2 during Oxidative Stress in HT22 Cells (*M*±*m*)

Group	MTT reductase activity (% of control)	
	without quercetin	with quercetin
Control	100.0±2.5	100±0
Adaptation to 3 μM H_2O_2	96.1±3.4	96.9±3.6
Oxidative stress (600 μM H_2O_2)	73.4±3.8*	80.4±2.2
Adaptation to 3 μM H_2O_2 and oxidative stress (600 μM H_2O_2)	93.0±0.4$^+$	57.8±1.9^x

Note. p<0.001: *compared to the control; $^+$compared to oxidative stress; $^x p$<0.001 and p<0.01 compared to the control and oxidative stress, respectively.

These data show that adaptation of HT22 mouse hippocampal neurons decreases damage induced by oxidative stress (600 μM and 1.5 mM H_2O_2) by 100 and 50%, respectively.

HSP synthesis in the organism plays an important role in the protective effect of adaptation [8]. HSP accumulation was studied during adaptation of hippocampal neurons to 3 μM H_2O_2. Figure 2 illustrates the results of Western blot analysis of HSP32 content in HT22 cells. Single treatment with 3 μM H_2O_2 slightly increased HSP32 content in cells, while 3-day adaptation increased the content of this protein by 2 times. Oxidative stress (exposure with 1.5 mM H_2O_2) increased induction of HSP32 in these cells by 16.27 times compared to the control. Since HSP32 is a marker of oxidative stress [10], it can be concluded that adaptation of cells is accompanied by moderate activation of oxidative stress. Treatment with H_2O_2 in high doses causes severe oxidative stress, which results in cell

damage. Preadaptation of HT22 cells to 3 μM H_2O_2 decreased HSP32 content during subsequent exposure to damaging oxidative stress (by 37.33±13.5% compared to treated cell with no preadaptation). HSP32 is a marker of oxidative stress. Hence, preincubation of HT22 cells with H_2O_2 in non-damaging concentrations decreases the severity of this stress.

Study of HSP70 synthesis in HT22 cells showed that these cells exhibit high basal expression of the protein under control conditions (Fig. 3, *a*, *b*). Single treatment with 3 μM H_2O_2 was followed by a decrease in HSP70 content in MT22 cells by 2.3±0.3 times compared to the control. Adaptation contributed to increased induction of this protein compared to the control. However, HSP70 induction in nerve cells decreased by 28.7±0.1 times after exposure to oxidative stress induced by 1.5 mM H_2O_2. Three-day preadaptation of HT22 cells to 3 μM H_2O_2 protected these cells from severe oxidative stress, which was related to the increased induction of HSP70 (by 15.2±0.2 times compared to the cells exposed to oxidative stress with no preadaptation). Therefore, HSP70 is involved in the protective effect of adaptation to H_2O_2. Our results are consistent with published data that HSP70 plays a protective role during oxidative stress accompanied by neurodegenerative changes in the organism [11,14].

Experiments with HSP70 synthesis inhibitor quercetin (50 μM) were performed to evaluate the role of HSPs in the protective effect of adaptation to H_2O_2 [13]. Administration of quercetin in this dose for 3 days had no effect on MTT reductase activity in cells under control conditions. However, the protective effect of adaptation (MTT test) was not observed after treatment with quercetin. Preadaptation did not protect neurons from oxidative stress after quercetin administration. At the same time, experiments without quercetin indicate that adaptation of neurons to H_2O_2 has a strong protective effect on their survival (Table 1, Fig. 1). Hence, HSPs play an important role in the protective effect of adaptation to H_2O_2 in isolated cells not exposed to neurohormonal influences.

Our study demonstrates the possibility for adaptive protection from oxidative stress. Adaptation of cells has a direct protective effect, which is consistent with published data [3]. We conclude that adaptation of cells can be realized without no involvement of neuroendocrine factors.

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