ISSN 0026-8933, Molecular Biology, 2017, Vol. 51, No. 1, pp. 148–154. © Pleiades Publishing, Inc., 2017.
Original Russian Text © V.A. Lakunina, K.M. Burnysheva, V.A. Mitkevich, A.A. Makarov, I.Y. Petrushanko, 2017, published in Molekulyarnaya Biologiya, 2017, Vol. 51, No. 1, pp. 172–179.

MOLECULAR CELL BIOLOGY

UDC 577.1:577.32

Changes in the Receptor Function of Na,K-ATPase during Hypoxia and Ischemia

V. A. Lakunina, K. M. Burnysheva, V. A. Mitkevich, A. A. Makarov, and I. Y. Petrushanko*

Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Moscow, 119991 Russia

**e-mail: irina-pva@mail.ru*

Received June 6, 2016; in final form, June 24, 2016

Abstract—Na,K-ATPase maintains sodium and potassium homeostasis. It is the only known receptor for cardiotonic steroids such as ouabain. Binding of ouabain to Na,K-ATPase leads to the activation of Src kinase and the subsequent initiation of intracellular signaling pathways, including the induction of apoptosis. Changes in Na,K-ATPase activity is one of the earliest responses to hypoxia and is most critical for cell survival. However, it is not known how the hypoxia affects the functioning of Na,K-ATPase as a receptor. We have shown that, under the conditions of hypoxia and ischemia, ouabain is less toxic for murine fibroblast cells (SC-1 cell line) and ouabain does not cause an increase in the level of reactive oxygen species, which is typically observed at 20% pO_2. Under hypoxia, the treatment of cells with ouabain also does not lead to the activation of Na,K-ATPase-associated Src kinase. Thus, at low oxygen content, the receptor function of Na,K-ATPase is altered, and cells become less sensitive to cardiotonic steroids. The decrease in sensitivity to cardiotonic steroids, which is evident at hypoxic conditions, should be taken into account in clinical practice. At the same time, in the presence of ouabain the cells are less sensitive to hypoxia, which indicates that cardiotonic steroids can be protective in acute ischemia.

Keywords: Na,K-ATPase, receptor function, ischemia, hypoxia, ouabain, Src kinase

DOI: 10.1134/S0026893317010101

Na,K-ATPase is a transmembrane protein that creates a gradient of ions that is critical for the cell viability of any animal cell. The enzyme transports three Na ions out of the cell and two K ions into the cell against their electrochemical gradients and uses the energy of ATP. During the enzymatic cycle, Na,K-ATPase undergoes two main E1–E2 conformational transitions as follows: E1 with high affinity for Na^+ and ATP and E2 with high affinity for K^+. The monomer of Na,K-ATPase is composed of two subunits, i.e., the (α) catalytic and (β) regulatory. The α-subunit contains binding sites for metal ions, actuator, and nucleotide-binding domains, as well as the P-domain, which undergoes phosphorylation during the catalytic cycle. The β-subunit is required for embedding the α-subunit into the membrane and the occlusion of potassium ions. This subunit contains only a single transmembrane loop and a heavily glycosylated extracellular domain [1].

In addition to ion-pumping function, Na,K-ATPase also functions as the receptor for cardiotonic steroids (CTSs), specific inhibitors of the Na,K-ATPase. CTSs were detected and isolated from plants more than 200 years ago; since then, they have been widely used in clinical practice in patients with chronic heart failure [2]. The CTS molecule binds to the extracellular fragment of the α-subunit and stabilizes the enzyme in the E2P conformation and disrupts the cycle of the Na,K-ATPase [3, 4]. After inhibiting the enzyme, the intracellular Na^+ concentration increases, which leads to the inhibition of the Na^+/Ca^+-exchanger and slows the transmembrane exchange of Na^+ and Ca^{2+}. This results in an increased concentration of Ca^{2+} in the cell, which increases the force of myocardial contractions [5].

Later, endogenous analogues of CTSs were also found in vertebrates [6]. In particular, endogenous ouabain and marinobufagenin were identified in humans [7, 8]. CTSs present in vertebrate blood plasma at a nanomolar concentration do not inhibit the Na,K-ATPase, but act as hormones triggering apoptosis signaling pathways or cell. Thus, the Na,K-ATPase is not only an ion pump, but also as the receptor for CTSs [2].

At present, it has been suggested that binding of CTSs triggers signal transmission mediated by interaction between the Na,K-ATPase and Src kinase. Src kinase is included in the Src kinase family of tyrosine kinases not coupled to receptors but involved in the regulation of the growth, differentiation, and survival

Abbreviations: CTS, cardiotonic steroid; ROS, reactive oxygen species; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide.

of cells, as well as cytoskeletal organization, secretion, ion channel functioning, and many other cellular processes [9, 10]. Src kinase activity is regulated by phosphorylation of Y416 and Y527 tyrosine residues. Y527 phosphorylation causes conformational changes in the protein molecule resulting in the inactivation of Src kinase [11, 12]. Dephosphorylation of Y527 increases Src kinase activity and the activity of Src kinase enhances stronger after phosphorylation of Y416 [13]. Src kinase binds directly to Na,K-ATPase, forming a signaling complex. The Src kinase in association with Na, K-ATPase is inactive, but binding ouabain freed the kinase domain from the Na,K-ATPase/Src kinase complex and restored the activity of Src kinase [14]. In addition, binding ouabain to the Na,K-ATPase activates phosphorylation of Y416 [9] and further increases the activity of Src kinase.

CTSs are involved in the regulation of many physiological and pathological conditions. Ouabain is one of the most widely used CTSs in medical research and can protect the heart against ischemia-reperfusion injury via activating signaling pathways involving Src kinase and reactive oxygen species (ROS) [15].

The Na,K-ATPase is highly sensitive to the intracellular redox state and oxygen content [16, 17]. The activity of the enzyme was suppressed considerably under hypoxic conditions and oxidative stress [17–20] due to the glutathionylation of regulatory cysteine residues in the α-subunit [20]. This modification is fully reversible and plays an important role in the adaptation of cells, preventing irreversible ATP depletion in the cell [20]. However, whether the receptor function of Na,K-ATPase changes in hypoxia has not yet been elucidated.

MATERIALS AND METHODS

Cell cultures. The SC-1 cell line of murine embryonic fibroblasts was used. Cells were grown in Dulbecco's modified Eagle's culture medium containing 4.5 g/L glucose (Invitrogen, United States), 10% fetal calf serum (Invitrogen), 2 mM glutamine (PanEco, Russia), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), at 37°C under 5% CO_2. Cells were placed in flasks (25 cm^2) for adhesive cell cultures (Corning-Costar).

Immunoblotting analysis of Src kinase phosphorylation. Cells were detached from the plate using trypsin, washed in PBS, centrifuged (100 *g*, 10 min), and the pellet was frozen. To lyse the cells, 200 μL of RIPA buffer was added to the pellet (2.5–3 million cells) containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet-P40, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate. The mixture was incubated at +4°C and stirring for 1 h, after which the samples were centrifuged and the supernatant was collected. Protein concentration in the lysates was measured by the Lowry method. Then, each of the samples was loaded on 10% polyacrylamide gel and the Laemmli method of gel electrophoresis was performed. Then, in a buffer containing 25 mM Tris, 192 mM glycine, and 20% ethanol, the proteins were transferred from the gel to PVDF-membrane (BioRad) at 250 mA for 1.5 h. The membranes were blocked in 5% skim milk (BioRad) in PBST for 30 min and incubated with primary antibodies to Src kinase (2108s, CellSignalling) at a dilution of 1 : 1000, to Src kinase phosphorylated at Tyr527 (2105s, CellSignalling) at a dilution of 1 : 1000, and with antibodies to Src kinase phosphorylated at Tyr416 (2101s, CellSignalling) at a dilution of 1 : 500. The membranes were then hybridized with a solution of secondary horseradish peroxidase-conjugated antibodies. To viualize chemiluminescence, the membrane was stained using a SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Thermo Scientific), the signal was detected using the Bio-Rad ChemiDoc MP apparatus. Each membrane was then incubated with a solution of antibodies to β-actin (AM4302, Ambion), stained with secondary antibodies and the signal was visualized. The analysis of immunoblotting data was performed in the ImageLab software (Bio-Rad). The intensities of each band of Src kinase isoforms were normalized to the intensity of the actin band in the corresponding membrane, and then the value obtained for each of the phosphorylated isoforms was normalized to the value obtained for total Src kinase.

Assessment of cell survival under hypoxic conditions. To determine cell survival under hypoxic conditions (0.05% oxygen), the cells were plated on 96-well plates and placed in the sealed oxygen-impermeable chamber, which was filled with ready gas mixtures from balloons containing 0.05% oxygen, 5% carbon dioxide, and nitrogen. Cell survival was assessed based on their respiratory activity using the MTT test (Invitrogen). MTT dye was diluted in phosphate buffer to a concentration of 12 mM. Ten microliters of dye were added in each well, mixed, and incubated for 3.5 or 24 h at a given oxygen concentration and a temperature of 37°C. Then, 150 μL DMSO were added to each well, mixed, and incubated for 10 min at 37°C. Thereafter, each sample was mixed and the absorbance was measured at 540 nm.

Flow cytometry. Intracellular parameters were assessed with flow cytometry. For this, the cells were suspended. Cells grown in 12-well plates were detached from the support using trypsin. For this, the growth medium was collected, cells were washed with Versen solution; the monolayer was poured with a solution for detaching cells (0.25% sterile trypsin solution with Hanks' salts (without Ca^{2+} and Mg^{2+}) and incubated at 37°C for 1–2 min, shaking periodically. When the cells were completely detached from the plate, 500 μL of complete medium was added and thoroughly dispersed. The cells were transferred into sealed flasks with a stopper that has inlet and outlet openings of small diameters (less than 2 mm).

Flasks with cell suspension were placed on a water bath at 37°C. Inlet and outlet openings of flasks were sequentially connected to tubing with three flasks in one series connection; the first of the flasks was supplied with gas from a balloon with a fixed oxygen concentration (5% CO_2, 0.05% O_2, 94.95% N_2). Before entering the first flask gas from a balloon passed through three similar flasks containing water of high purity (Millipore) standing on a bath to provide the cells with inflowing gas mixture of required humidity and temperature. The output tubing of each flask was connected to a flask with water allowing to record gas flow in the system. After 10 min, ouabain was added in varying concentrations. Cells were incubated for 30 min or 3.5 h with continuous flow of gas and stirring.

Cytometric analysis was performed using the GALLIOS flow cytometer (Beckman Coulter). Before staining, the cells were centrifuged, the supernatant removed, and the cells were resuspended in 100 μL of PBS. The level of ROS was evaluated using dihydrorhodamine 123 (DHR 123) (Invitrogen, Ex/Em = 507/525), which can detect ROS not only in the cytosol, but also in mitochondria. To stain cells, DHR 123 was added to a final concentration of 10 μM and incubated for 30 min at 37°C in the dark. Glutathione levels were evaluated using ThiolTracker (Ex/Em 405/525 nm) (Invitrogen). To stain the cells, ThiolTracker was added at a final concentration of 7.5 μM and incubated for 30 min at 37°C in the dark. To determine the cells with damaged membrane, propidium iodide was added 1 min prior to measurement at a concentration of 10 μg/mL (Sigma, United States, Ex/Em = 535/617 nm). Glutathione and ROS levels were measured based on average intensity of green fluorescence in cells not stained with propidium iodide.

Statistical analysis. The mean values ± standard deviations are given. A comparison of the group data was performed using the Student's *t*-test; value $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Cytotoxic Effect of Ouabain Decreases under Hypoxia and Ischemia

Toxic effects of ouabain under hypoxia and ischemia were assessed using the SC-1 cell line of murine fibroblasts. SC-1 cells contain α1-isoform of Na,K-ATPase. In rodents, the affinity of this isoform to ouabain is three orders of magnitude lower than that of the isoform in humans and other mammals. The effect of ouabain on the receptor function of Na,K-ATPase was evaluated at concentrations of 50, 250, and 500 μM, at which the toxic effects of ouabain are low (Fig. 1). Previously, we have shown that, under hypoxic conditions, in as little as 3.5 h, the level of glutathionylation of the α-subunit of Na,K-ATPase in SC-1 cells increases by two times [21]. The influence of hypoxia and ischemia on the sensitivity of SC-1 cells to ouabain was determined using the MTT test based on the change in cell viability under different conditions after 24 h of incubation with ouabain. Hypoxia conditions were simulated as follows: 0.05% pO_2, pH 7.4, 1 g/L of glucose, while ischemia conditions were simulated as follows: 0.05% pO_2, pH 6.2, glucose 0 g/L. Control cells were at 20% pO_2. After incubation under hypoxic conditions for 24 h, cell viability was reduced 2 times (Fig. 1a). Addition of ouabain in these conditions increases cell viability, in contrast to cells maintained at 20% pO_2, whose viability reduced under the action of ouabain (Fig. 1a). At 20% pO_2, pH 6.2, and the absence of glucose, the cytotoxic effects of ouabain are enhanced and appear at lower concentrations. At a concentration of 500 μM, ouabain causes a 37% decrease in cell viability (Fig. 1b). A reduction in the oxygen content at pH 6.2 and the absence of glucose reduce cell viability by 80%. However, under these conditions simulating ischemia, cytotoxic effect of ouabain is not observed.

It is known that ROS levels can rise in the cells in the presence of ouabain [22]. Under conditions of 20% pO_2, SC-1 cells show a dose-dependent increase in ROS as early as 3.5 h after incubation with ouabain (Fig. 2). At a concentration of 500 μM ouabain, level of ROS increased by almost two times. The same increase in ROS was also identified during incubation under 20% pO_2 in medium without glucose at pH 6.2 (Fig. 2). At the same time, under hypoxic conditions, the ROS level increases to a lesser extent under the influence of ouabain and hardly changes under ischemia. Thus, the cell response to ouabain changes at low oxygen. Under hypoxia, ouabain has a protective effect (Figs. 1, 2). The observed effect explains cardioprotective influence of ouabain exposure against myocardial tissue injury after myocardial infarction [15]. This may be associated with altered affinity of ouabain/Na,K-ATPase complex to intracellular ligands under low oxygen levels.

Ouabain Lowers Level of Activated Src Kinase under Hypoxia

The influence of ouabain on Src kinase activity was assessed based on the change in the level of inhibitory (Y527) and activating (Y416) phosphorylation with Western blotting (Fig. 3). Inhibitory phosphorylation of Src kinase level decreases (Fig. 3b) and activating phosphorylation level increases (Fig. 3c) in SC-1 cells at 20% pO_2 under the influence of ouabain at a concentration of 250 and 500 μM. An increase in the level of activating phosphorylation under the influence of ouabain was shown previously for other types of cells [9, 14, 23]. At hypoxia (0.05% pO_2) the level of activating phosphorylation is significantly higher than at 20% pO_2 (Fig. 3b), which was also shown in samples of the rat pulmonary artery [10]. The addition of ouabain under hypoxic conditions causes a reduction in activating phosphorylation level of Src kinase more than

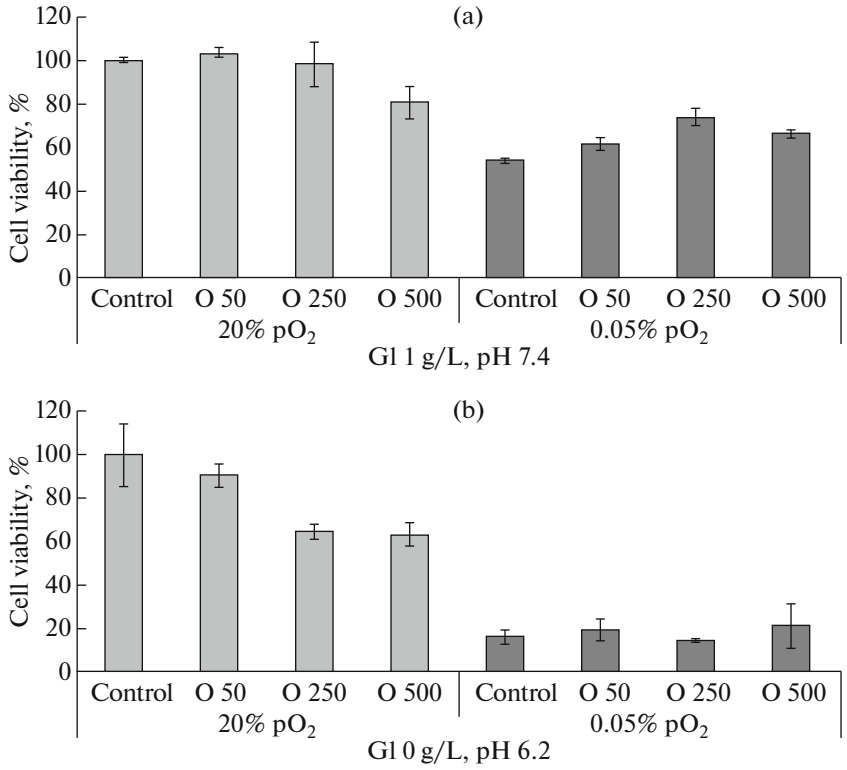

Fig. 1. Changes in viability of SC-1 cells at 20 and 0.05% oxygen under the influence of ouabain (O) at different concentrations (a) under normal (1 g/L) glucose (Gl) and pH 7.4 and (b) in the absence of glucose and pH 6.2 simulating ischemic conditions at 0.05% pO_2 after 24 h of incubation.

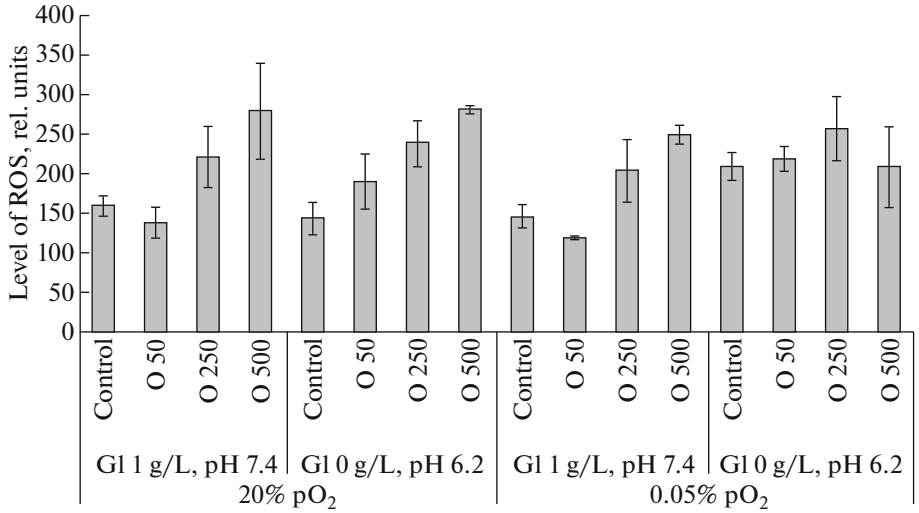

Fig. 2. Changes in the level of ROS during incubation of SC-1 cells with 50, 250, and 500 μM ouabain (O) at 20 and 0.05% oxygen (hypoxia) with different levels of glucose (Gl) and various values of pH for 3.5 h. Low level of oxygen, absence of glucose, and pH 6.2 values simulate ischemic conditions.

2 times, while inhibitory phosphorylation level hardly changes. Thus, ouabain cancels an increase in the level of activated form of Src kinase induced by low oxygen content. Based on the data, it can be concluded that, at a reduced oxygen content, Na,K-ATPase does not lose the ability to bind to ouabain, but the effect of ouabain on the receptor functioning of the enzyme changes substantially compared to its effect at 20% pO_2. The reason for this may be the redox-modification of cysteine residues in the Na,K-ATPase molecule, in par-

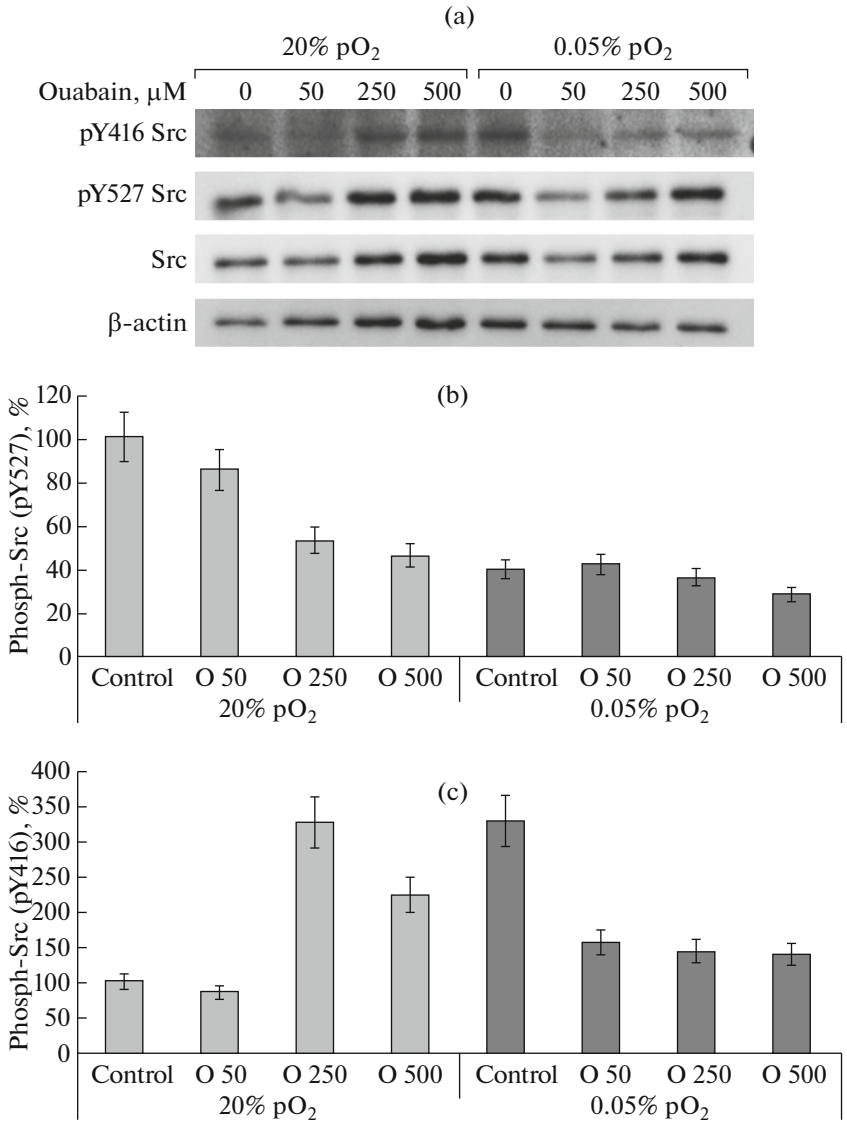

Fig. 3. The level of phosphorylation of Src kinase after incubation of SC-1 cells for 30 min in the presence of various concentrations of ouabain (50, 250, and 500 μM) with normal glucose (1 g/L), pH 7.4, 20 and 0.05% oxygen. (a) Immunoblotting with antibodies to pY416Src, pY527Src, total Src, and β-actin. (b) and (c) The results of quantitative immunoblot analysis. Phosphorylation level of Src kinase in cells incubated at 20% oxygen without ouabain is taken as 100%. (b) Changes in the level of inhibitory pY527Src phosphorylation. (c) Changes in the level of activating phosphorylation of pY416Src.

ticular glutathionylation, which occurs during hypoxia [21] and can affect the interaction of Na,K-ATPase with ouabain.

Redox modifications of proteins arise due to changes in the redox state of cells. To evaluate the redox state we determined levels of ROS and GSH in cells after ouabain treatment for 30 min (Fig. 4). During hypoxia, the ROS level is higher than in the presence of 20% pO_2 (Fig. 4a). This is consistent with the data, according to which an increase in ROS levels in cells under hypoxia occurs in the first 15–30 min of incubation [24]. In the presence of ouabain under 20% pO_2, the level of ROS increases in the cells, whereas under hypoxia ROS level decreases (Fig. 4a). GSH level is higher under hypoxia than at 20% pO_2 (Fig. 4b). This agrees with the data obtained previously using SC-1 cells that were incubated over 3.5 h under hypoxic conditions [21]. Level of glutathionylation of the Na,K-ATPase increased [21]. The addition of ouabain leads to a decrease in GSH level under hypoxia and its increase at 20% pO_2 (Fig. 4b). Thus, the presence of ouabain influences the redox state of cells during hypoxia and, therefore, on the redox-dependent modification of proteins.

Based on the data, it can be concluded that the cell response to CTSs changes at a low oxygen content. In the presence of ouabain, cells become less sensitive to the low oxygen supply. This may be associated with the

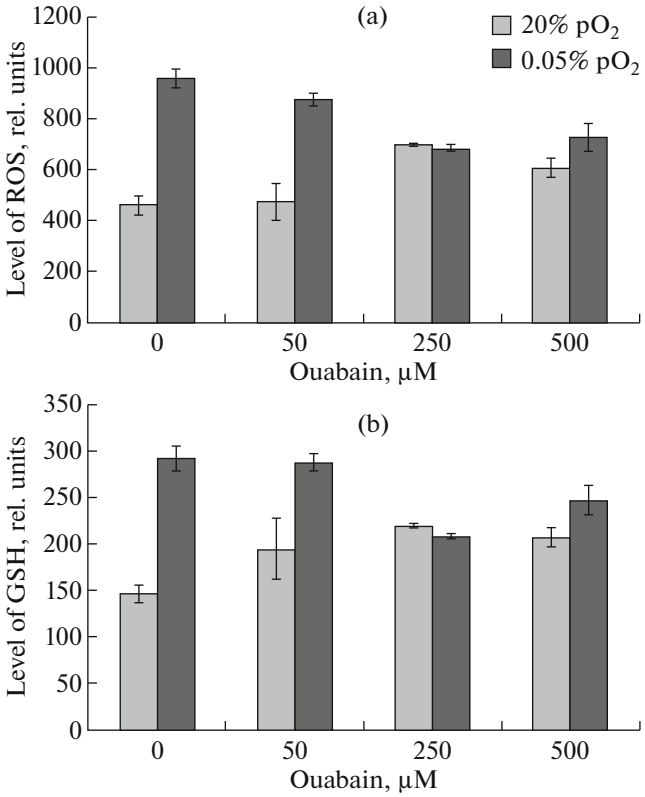

Fig. 4. (a) Changes in the level of ROS and (b) reduced glutathione (GSH) under the influence of ouabain at different concentrations after 30 min of incubation in the presence of 20 and 0.05% oxygen.

decreased consumption of intracellular ATP on functioning of the Na,K-ATPase, the influence of ouabain on the redox state of cells, and changes in the activity of Na,K-ATPase ligands, in particular, Src kinase. Thus, the changes in the effects of CTSs on cells under ischemic conditions should be taken into account in clinical practice. It can be suggested that CTSs will be protective in acute ischemic conditions.

ACKNOWLEDGMENTS

This study was supported by the Russian Science Foundation (project no. 14-14-01152).

REFERENCES

1. Kaplan J.H. 2002. Biochemistry of Na,K-ATPase. *Annu. Rev. Biochem.* **71**, 511–535.
2. Reinhard L., Tidow H., Clausen M.J., Nissen P. 2013. Na^+,K^+-ATPase as a docking station: protein-protein complexes of the Na^+,K^+-ATPase. *Cell. Mol. Life Sci.* **70**, 205–222.
3. Laursen M., Yatime L., Nissen P., Fedosova N.U. 2013. Crystal structure of the high-affinity Na^+,K^+-ATPase-ouabain complex with Mg^{2+} bound in the cation binding site. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 10958–10963.
4. Cornelius F., Kanai R., Toyoshima C. 2013. A structural view on the functional importance of the sugar moiety and steroid hydroxyls of cardiotonic steroids in binding to Na,K-ATPase. *J. Biol. Chem.* **288**, 6602–6616.
5. Kharkevich D.A. 2010. *Farmakologiya (Pharmacology)*. Moscow: GEOTAR-Media.
6. De Angelis C., Haupert G.T., Jr. 1998. Hypoxia triggers release of an endogenous inhibitor of Na^+,K^+-ATPase from midbrain and adrenal. *Am. J. Physiol.* **274**, F182–F188.
7. Ludens J.H., Clark M.A., DuCharme D.W., Harris D.W., Lutzke B.S., Mandel F., Mathews W.R., Sutter D.M., Hamlyn J.M. 1991. Purification of an endogenous digitalislike factor from human plasma for structural analysis. *Hypertension.* **17**, 923–929.
8. Fedorova O.V., Doris P.A., Bagrov A.Y. 1998. Endogenous marinobufagenin-like factor in acute plasma volume expansion. *Clin. Exp. Hypertens.* **20**, 581–591.
9. Haas M., Wang H., Tian J., Xie Z. 2002. Src-mediated inter-receptor cross-talk between the Na^+/K^+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. *J. Biol. Chem.* **277**, 18694–18702.
10. Knock G.A., Snetkov V.A., Shaifta Y., Drndarski S., Ward J.P., Aaronson P.I. 2008. Role of src-family kinases in hypoxic vasoconstriction of rat pulmonary artery. *Cardiovasc. Res.* **80**, 453–462.
11. MacAuley A., Cooper J.A. 1989. Structural differences between repressed and derepressed forms of p60c-src. *Mol. Cell. Biol.* **9**, 2648–2656.
12. Xu W., Harrison S.C., Eck M.J. 1997. Three-dimensional structure of the tyrosine kinase c-Src. *Nature.* **385**, 595–602.
13. Cowan-Jacob S.W., Fendrich G., Manley P.W., Jahnke W., Fabbro D., Liebetanz J., Meyer T. 2005. The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. *Structure.* **13**, 861–871.
14. Tian J., Cai T., Yuan Z., Wang H., Liu L., Haas M., Maksimova E., Huang X.Y., Xie Z.J. 2006. Binding of Src to Na^+/K^+-ATPase forms a functional signaling complex. *Mol. Biol. Cell.* **17**, 317–326.
15. Pasdois P., Quinlan C.L., Rissa A., Tariosse L., Vinassa B., Costa A.D., Pierre S.V., Dos Santos P., Garlid K.D. 2007. Ouabain protects rat hearts against ischemia–reperfusion injury via pathway involving src kinase, mitoKATP, and ROS. *Am. J. Physiol. Heart Circ. Physiol.* **292**, H1470–1478.
16. Bogdanova A., Petrushanko I., Boldyrev A., Gassmann M. 2006. Oxygen- and redox-induced regulation of the Na/K-ATPase. *Curr. Enzyme Inhibition.* **2**, 37–59.
17. Petrushanko I.Y., Bogdanov N.B., Lapina N., Boldyrev A.A., Gassmann M., Bogdanova A.Y. 2007. Oxygen-induced regulation of Na/K-ATPase in cerebellar granule cells. *J. Gen. Physiol.* **130**, 389–398.
18. Petrushanko I., Bogdanov N., Bulygina E., Grenacher B., Leinsoo T., Boldyrev A., Gassmann M., Bogdanova A. 2006. Na-K-ATPase in rat cerebellar granule cells is

redox sensitive. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R916-925.

19. Rasmussen H.H., Hamilton E.J., Liu C.C., Figtree G.A. 2010. Reversible oxidative modification: Implications for cardiovascular physiology and pathophysiology. *Trends Cardiovasc. Med.* **20**, 85–90.
20. Petrushanko I.Y., Yakushev S., Mitkevich V.A., Kamanina Y.V., Ziganshin R.H., Meng X., Anashkina A.A., Makhro A., Lopina O.D., Gassmann M., Makarov A.A., Bogdanova A. 2012. S-glutathionylation of the Na,K-ATPase catalytic alpha subunit is a determinant of the enzyme redox sensitivity. *J. Biol. Chem.* **287**, 32195–32205.
21. Petrushanko I.Yu., Simonenko O.V., Burnysheva K.M., Klimanova E.A., Dergousova E.A., Mitkevich V.A., Lopina O.D., Makarov A.A. 2015. The ability of cells to adapt to low-oxygen conditions is associated with glutathionylation of Na,K-ATPase. *Mol. Biol.* (Moscow). **49** (1), 153–160.
22. Smolyaninova L.V., Dergalev A.A., Kulebyakin K.Y., Carpenter D.O., Boldyrev A.A. 2013. Carnosine prevents necrotic and apoptotic death of rat thymocytes via ouabain-sensitive Na/K-ATPase. *Cell. Biochem. Funct.* **31**, 30–35.
23. Kominato R., Fujimoto S., Mukai E., Nakamura Y., Nabe K., Shimodahira M., Nishi Y., Funakoshi S., Seino Y., Inagaki N. 2008. Src activation generates reactive oxygen species and impairs metabolism-secretion coupling in diabetic Goto-Kakizaki and ouabain-treated rat pancreatic islets. *Diabetologia.* **51**, 1226–1235.
24. Hernansanz-Agustin P., Izquierdo-Alvarez A., Sanchez-Gomez F.J., Ramos E., Villa-Pina T., Lamas S., Bogdanova A., Martinez-Ruiz A. 2014. Acute hypoxia produces a superoxide burst in cells. *Free Radic. Biol. Med.* **71**, 146–156.

Translated by M. Novikova