

The Effect of Extracellular Glutathione on the Regulation of Thymocyte Volume in Rats under Conditions of Hypoosmotic Stress.

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Abstract: Glutathione (GSH) is a negatively charged tripeptide, which is a major determinant of the cellular redox state and defense against oxidative stress. It is assembled inside and degraded outside the cells and is released under various physiological and pathophysiological conditions. The GSH release mechanism is poorly understood at present. In our experiments, thymocytes immersed in a hypo osmotic medium effectively restored their volume by $78.1 \pm 5.6\%$ ($n = 6$) in the control. Glutathione already at a concentration of $0.1 \mu\text{M}$ significantly suppressed the regulatory decrease in the volume of thymocytes, however, even at high concentrations of the substance, the effect was incomplete, and the cells were able to restore their volume by $69.05 \pm 1.3\%$ ($n = 6$) at $10 \mu\text{M}$ and by $65.06 \pm 1.4\%$ ($n = 6$) at $100 \mu\text{M}$. The semi-maximal effect was observed at $1.3 \pm 0.1 \mu\text{M}$.

Keywords: Glutathione, cell, thymocyte, hypo osmotic

Introduction

The metabolites that make up the cytoplasm of the cell are involved in many processes necessary for the physiology of the cell. However, under certain physiological conditions, they are released outside the cell and, like signaling molecules, transmit information about the physiological state of the cell, which is the emitter (that is, the source of the signal), to other surrounding cells. An example of this is the ATP molecule. This substance is not only the main source of energy for the cell, but is also released outside the cell under various stressful conditions (for example, metabolic stress, osmotic stress, mechanical irritation, etc.). Almost all plasma membrane cells contain special purine energy receptors that receive ATP. When they bind to extracellular ATP, a purinergic signal is transmitted within the cell - a process called purinergic signaling. Glutamic acid is an example of another signaling molecule. This low molecular weight substance is involved in the biosynthesis of proteins in the cytoplasm of the cell. However, in some cases of stress, especially in the case of overactive brain, glutamate is released in cells and affects glutamatergic receptors present in other cells. This process can also lead to the death of brain neurons.

Glutathione is a universally important antioxidant in living organisms and is involved in several biological processes. It is present in all organs, including the liver, kidneys, pancreas, cornea and is found in higher concentrations than other organs. It also has strong antiviral properties that are important for the immune system. It is a powerful anti-cancer agent. It is involved in the liver detoxification process. Glutathione is an antioxidant that protects proteins and maintains the redox potentials of cells, which are also important for nucleic acid synthesis and DNA building[5]. Glutathione slows down the aging process. It protects the integrity of red

blood cells, maintains normal brain function, and regulates the functional activity of lymphocytes during the body's immune response.

In particular, recovered glutathione is found in many plants, microorganisms and various living cells. Its concentration in the body is higher than that of other organic substances, and the concentration in the cytosol is 1-2 mM. Glutathione restores and isomerizes disulfide bonds, affects the activity of enzymes and proteins, and is a store of cysteine in the body. [6].

An important antioxidant property of glutathione is its participation in physiological processes in the body. The antioxidant function of the digestive system is to prevent the entry of glutathione into the gastrointestinal tract through endogenous and nutrient substances, interact with the mucous membrane of the small intestine, and protect against free radical oxidation products.

The liver is the most important organ in mammals that synthesizes GSH. The liver synthesizes 90% of glutathione for physiological and biochemical processes in the body. During fasting, the amount of glutathione in the liver decreases by 2 times and returns to normal after eating. Glutathione production depends on the amount of cysteine in the food. The transfer of glutathione from the liver to blood plasma and bile is stimulated by glycogen and vasopressin. [2]. When glutathione synthesis is suppressed, its amount in the liver, blood plasma and the body as a whole decreases. A decrease in the activity of the enzyme gamma-glutamyl transpeptidase in skeletal muscle preserves glutathione in plasma, and in the liver and kidneys increases the activity of gamma-glutamyl transpeptidase by reducing glutathione in plasma [9].

In the body, bile secretes various glutathione and removes toxins against xenobiotics. Glutathione-S-transferase catalyzes the reaction between electrophilic metabolites of glutathione and most xenobiotics, in which the hydrophilicity of the ligands increases and is transported to the liver.[1]. Conjugated enzymatic degradation and degradation of 3 enzymes:

- 1- Fragments of the gamma-glutamyl fragment of 1-gamma-glutamyltransferase ("back");
- 2- 2-dipeptidase glycine;
- 3- 3-N-acyltransferase acetylates cystine conjugates with acid to mercapturic acid.

Usually, the state of the oxidized dimer of GSSG occurs less frequently in mammals than the state of reduced glutathione in blood plasma. Oxidative stress leads to the formation and deposition of GSSG in the liver. An increase in the level of GSSG leads to oxidation and inactivation of thiol groups of plasma proteins and proteins of the basolateral membrane of tissue cells. [2].

Glutathione is produced by the consumption of glutamic acid, cysteine and glycine, two ATP molecules in two enzymatic steps. Glutathione is involved in many biological processes, including protein and DNA synthesis, molecular transport, and redox signaling. In particular, the ability of glutathione to reduce free radicals and reactive oxygen species is important[6].It is an endogenous antioxidant that maintains oxidant levels in the intracellular environment. Glutathione is oxidized by the enzyme glutathione peroxidase during oxidation by reactive oxygen compounds to form GSSH and water. During the reduction process, glutathione reductase forms glutathione from glutathione disulfide. This process keeps active oxygen compounds at the physiological level. Consequently, glutathione plays an important role in the redox system of the cell. GSH/GSSH levels are an indicator of the redox status of cells.

Reduced GSH plays an important role in many cellular biochemical processes. In particular, in the synthesis of GSH DNA, in the control of the cell cycle, in the fight against oxidative stress, in the cellular processes of cell repair, in the detoxification of endogenous and exogenous reactive substances, in the detoxification of cyanide, in the acylation of thiols [8]. Additional roles of this tripeptide play a role in gene expression, endogenous and exogenous molecular transport [9]. Synthesis of GSH, enzymatic catalysis of its derivatives, and control of transport in the plasma membrane together form the γ -glutamyl cycle [2]. GSH is synthesized in every cell, but as noted above, the liver is the main quantitative region.

ATP-dependent cytosolic enzymes are synthesized by γ -glutamylcysteine synthetase and glutathione synthetase within the GSH cell. It occurs mainly in the intracellular form (98%) in the reversible form (GSH), and sometimes in the oxidized form (GSSG) and in other forms [4]. After synthesis, GSH is released into other intracellular organelles, including mitochondria and endoplasmic reticulum [2]. GSH released into the extracellular environment is used by other cells and tissues. Unlike intracellular GSH synthesis, GSH degradation occurs outside the cell and outside the cells, also known as γ -glutamyl transpeptidase or γ -glutamyl transferase. γ -glutamyl transpeptidase is abundantly present in many epithelial tissues on the apical surface, including the liver and fat cells [1].

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 $\leq 0.1\%$. The osmolality of all solutions was measured using a freezing-point depression osmometer (OM802: Vogel, Kevelaer, Germany).

Cells

Cell isolation was performed as described previously [2]. Briefly, the 6–8 weeks old rats were anesthetized 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 μ m-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 $(1-15) \times 10^6$ cells/ml. The cell suspension was kept on ice for ≤ 5 h and contained no more than 5% of damaged cells as assayed by trypan blue exclusion.

Checking changes in thymocyte count using light conduction

The registration of cell size by light transmittance is based on the fact that the cell suspension scatters transient light, resulting in a lower light intensity (I) than the intensity of the incoming light[8]. The light transmittance of a cell suspension is determined by the following formula:

$$T = (I / I_0) \times 100\% \quad (1)$$

In the experiment, the method of recording the size of the cell according to the light transmittance was used [8]. Changes in the volume of thymocytes were recorded using an MKMF-1 microcolorimeter. A light filter with a maximum absorption of 610 nm was used. The signal measured in the microcolorimeter was amplified by an operational amplifier U5-11 and transmitted to a computer (Pentium IV) via a GO! Analog-to-digital converter! Link (Qubit Systems, Canada) with a frequency of 100 Hz using a special program Logger Lite (Qubit Systems, Canada).

In the experiments, a bottle of 900 μ L with normal Ringer's solution (control) or another test solution was placed in a cuvette. The glass cuvette is connected to a water thermostat U-3 (Germany). After the temperature was restored, a cell suspension (100 ml / ml, 100 μ l) was placed in the cell. The experiment lasted 15 minutes.

RVD, (*Regulatory Volume Decrease*) is calculated using the following formula:

$$RVD = [(T_{max} - T_{15}) / (T_{max} - T_0)] \times 100\% (2)$$

Here T_0 and T_{max} are the initial and maximum values of light transmission, T_{15} is the light transmission measured 15 minutes after hypotonic shock.

Complete restoration of cell volume to the initial level is $RVD = 100\%$. $RVD = 0$ when volume control is completely stopped.

Under control conditions, the RVD index is 60-90%, depending on the type of cell, the size of the osmotic gradient, temperature and other experimental conditions.

Results

The role of glutathione in cell-to-cell signaling has been the subject of much debate in recent years. Of course, the main function of glutathione is to keep the internal environment of the cell strong and to protect the cell from oxidative stress. Glutathione is mainly present in the cytoplasm, but a certain amount of glutathione is also observed in interstitial fluids and blood plasma. The extraction and extracellular function of this extracellular glutathione is poorly understood to date. The cells of a living organism are highly organized and self-regulate in changing environmental conditions. Maintaining the integrity of the cell is closely related to the active control of the constancy of its volume. The study of the mechanisms for controlling the volume of living cells is one of the central tasks of modern biophysics and cell physiology. In our experiments, we studied the effect of extracellular glutathione on thymocyte cell volume control systems. In this case, we exposed the thymocyte cell suspension to various concentrations of glutathione for 15 min.

Composition in our experiments (mM): 135 NaCl, 5 KCl, 10 HEPES, 2 CaCl_2 , 1 MgCl_2 , 5 glucose, pH 7.4 (290 ± 2 mOsm/kg H_2O) from ordinary Ringer's solution and its composition (mM): 5 KCl, 10 HEPES, 2 CaCl_2 , 1 MgCl_2 , 5 glucose, pH 7.4 (40 ± 2 mOsm/kg H_2O) we used N-

buffered solution. Vistar thymocyte cells weighing 100–150 g, fed under normal conditions in vivariums, were isolated from the thymus of 6–8-week-old white rats by a standard method and observed for 15 minutes using spectrophotometry of the cell volume control system.

In the control, the cell volume returned to $78.09 \pm 5.6\%$; a significant effect on this process was observed starting with a glutathione concentration of $0.1 \mu\text{M}$. At a glutathione concentration of $1 \mu\text{M}$, the cell volume returned to $71.12 \pm 1\%$, $69.05 \pm 1.3\%$ at $10 \mu\text{M}$, and $65.06 \pm 1.4\%$ at $100 \mu\text{M}$ (Figure 1). The kinetics of changes in cell volume under control and at certain concentrations of glutathione is shown in Figure 1. The ordinate is the light transmittance of the cell suspension, expressed as a percentage, the abscissa is the time.

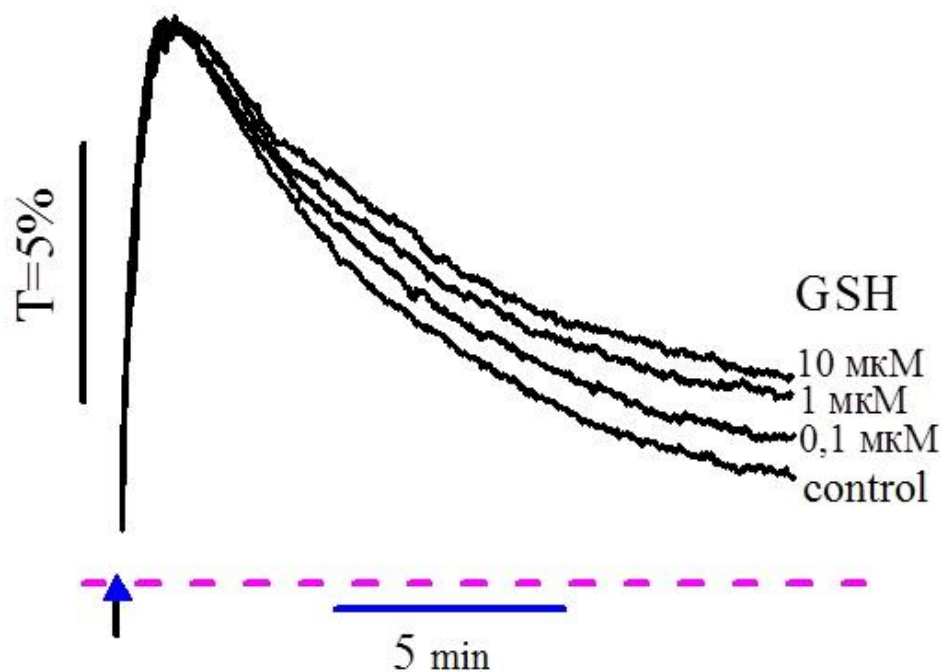
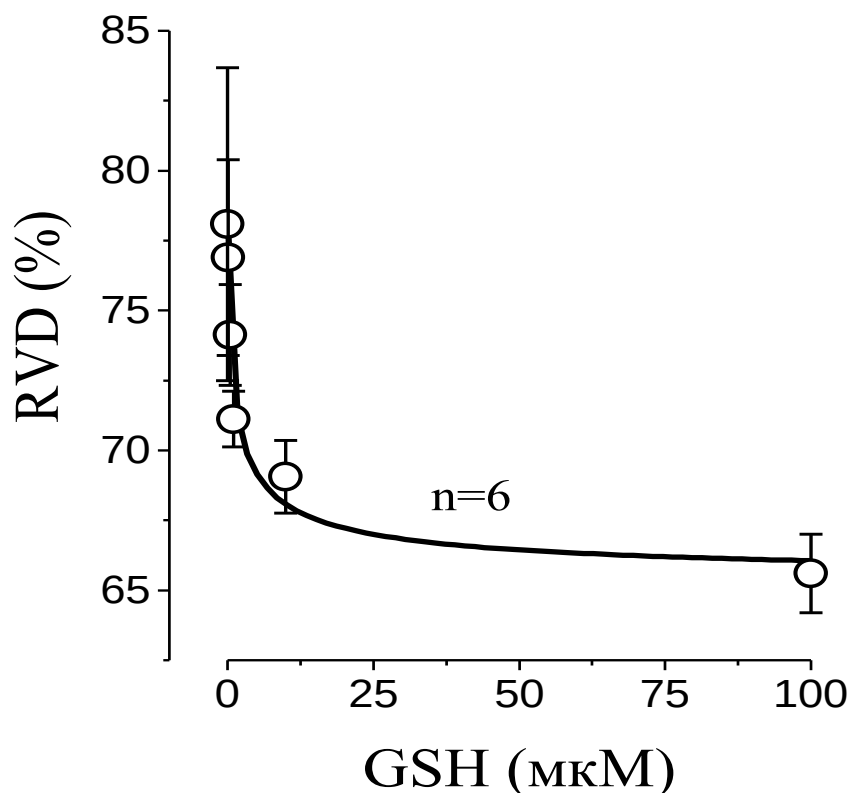


Figure 1. Measurement of the effect of extracellular glutathione on the regulation of cell volume by thymocytes under conditions of hypoosmotic stress ($147 \text{ mOsm} / \text{kg H}_2\text{O}$) using the light transmission method.

The resulting “dose-response” line is shown in Figure 2. At the same time, it is clear that the regulation of thymocyte depletion depends on the dose of glutathione. However, even at the highest concentrations, regulation of cell volume is only partially blocked. This result indicates that the effect of glutathione is modulation of volume control systems (especially calcium, potassium and chlorine transport systems), but not complete blockage. The half-maximum concentration is $1.3 \pm 0.1 \mu\text{M}$, which indicates that the observed process is also observed at physiological concentrations of glutathione in the thymus. The result shows that extracellular glutathione acts as an effective immunomodulator.



On the ordinate, the control of cell volume is expressed as a percentage; on the abscissa, the amount of glutathione is expressed in microns. In all cases, $P < 0.05$ ($n = 5$) relative to the control without glutathione. The curve is based on Hill's equation with a half-maximum effect of 1.3 ± 0.1 mM and a Hill coefficient of 0.64 ± 0.31 .

Figure 2. The effect of extracellular glutathione on the control of thymocyte volume under conditions of hypoosmotic stress (147 mOsm / kg H₂O).

In the course of our research, the effect of extracellular glutathione on the physiological functions of the cell was observed. The effect of extracellular glutathione on the control of thymocyte volume under conditions of hypoosmotic stress and the effect of extracellular glutathione on the resistance of human erythrocytes to colloid-osmotic lysis were studied. It has been shown that extracellular glutathione significantly affects the regulation of thymocyte cell volume during hypoosmotic stress.

This result indicates that extracellular glutathione acts as an effective modulator of the immune system.

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