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The Ability of Cells to Adapt to Low-Oxygen Conditions Is Associated with Glutathionylation of Na,K-ATPase

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Abstract—The decrease in the oxygen content of tissues, which is observed in a number of pathological processes, inevitably leads to damage. One of the main causes of cell damage and death in hypoxia is the failure of the systems that maintain the ionic balance. Na,K-ATPase is the main ion-transporting protein in the plasma membrane of animal cells, and its inhibition at low concentrations of oxygen is one of the earliest and most critical events for cell viability. Scientists are currently conducting an active search for regulators of Na,K-ATPase activity. Cardiac glycosides traditionally used for this purpose induce severe side effects, which necessitates the search for alternative inhibitors of Na,K-ATPase. We have previously found that glutathionylation of the Na,K-ATPase catalytic subunit leads to a complete inhibition of the enzyme. The present study demonstrates that the substances that increase the level of glutathionylation in Na,K-ATPase, namely, ethyl glutathione (et-GSH), oxidized glutathione (GSSG), and *N*-acetylcysteine (NAC), enhance cell survival under low-oxygen conditions, prevent ATP depletion, and normalize the redox status of the cells. The following concentration range in which these substances have the maximum protective effect and no pronounced cytotoxic properties was determined to be as follows: 0.2–0.5 mM et-GSH, 0.2–1 mM GSSG, and 10–15 mM NAC. These results demonstrate the prospects of developing methods of protecting tissues from damage under low-oxygen conditions that are based on changes in Na,K-ATPase glutathionylation.

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Keywords: Na,K-ATPase, hypoxia, ischemia, ATP depletion, protection against oxidation, glutathionylation, intracellular redox status

Hypoxia is a reduction of the oxygen content in tissues below the physiological level that always accompanies ischemia. This is observed in a number of pathological processes, including cardiovascular and neurovascular processes, the development of which can lead to myocardial infarction and brain hemorrhage. Hypoxia that inevitably causes tissue damage is also observed in diabetes, various lung pathologies, and surgical procedures [1, 2]. Acute and chronic myocardial and cerebral ischemia are among the major causes of death and disability in Russia's population. Restoring blood flow to hypoxic tissues can cause additional damage because of the development of oxidative stress [1].

One of the main causes of cell damage and death in hypoxia is the dysregulation of ion-transporting systems and the ionic balance required for the normal functioning of the cells [3–7]. Na,K-ATPase is the main ion-transporting protein in the plasma membrane of animal cells; it creates the Na^+ and K^+ gradient required for cell viability. Functional monomer of the Na,K-ATPase comprises a catalytic α-subunit and a regulatory β-subunit. The α-subunit contains the binding sites for ATP, Na^+, and K^+; it is responsible for the ATP hydrolysis and the transport of ions. Regulatory β-subunit is required for membrane integration of the α-subunit and for the occlusion of potassium ions [8]. Maintaining the function of Na,K-ATPase requires a sufficient amount of intracellular ATP; in the muscle tissue, it uses more than 20% of the intracellular ATP and, in the brain, it uses more than 80% [3]. The inhibition of the Na,K-ATPase in hypoxia is considered to be one of the earliest events in hypoxia, and the event is critical for cell viability [7, 9, 10]. Short-term enzyme inhibition is adaptive in nature; for instance, it allows one to save ATP, the formation of which is reduced during hypoxia. It also increases the intracellular sodium content, inhibits the Na^+/Ca^{2+} exchanger, and increases the concentration of intracellular calcium. In cardiomyocytes, it increases the heart rate, which improves the blood supply to the tissues [11]. However, the prolonged disturbance of the ion balance that results from the inhibition of the Na,K-ATPase causes cell death.

The effect of cardiac glycosides (ouabain, digoxin, etc.) that are currently used to stimulate cardiac activ-

ity is based on the Na,K-ATPase inhibition [12]. These drugs are used to treat congestive cardiac failure, cardiogenic shock, and cardiac arrhythmias. The inhibition of Na,K-ATPase by cardiac glycosides reduces the damage to myocardial tissue during ischemia/reperfusion [13]. However, not only do the cardiac glycosides inhibit the Na,K-ATPase, but they also trigger the activation of a number of signaling pathways in cells, and their long-term use leads to the development of various pathologies, such as hyperkalemia and cardiac hypertrophy [14, 15]. Moreover, it is rather difficult to select effective doses of cardiac glycosides that cause no toxic manifestations. By inhibiting the Na,K-ATPase, the cardiac glycosides do not reduce the risk of its irreversible damage by reactive oxygen species (ROS) in hypoxia, and the enzyme reactivation under these conditions is hindered.

Currently, there is an active search for the alternative regulators of the Na,K-ATPase activity. We hypothesized that tripeptide glutathione may act as a physiological redox-sensitive regulator of Na,K-ATPase. The addition of glutathione not only protects the thiol groups of proteins from irreversible oxidation and loss of function, it also regulates the activity of a number of proteins [16]. We have shown that the level of glutathionylation of the Na,K-ATPase α-subunit of in rat myocardium increases in hypoxia; it is accompanied by the enzyme inhibition that result from the blockage of the ATP binding [17]. The Na,K-ATPase glutathionylation in hypoxia can be regarded as a physiological mechanism of cell protection, which prevents the irreversible oxidation of the protein and reduces the ATP consumption by the cell. In this study, the glutathionylation of the Na,K-ATPase α-subunit in cell cultures under the conditions of oxygen deficiency was studied. It was shown that the substances that an in increase in the level of Na,K-ATPase glutathionylation, ethyl glutathione (et-GSH), oxidized glutathione (GSSG), and *N*-acetylcysteine (NAC) enhance cell survival in hypoxia, prevent the drop of ATP content, and normalize the intracellular redox status. The range of concentrations in which these substances exert maximum protective effect and demonstrate no significant cytotoxic properties have been defined. The prospects of developing the methods of tissue protection against damage under oxygen-deficient conditions that are based on the changes in the Na, K-ATPase glutathionylation have been demonstrated.

EXPERIMENTAL

Cell Cultures

Mouse embryonic stem cells SC1 were used in this study. The cells were grown at 37°C with 5% CO_2 in DMEM medium with a high glucose concentration (4.5 g/L) (Invitrogen) made up of 10% fetal calf serum (Invitrogen), 2 mM glutamine (PanEko), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The cells were seeded on sterile Petri dishes (35 × 10 mm) for adhesion cell cultures (Corning-Costar) at 400000 cells per dish.

Substances That Alter the Status of Cell Redox

Chlorodinitrobenzene (CDNB, Sigma) and diethyl maleate (DEM, Sigma) are the substances that form conjugates with glutathione and lead to a decrease in its intracellular level; nitrosoglutathione (GSNO, Sigma) is a glutathione derivative that causes an increase in its intracellular level, but it also affects both glutathionylation and nitrosylation of proteins; *N*-acetylcysteine (NAC, Sigma) is a cysteine derivative that penetrates the cell, acts as a reducing agent, and increases glutathione pool; GSSG (AppliChem) is an oxidized glutathione, and ethyl glutathione (et-GSH, Sigma) is a cell-penetrating analogue of reduced glutathione. Moreover, buthionine-sulfoximine (BSO, Sigma), an inhibitor of glutathione synthesis, and L-NAME, an NO-synthase inhibitor (Sigma), were also studied. The cells were preincubated with these compounds for 60 min.

Detection of Level of Na,K-ATPase Glutathionylation by Immunoblotting

In order to estimate the effect of the studied substances on the Na,K-ATPase glutathionylation level, the cells were incubated with them for 1 h, after which the samples were centrifuged, and the pellet was frozen. The samples were thawed prior to the immunoblotting. We used 30 μL RIPA per 1 million cells. The obtained cell suspension was incubated for 30 min at +4°C with constant stirring. The cell lysate was centrifuged for about 1 min at 16000 g. The supernatant was collected and the protein concentration was determined by the Lowry method. Then, Laemmli electrophoresis and the electrotransfer of proteins from the gel to a nitrocellulose membrane were performed. The immunoblotting of cell lysates was performed using antibodies to glutathione from the protein set MAB5310 (Chemicon Millipore) at a dilution of 1 : 1000, as was previously described in [17]. Afterwards, the antibodies to glutathione were removed, and immunoblotting was performed to identify the catalytic α1-subunit using the C464-6 (Upstate Millipore) antibodies to α1-subunit at a dilution of 1 : 10000. The intensity of the catalytic subunit glutathionylation band was normalized by the amount of the subunit in the band. The immunoblotting visualization was performed using BioRad ChemiDoc XRS+ and Image Lab software.

Detection of Level of Na,K-ATPase Glutathionylation Using Immunoprecipitation

Immunoprecipitation was performed on lysates of SC-1 cells. The antibodies to the Na,K-ATPase α1-subunit (15 μg) were added to the cell lysate, and the samples were incubated overnight at +4°C and

constant agitation. The resulting immune complex was added to a tube containing protein A agarose, after which the samples were incubated for 2 h at +4°C and constant agitation. The samples were then centrifuged for 1 min at 5000 g, and the supernatant was removed. The precipitate was washed with phosphate-saline Dulbecco buffer for three times. Three tubes were filled by 10 mL 1 M Tris-HCl, pH 9.5 (neutralizing buffer). The volume of 190 μL 0.1 M glycine-HCl, pH 2.8 was added to the immune complexes. The tubes were gently shaken several times; then, their contents were placed in a neutralizing buffer. The samples were gently shaken and then centrifuged for 1 min at 5000 g. The supernatant was collected. This procedure was repeated two more times. The content of protein fractions was analyzed using polyacrylamide gel electrophoresis (PAGE) (Laemmli). Then, the Na,K-ATPase glutathionylation level was detected using immunoblotting.

Determination of Cell Survival under Hypoxic Conditions

The cells (96-well plates) were incubated in a medium containing less than 1% oxygen. For this purpose, a sealed box of oxygen-impermeable material was used; the box was filled with premixed gases from the cylinders containing nitrogen, 5% carbon dioxide, and exactly specified amount of oxygen. Cell survival was determined by the measurement of respiratory activity using the MTT assay (Invitrogen). MTT dye was diluted in phosphate buffer to a concentration of 12 mM. Ten microliters of the dye were added to each well on the plate; the solution was mixed and incubated for 4 h at a given oxygen concentration and a temperature of 37°C. Then, 150 μL dimethyl sulfoxide were added to each well, the sample was mixed and incubated for about 1 min at 37°C. Each sample was mixed once again, and the absorbance was measured at 540 nm.

Measurement of ATP Level

The ATP level in the cell lysates was determined by chemiluminescence in the luciferin–luciferase system; for this purpose, an ATP determination kit (Invitrogen) was used. Chemiluminescence intensity was recorded using a Plate Chameleon 425-106 plate analyzer (Hidex Ou).

Estimation of the Intracellular Parameters by Flow Cytometry

The cells were suspended for analysis. The cells grown in 12-well plates were removed from the culture substrate by trypsin treatment and plated into flasks, each of which was hermetically closed by a plug with an inlet and an outlet of small diameter (less than 2 mm). Cell growth medium was collected in order to remove the cells. Then, the cells were washed with a Versene solution (120 μL per 1.9 cm^2), covered with a monolayer of a cell-removal solution (a sterile solution of 0.25% trypsin containing Hanks salts without Ca^{2+} or Mg^{2+}) (120 μL per 1.9 cm^2), and incubated at 37°C. During incubation, the cells were periodically gently shacked for 1 or 2 min. When cells became completely separated from the substrate, they were supplied with 500 μL of a complete medium and thoroughly dispersed. After staining with trypan blue, the cells were counted in a Goryaev counting chamber under an inverted microscope. The cell suspension was placed into flasks.

Flasks containing the cell suspension were placed in a water bath (37°C). Before that, the inlets and outlets of the flasks were serially connected with hoses such that three flasks formed one connection. The gas from a gas cylinder containing 5% CO_2 with fixed contents of oxygen and nitrogen was fed to the first of the flasks. Before entering this flask, the gas passed through three flasks filled with high purity water (Millipore) that were incubated in the bath. Through this process, the gas mixture provided to the cells was of the necessary humidity and temperature. An outlet hose of each flask was connected to a flask with water, which enabled us to register the flow of gas in the system. Incubation was carried out for 3.5 h with stirring and a continuous gas stream.

Cytometric analysis was performed on a GALLIOS flow cytometer (Beckman Coulter). Before staining, the cells were centrifuged, and the supernatant was removed. The cells were then resuspended in 100 μL phosphate buffer. The level of ROS was assessed using dihydrorhodamine 123 (DHR) (Invitrogen, Ex/Em 507/525), which is capable of detecting ROS, not only in cytosol, but also in mitochondria. The cells were stained by adding DHR 123 to a final concentration of 10 μM [18] an incubating for 30 min at 37°C in the dark. The level of glutathione was evaluated using CMFDA (5-Chloromethylfluorescein Diacetate, Invitrogen, Ex/Em 488/535 nm) [18]. The level of NO was assessed using a DAF-FM-DA dye (4-Amino-5-Methylamino-2', 7'Difluorofluorescein Diacetate) [9]. DAF-FM-DA penetrates the cell, where its acetate groups are cleaved and DAF-FM-DA transforms into its fluorescent form (DAF-FM), which is incapable of penetrating the cell membrane. After its interaction with NO, the quantum yield of DAF-FM increases by a factor of 160 (Ex/Em DAF-FM, ~495/515 nm), which leads to a significant increase in fluorescence intensity and allows one to estimate the level of NO in the cell. For staining, the cells were incubated with 5 μM DAF-FM for 30 min at 37°C. To detect cells with damaged membranes, propidium iodide (Sigma, Ex/Em 535/617 nm) was added to a final concentration of 10 μg/mL 1 min before the measurement. The levels of glutathione, ROS, and NO were determined from an average intensity of

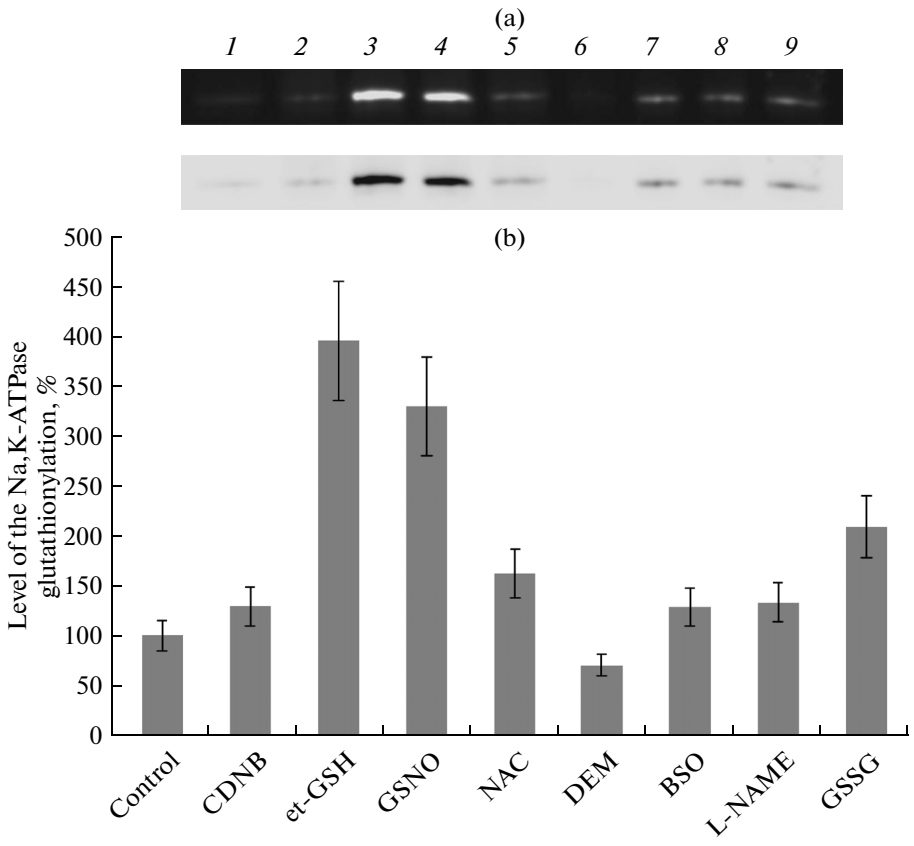

Fig. 1. Level of glutathionylation of the Na,K-ATPase α-subunit after the incubation of SC1 cells for 60 min in the presence of 0.3 mM CDNB, 5mM et-GSH, 0.2 mM GSNO, 5 mM NAC, 1 mM DEM, 0.1 mM BSO, 0.1 mM L-NAME, and 1 mM GSSG. (a) Immunobloting of the SC1 cell lysates with the antibodies against glutathionylated proteins for the cells pre-incubated with (*2*) CDNB, (*3*) et-GSH, (*4*) GSNO, (*5*) NAC, (*6*) DEM, (*7*) BSO, (*8*) L-NAME, and (*9*) GSSG. *1* is the control. Top panel: results of immunoblotting visualized by chemiluminescence. Bottom panel: inverted immunoblot image. (b) Results of immunoblot digital processing. Level of Na,K-ATPase glutathionylation in untreated cells is 100%. We present the average values obtained from three independent measurements performed in triplicate ± SD.

green fluorescence in the cells unstained with propidium iodide.

Statistical Analysis

The average values of standard deviation (SD) are given. A comparison of the data groups was performed using Student's t test and the values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Substances That Regulate the Redox Status of Cells on Degree of Na,K-ATPase Glutathionylation

Figure 1 presents data on changes in the level of glutathionylation of Na,K-ATPase catalytic subunit after cells were treated with substances that alter their redox status, including etGSH, NAC, GSNO, GSSG, BSO, and L-NAME. The inhibitor of glutathione synthesis, BSO, and the NO synthase inhibitor, L-NAME, did not alter the level of glutathionylation of the Na,K-ATPase, which is consistent with our earlier data on the absence of influence of these substances on the intracellular glutathione level [18]. Substances that cause the depletion of glutathione, CDNB, and DEM in cells also increase the level of protein glutathionylation. The increased glutathionylation of the Na,K-ATPase catalytic subunit was observed upon incubation with et-GSH, NAC, GSNO, and GSSG. The greatest increase was observed upon the incubation of the cells with et-GSH (Fig. 1). Previously, we have shown that et-GSH inhibits the activity of Na,K-ATPase in primary cultures of rat cerebellar neurons [18], but the reason for this was unclear. The results of this study, along with the data on the inhibition of enzyme activity by glutathionylation [17], show that glutathionylation is the cause of the enzyme inhibition upon the incubation with et-GSH.

Previously, we suggested that a lack of oxygen may be a physiological stimulus to increase glutathionylation of the Na,K-ATPase catalytic subunit n the cells [17]. The change in the glutathionylation of the Na,K-ATPase catalytic subunit in SC1 cells at different oxygen contents in the atmosphere (20, 0.2, and

0.05% O_2) was determined using immunoprecipitation with the antibodies against the catalytic subunit of Na,K-ATPase and subsequent immunoblotting with the antibodies against glutathione (Fig. 2). Upon a reduction in the oxygen content to 0.05 and 0.2%, the level of the enzyme glutathionylation increases two- and threefold, respectively. Furthermore, the activity of Na,K-ATPase should decrease because glutathionylation of cysteine residues near the nucleotides binding site disrupts the interaction between ATP and the enzyme [17]. The increase of the Na,K-ATPase glutathionylation during hypoxia is attributable to the reduced levels of ATP in the cell (Fig. 3), as we have previously shown that the efficiency of the enzyme glutathionylation increases with a decrease in the ATP level [17, 19]. Moreover, the ratio of oxidized and reduced glutathione in cells increases under hypoxia, which contributes to Na,K-ATPase glutathionylation [17]. For instance, it was demonstrated in the myocardial tissue [20]. ATP prevents the glutathionylation of the enzyme more efficiently than ADP and AMP [19]. Apparently, the glutathionylation of the Na,K-ATPase, which is the primary consumer of ATP, is a protective mechanism for preventing the depletion of ATP in cells at a low oxygen content.

Cell Treatment with Substances That Increase the Level of Glutathionylation Protects Cells from Damage in Hypoxia

The effects of et-GSH, GSSG, and NAC on the survival of SC1 cells in hypoxia (0.2% O_2) (Fig. 4) was assessed to find out how induced increase of the Na,K-ATPase glutathionylation affects cell viability. The incubation of cells for 3.5 h at a low oxygen content reduces their survival by 32%; however, the pretreatment of the cells with et-GSH, GSSG, and NAC reduces the adverse effects of hypoxia. The greatest protective effect of et-GSH is manifested at 0.5 mM et-GSH. In this case, cell viability was restored to the control level. When using et-GSH at the concentration of 1 mM or higher, the protective effect decreases, apparently due to the prooxidant activity that manifests itself at high et-GSH concentrations, as we have previously shown [18]. The antihypoxic effect of GSSG is most pronounced when the substance is used at a concentration of 0.5 mM, but the effect is considerably lower than that of et-GSH due to a low ability of GSSG to penetrate cell membrane. The protective effect of NAC is manifested at high concentrations of the substance (10–15 mM).

The ATP level in SC1 cells that were incubated for 3.5 h under hypoxic conditions (0.2% pO_2) was two times lower than in the control (Fig. 3). Preincubation with et-GSH, GSSG, and NAC prevents a reduction of the ATP level in cells under the conditions of oxygen deficiency. This confirms that the inhibition of Na,K-ATPase induced by glutathionylation decreases ATP consumption.

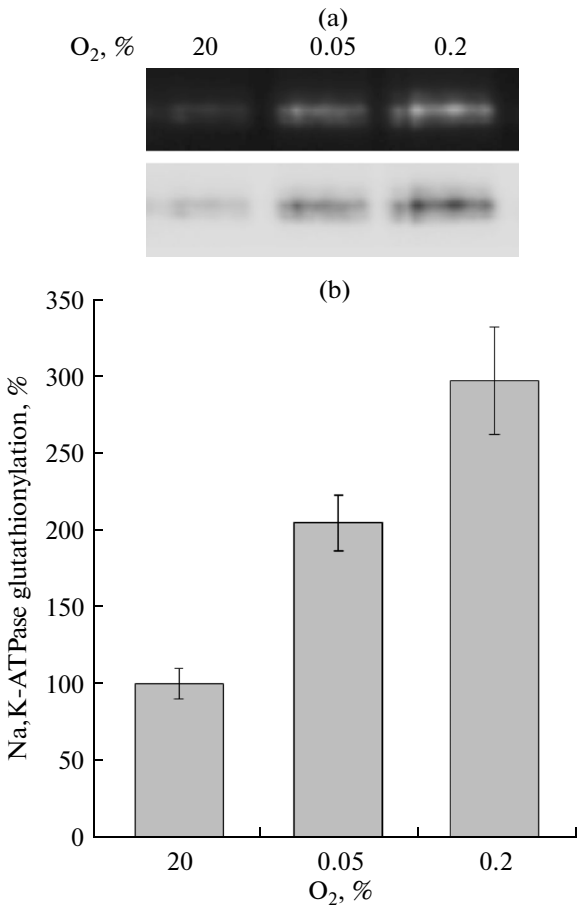

Fig. 2. Level of Na,K-ATPase glutathionylation in SC1 cells incubated at different oxygen contents (20, 0.05, and 0.2%). (a) Top panel: immunoblotting results visualized by chemiluminescence. (b) Results of the immunoblot digital processing. 100% is the level of Na,K-ATPase glutathionylation at 20% O_2. The incubation was performed for 3.5 h at 37°C. We present the average values obtained from three independent measurements performed in triplicate ± SD.

To understand how the protective action of et-GSH, GSSG, and NAC is implemented, the impact of these substances on the redox status of SC1 cells was analyzed. Cytofluorimetric analysis showed that preincubation with et-GSH (0.5 mM), GSSG (0.5 mM), and NAC (10 mM) normalizes the redox status of the cells under hypoxic conditions (Fig. 5). Figure 5 shows the increase in the levels of ROS, intracellular glutathione, and nitric oxide in the SC1 cells incubated for 3.5 h in a medium containing 0.05% O_2. The cells that were preincubated with et-GSH, GSSG, and NAC for 60 min under standard conditions (20% O_2) maintained normal levels of glutathione in hypoxia. Moreover, if et-GSH and GSSG were used, the levels of ROS and nitric oxide also decreased. NAC had the lowest impact on the redox status of the cells under hypoxic conditions.

A decrease in the oxygen content in the medium leads to a drop in the ATP level in cells and tissues

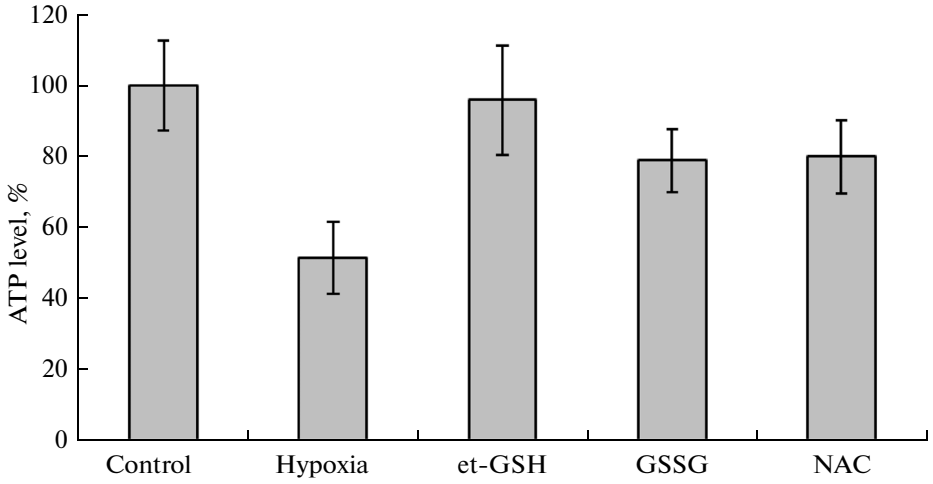

Fig. 3. Changes in ATP level in SC1 cells after their incubation at 0.2% O_2 for 3.5 h. Cells were preincubated for 60 min in the presence of 0.5 mM et-GSH, 10 mM NAC, or 0.5 mM GSSG. We present the average values obtained from three independent measurements performed in triplicate ± SD.

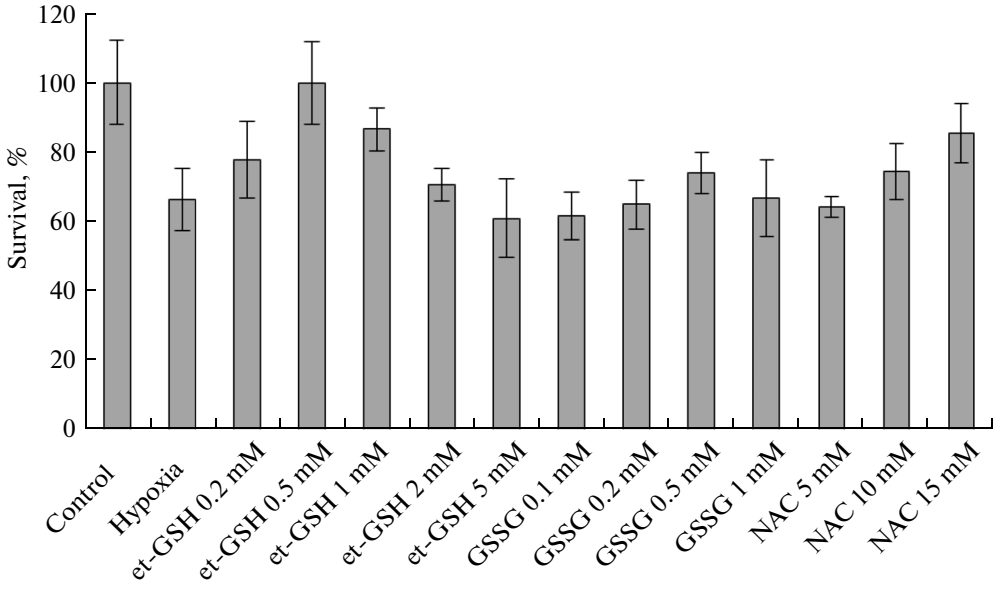

Fig. 4. Survival of SC1 cells after incubation at 20% (control) and 0.2% O_2 (hypoxia) for 3.5 h, 37°C. Cells were preincubated for 60 min with et-GSH (0.2, 0.5, 1.2, and 5 mM); GSSG (0.1, 0.2, 0.5, and 1 mM); and NAC (5, 10, and 15 mM). We present the average values obtained from three independent measurements performed in triplicate ± SD.

[9, 21]. In the hypoxia-resistant animals, the ATP level is maintained by the inhibition of Na,K-ATPase and channel arrest, which insures the inactivation of the ion channels, thereby preventing the leakage of ions from the cells. The cells of most mammals, including human, are incapable of the channel arrest. Therefore, the prolonged inactivation of the Na,K-ATPase is dangerous for them. The inhibition of Na,K-ATPase at a low oxygen content is observed in cells of different types. It can be the cause of pathological events, such as the loss of muscle tissue excitability and neuronal activity, swelling, and necrotic death. However, in severe hypoxia, the inhibition can promote cell survival by reducing the consumption of ATP [10]. We have found that the inhibition of the enzyme in hypoxia is caused by the glutathionylation of its catalytic subunit, which is induced by changing the redox status of the cells and the drop in the level of ATP [17]. We believe that the short-term shutdown of the enzyme by glutathionylation, which is reversed by glutaredoxin upon the normalization of the redox status [16, 17], increases the cell's ability to adapt to low

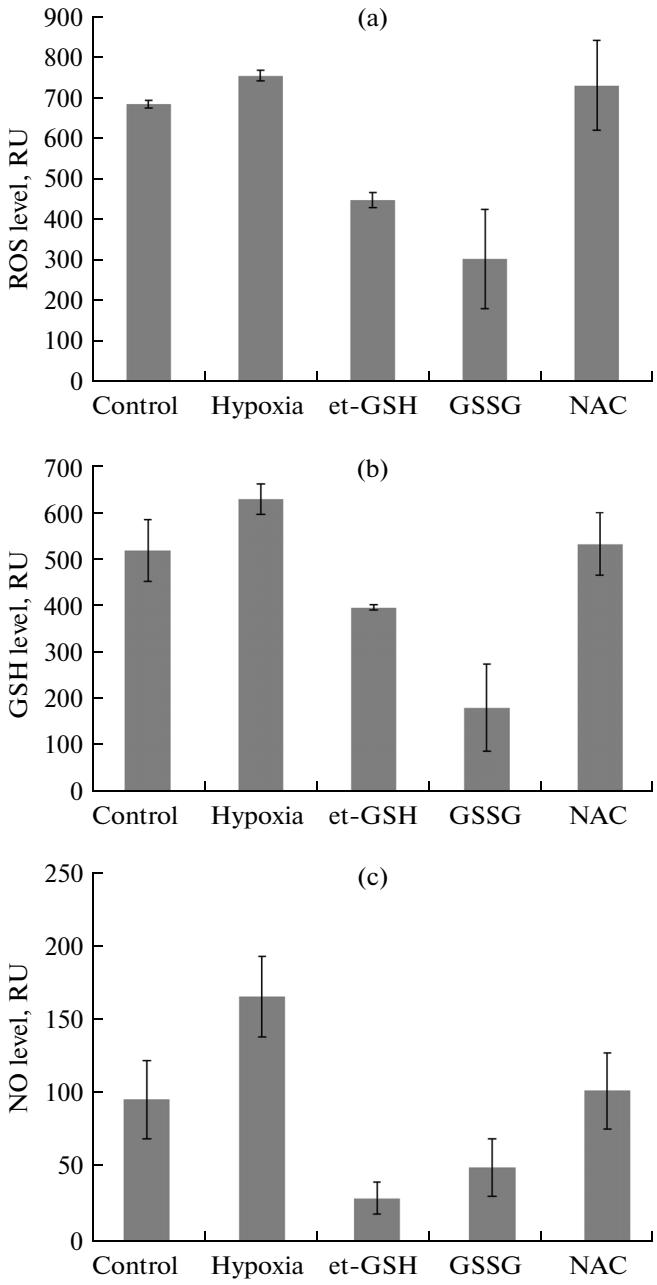

Fig. 5. Changes in the level of (a) ROS, (b) intracellular glutathione, and (c) nitrogen oxide in SC1 cells after incubation with 0.05% O_2 for 3.5 h. Cells were preincubated for 60 min with 0.5 mM GSH, 10 mM NAC, or 0.5 mM GSSG. We present the average values obtained from three independent measurements performed in triplicate ± SD.

oxygen conditions. According to the data obtained in the present study, preincubation with the substances that cause the increase in the Na,K-ATPase glutathionylation reduces cell damage in subsequent hypoxia. These substances may have multiple targets; thus, the glutathionylation of the Na,K-ATPase catalytic subunit can probably be considered one of the factors that increase cell survival during hypoxia and normalize their redox status. The increase in the Na,K-ATPase glutathionylation may be achieved by treating cells with et-GSH, GSSG, or NAC. The protective effect of these substances is maximal and the cytotoxic activity is unpronounced at the following concentrations: 0.2–0.5 mM etGSH, 0.2–1 mM GSSG, and 10–15 mM NAC. These results indicate that the controlled glutathionylation of the Na,K-ATPase can be promising for protection from tissue damage in the conditions of oxygen deficiency.

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