DEPENDENCE OF SOLUTION ION CONTENT ON GLUTATION OUT OF THYMOTS UNDER HYPOOSMOTIC STRESS

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ABSTRACT

Glutathione is important in many cellular processes, its concentration inside and outside the cell is always constant. Intracellular glutathione concentrations are controlled by two mechanisms: the rate of synthesis and the rate of excretion from the cell. To date, it has been studied that a number of proteins are responsible for the release of glutathione from mammalian cells, and it has been found that glutathione transporters have specific properties and perform a number of functions. All types of ABCC / MRP membrane proteins mediate GSH transport and homeostasis. MRP proteins not only mediate GSH release, but also transport GSH oxidation products (glutathione disulfide (GSSG), metal complexes of glutathione), as well as other glutathione conjugates. In our experiments, we studied the effect of solution composition on glutathione release from the cell. Therefore, we replaced the sodium ion with NMDG (N-methyl-D-glucamine) and potassium ion, sodium chloride with sodium glutamate, and used BAPTA solution to determine the effect of calcium on glutathione output in the intracellular environment.

***Keywords**: glutathione; rat thymocyte; cells; hypoosmotic stress*

Introduction

Glutathione is a universal, important antioxidant in living organisms and is involved in many biological processes. It is present in all organs, including the liver, kidneys, pancreas, the cornea and pearl of the eye, in higher concentrations than other organs. The liver cell contains relatively more glutathione (around 10 mM), which is due to the high consumption of molecules in the detoxification of xenobiotics, where xenobiotics are released from cells in the form of glutathione conjugates using specific adenosine triphosphatase. [2]. Small amounts of glutathione can also be found in the intercellular environment. The concentration of glutathione in the interstitial fluid is a few µM, while in plasma this amount is usually twice as high. At the same time, the concentration of glutathione in the thin layer of fluid covering the lung epithelium is about 400 µM, which is twice as much as in normal interstitium. This is caused by intense gas exchange in the surface layer of the lung epithelium, resulting in strong oxidative stress. Therefore, the epithelial cells of the alveoli secrete glutathione into the extracellular environment, thereby protecting themselves from free radical oxidation [1].

Glutathione has important antiviral properties in the immune system. It is a powerful agent against cancer. It is involved in the detoxification process in the liver. Glutathione is an antioxidant that protects proteins and maintains cellular oxidation-reduction potential, which is also important in nucleic acid synthesis and DNA construction [3]. Glutathione slows down the aging process. It protects the integrity of red blood cells in the blood, maintains the normal function of the brain, regulates the functional activity of lymphocytes in the body's immune response. Basically, reversible glutathione is found in many plants, microorganisms, and various living cells. The amount in the body is higher than in other organic substances and the concentration in cytosol is 1-2 mM. Glutathione regenerates and isomerizes disulfide bonds, affects the activity of enzymes and proteins, is a reserve of cysteine in the body [4]. An important antioxidant property of glutathione is reflected in its participation in physiological processes in the body. The antioxidant function of the digestive system is explained by the consideration of the pathways of glutathione entering the gastrointestinal tract through endogenous and nutrients, where it interacts with the mucous membrane of the

small intestine and is protected from free radical oxidation products.

The most important organ in mammals that synthesizes GSH is the liver.. The liver synthesizes 90% glutathione for physiological and biochemical processes in the body. During starvation, the amount of glutathione in the liver is reduced by 2 times and returns to its normal state after eating. The formation of glutathione depends on the amount of cysteine in the food. The transition of glutathione from the liver to blood plasma and bile is stimulated by glycogen and vasopressin. When glutathione synthesis is inhibited, the amount in the liver, blood plasma, and the body as a whole decreases.

Skeletal muscles retain glutathione in plasma by reducing the activity of the enzyme gamma-glutamyl transpeptidase, and in the liver and kidneys increase the activity of gamma-glutamyl transpeptidase due to a decrease in the level of glutathione in plasma [8]. Glutathione is formed as a result of the consumption of two ATP molecules at two enzymatic stages of glutamic acid, cysteine and glycine. Glutathione is involved in many biological processes, including protein and DNA synthesis, molecular transport, and redox signaling. Of particular importance is the ability of glutathione to return free radicals and active forms of oxygen [6]. It is an endogenous antioxidant that maintains oxidant levels in the intracellular environment. Glutathione is oxidized in the presence of the enzyme glutathione peroxidase during oxidation with active compounds of oxygen to form GSSH and water. During the reduction process, glutathione reductase produces glutathione from glutathione disulfide. This process maintains the active compounds of oxygen at the physiological level. Therefore, glutathione plays an important role in the cell redox system. GSH / GSSH levels are an indicator of the state of cell oxidation reduction[5].

It was found that 50-60% of glutathione in the liver is transported through bile. Glutathione in bile fluid is an important reducer in the metabolic changes of peroxidated fats in the small intestine [3]. The antioxidant system of glutathione protects the cell from oxidative stress. This protection is mainly mediated by 3 different enzymatic pathways. These are superoxide dismutase, catalase and glutathione peroxidase. In this case, superoxide radicals, hydrogen peroxide and organic hydroperoxides are returned.

Materials and Methods

Solutions and chemicals. The normal isotonic Ringer solution contained (in mM): 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 5 Na-HEPES, 6 HEPES, and 5 glucose (pH 7.4, 290 mosmol/kg-H2O). Hypotonic solutions were prepared by mixing the normal Ringer solution with a HEPES-buffer solution containing (in mM): 5 KCl, 2 CaCl2, 1 MgCl2, 5 Na-HEPES, 6 HEPES, and 5 glucose (pH 7.4, 38 mosmol/kg-H2O). Nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase were from Oriental Yeast (Tokyo, Japan). Other drugs were stored as 1000-times stocks in DMSO and added to the experimental solution immediately before use. DMSO did not have any effect, when added alone at a concentration of $\leq 0.1\%$. Osmolality of all solutions was measured using a freezing-point depression osmometer (OM802: Vogel, Kevelaer, Germany).

Cells. Cell isolation was performed as described previously [24–26].Briefly, the 6–8 weeks old rats were anaesthetized with halothane or diethyl ether and painlessly euthanized by cervical dislocation; the thymi were dissected and carefully washed with an ice-cold Ringer solution. The thymi were then minced using fine forceps and passed through a 100 mm-nylon mesh. The suspension was centrifuged at 1000 g for 5 min, the pellet was washed two times with the normal Ringer solution and resuspended in this medium at a cell density of (1–15)6108 cells/ml. The cell suspension was kept on ice for $\leq$ 5 hand contained no more than 5% of damaged cells as assayed by trypan blue exclusion.

Glutathione release assay. The bulk extracellular GSH concentration was measured by an enzymatic recycling method by reduction of 5,59-dithiobis-2-nitrobenzoic acid (DTNB, Ellmans reagent) in yellow-colored 5-thio-2-nitrobenzoic acid (TNB) as described elsewhere [7-8]. Briefly, the cell suspension was diluted 1:10 with the normal or hypotonic Ringer solution and incubated at 25 uC (if not indicated specifically). In some experiments,

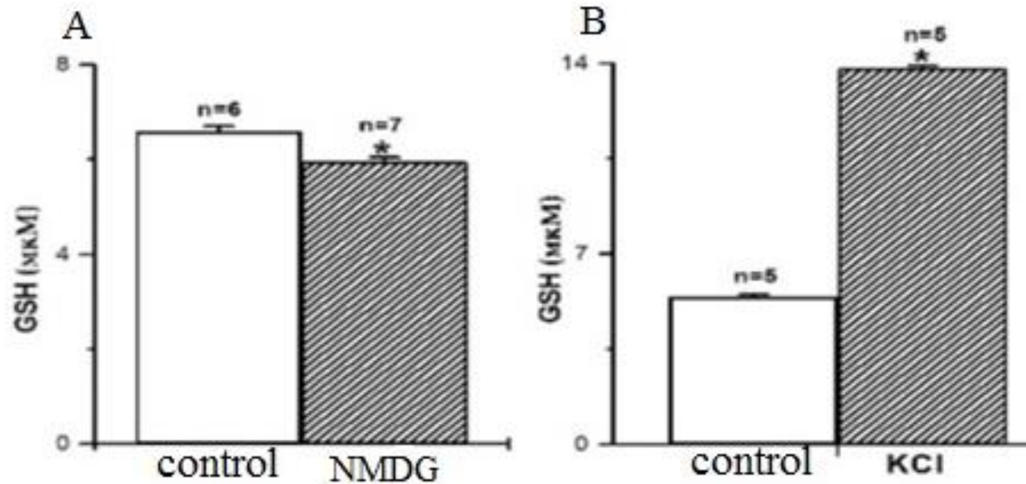
the cells were exposed to the normal Ringer solution supplemented with 500 mM mannitol. At the specified time points, the cell suspension was centrifuged at 1000 g for 10 min, and 125 ml aliquots of the supernatants were collected for a photometric assay. The aliquots were mixed with 375 ml of a cocktail containing (in mM): 133 MES (2-(morfolino) ethanesulphonic acid), 33 KH2PO4, 0.66 EDTA, 0.11 NADPH, and 0.2 DTNB (pH 6.0). The cocktail was prepared on the day of experiment and was additionally supplemented with 0.25 U/ml glutathione reductase (EC 1.6.4.2) immediately before use. The mixture was incubated in dark for 25 min at room temperature and the optical density was measured at 412 nm. The GSH concentration was calculated from a standard calibration curve obtained using the same procedure performed with pure GSH in a range from 0 to 16 mM. When required, drugs were added to the normal or hypotonic solutions to give the final concentrations as indicated. The drugs at the concentrations employed in the present study had no significant effect on the assay reaction. In some experiments, treatment with a GSH scavenger, 2- vinylpyridine (2-VP), was performed with supernatants for 60 min at room temperature. Since at the used concentration (30 mM), 2-VP had a mild inhibiting effect on the enzymatic reaction, the error caused by this effect was accounted for by calibration using oxidized glutathione (GSSG) as a substrate.

Results

Glutathione is important in many cellular processes, its concentration inside and outside the cell is always constant. Intracellular glutathione concentrations are controlled by two mechanisms: the rate of synthesis and the rate of excretion from the cell. To date, it has been studied that a number of proteins are responsible for the release of glutathione from mammalian cells, and it has been found that glutathione transporters have specific properties and perform a number of functions. All types of ABCC / MRP membrane proteins mediate GSH transport and homeostasis. MRP proteins not only mediate GSH release, but also transport GSH oxidation products (glutathione disulfide (GSSG), metal complexes of glutathione), as well as other glutathione conjugates. Although the functions of glutathione in the intracellular environment are now clear, its pathways out of the cell have not been determined. Therefore, in our experiments, we studied the effect of ions on the process of glutathione exit from the cell. In our experiments, we replaced the sodium ion in the solution with NMDG (N-methyl-D-glucamine) and the potassium ion. While 6.58 ± 0.12 μM glutathione was isolated in the control, a 5.95 ± 0.09 μM glutathione yield was observed in the NMDG solution. It can be seen that NMDG reduces glutathione release from the cell (Fig. 1A), and this decrease indicates the involvement of a sodium-dependent transport mechanism in glutathione output. We continued the experiments by replacing the sodium ion in the solution with the potassium ion. At the same time, 7.43 ± 0.12 μM glutathione was observed in 100 ml / ml cell for 20 min under control, and 15.01 ± 0.18 μM in potassium solution (Fig. 1B). The result obtained differs from the above result in the membrane potential in a high-potassium environment because the intracellular negative electrical potential must help glutathione, which has a negative charge, to leave the cell. Presumably, swelling of thymocytes at high concentrations of potassium (as described in this literature) may have led to an increase in glutathione output.

Effect of glutathione release from thymocyte cells under hypoosmotic stress under exchange of sodium ion (A) NMDG and (B) potassium ion in solution. The number of cells is 100 million / ml. On the ordinate axis - the glutathione output from the cell is expressed in mkM, on the abscissa axis - the experimental groups are represented. Relative to control in all cases.

Figure1:



In the next phase of our experiments, we used sodium glutamate instead of sodium chloride in the solution to determine the role of the chloride ion in the solution. In this experiment, a glutathione output of 7.1 ± 0.22 µM was observed from a 100 ml / ml cell for 20 min under control, and 7.9 ± 0.19 µM from glutamate solution (Figure 2). The fact that the amount of glutathione is not reduced, and, conversely, slightly increased, indicates that the mechanism of glutathione release is not related to the process of chlorine metabolism.

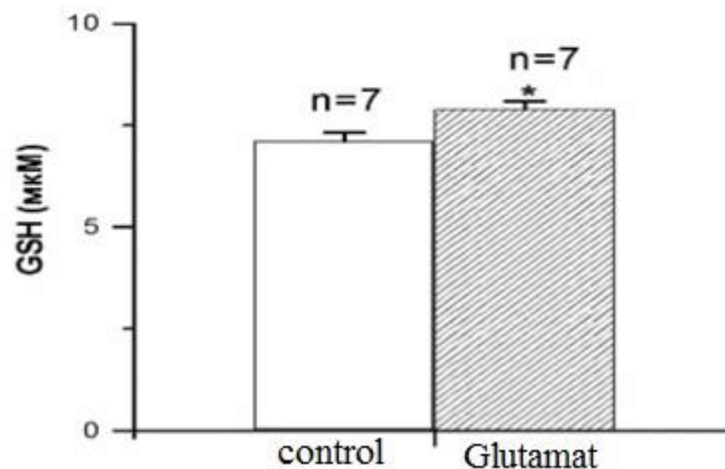


Figure 2. The effect of glutathione release from thymocyte cells under hypoosmotic stress under the exchange of chloride ion in solution for glutamate.

The number of cells is 100 million / ml. On the ordinate axis - the glutathione output from the cell is expressed in mkM, on the abscissa axis - the experimental groups are represented. In all cases, P <0.05 (n = 7) relative to the control.

To determine the role of the calcium ion in the glutathione release process, we removed the calcium ion from the solution. According to the results obtained, 6.03 ± 0.25 µM glutathione was released in the control, while 7.17 ± 0.13 µM glutathione was released in the calcium-free environment. In the next part of our experiment, we used a 100 µM BAPTA solution to determine the effect of calcium in the intracellular environment on glutathione output. BAPTA binds intracellular calcium and prevents it from participating in the process (Figure 3). Under these conditions, glutathione release did not change significantly. The result obtained suggests that the participation of calcium ions in the

mechanism of glutathione release under hypoosmotic stress is not significant.

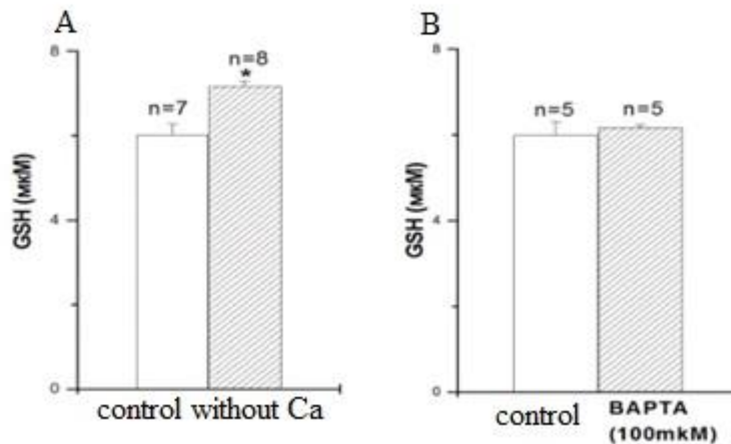


Figure 3. The effect of removal of calcium ions from the solution on the release of glutathione from thymocyte cells under conditions of hypoosmotic stress and chelation with the help of BAPTA complex.

The number of cells is 100 million / ml. On the ordinate axis - the glutathione output from the cell is expressed in mkM, on the abscissa axis - the experimental groups are represented. In all cases, P <0.05 (n = 7) relative to the control.

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