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Release Of Glutathione From Cells Under Normal And 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, freshly isolated rat thymocytes were found to release GSH under normal isotonic conditions at a low rate of 0.8260.07 attomol/cell/min and that was greatly enhanced under hypoosmotic stimulation to reach a level of 6.160.4 attomol/cell/min. The swelling-induced GSH release was proportional to the cell density in the suspension and was temperature-dependent with a relatively low activation energy of 5.460.6 kcal/mol indicating a predominant diffusion mechanism of GSH translocation. The glutathione release rate from rat thymocytes was found to be 4-10 times higher compared to the human red blood cells. Cultured melanoma cells also exhibited substantial release of glutathione both in normal and hypoosmotic stress conditions.

Keywords: Glutathione, cell, thymocyte, hypoosmotic

Introduction

Glutathione (GSH) is a tripeptide (c-L-glutamyl-L-cysteinyl glycine) bearing one net negative charge. Our molecular modeling (see [3, 6] for calculation method) yielded an effective GSH radius of 0.52–0.56 nm, implying that the molecule could be accommodated or even passed by VRACs. Therefore, we hypothesized that activation of the maxi-anion and/or VSOR channels could result in a release of this key regulator of cellular oxidation/reduction status. GSH is a ubiquitous and most prevalent intracellular thiol tripeptide found throughout the body and involved not only in maintaining the cytosolic redox potential and defense against the oxidative stress but also in many other cellular processes including protein and nucleic acid synthesis, regulation of cell cycle,

proliferation, exocrine secretion and thermotolerance [1,2]. In most cells, the cytosolic concentration of GSH is in the range of 1–10 mM (.98% in the thiol-reduced form), whereas micromolar

concentrations are found in the plasma [3,9]. The biosynthesis of GSH occurs intracellularly, whereas it is degraded exclusively outside the cells via cleavage by an ectoenzyme, ɤ-glutamyl transpeptidase, and by dipeptidase [8]. Therefore, constant delivery of GSH and its S-conjugates to the cellular exterior is an important step for the c-glutamyl cycle, which constitutes the cyclic process connecting between the GSH metabolism and the transport of amino acids. GSH release has been reported to be induced or enhanced by a variety of stimuli including hypoosmotic stress [7,10]

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[4].

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.[118; 380-6]. 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 cell repair, in the detoxification of endogenous and exogenous reactive substances, in the detoxification of cytocyanide cystocystocystitis, 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 ≤ 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 [24–26]. 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 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 ≤ 5 h and 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 a reduction of 5,59-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) in yellow-colored 5-thio-2-nitrobenzoic acid (TNB) as described elsewhere [4,5]. 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-(morpholino) ethane sulphonic acid), 33 KH2PO4, 0.66 EDTA, 0.11 NADPH, and 0.2 DTNB (pH 6.0). The cocktail was prepared on the day of the 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.

Results

In our experiments, we recorded noticeable amounts of glutathione in the extracellular medium even in the absence of stimulation during the incubation of thymocytes under normal isosmotic conditions. The basic yield of glutathione was 0.29 ± 0.07 μM with a cell concentration in the suspension of 12.5 million / ml and 1.11 ± 0.04 μM in a suspension containing 100 million / ml cells after 10 min incubation. Under conditions of hypoosmotic stress (147 mOsm / kg H2O), we observed a sharp increase in the release rate of glutathione, which amounted to 1.23 ± 0.09 μM and 6.37 ± 0.04 μM in suspension with a cell concentration of 12.5 million / ml and 100 ppm after 10 min incubation, respectively.

The dependence of the glutathione content in the extracellular medium on the number of cells in the suspension was close to linear under normal isotonic conditions and under hypoosmotic stress, as shown in (Fig. 1A). This is evidence that thymocytes are the source of glutathione in the extracellular environment in our experimental conditions.

The kinetics of the release of glutathione markedly differed in isotonic and hypotonic conditions. So, if the basic yield of glutathione under normal conditions gradually increased in time, then under hypoosmotic stress, we observed a sharp jump-like increase in the content of glutathione in the medium at the initial moment, which was then replaced by a smooth increase to an approximately constant level, which was reached after about 20 minutes incubation (Fig. 1B).

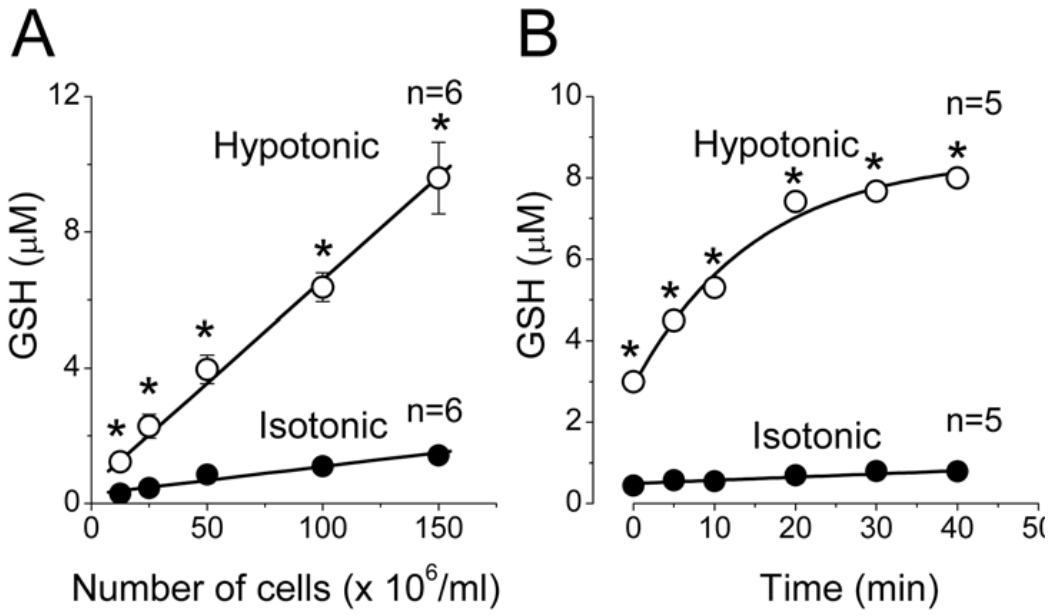

Fig. 1.Glutathione release in normal isotonic medium (light symbols) and under conditions of hypoosmotic stress (147 mOsm/kg H2O; dark symbols) depending on the concentration of cells in suspension (A: incubation time 10 min) and time (B: cell concentration 100 mln/ml). The experiments were carried out at 25°C. In all cases,P <0.05 relative to the norm (control).

Such two-phase kinetics may indicate the presence of at least two mechanisms for the release of glutathione from thymocytes with different kinetic parameters. The above results indicate a massive release of glutathione from thymocytes into the extracellular environment both under normal conditions and during hypoosmotic stress. Is a similar system for the controlled release of glutathione present in other cell types? To answer this question, we investigated the output of glutathione from normal human red blood cells after 20 minutes of incubation in an environment with different tonicities with varying numbers of cells in suspension. It was found that the concentration of glutathione in the extracellular medium in a

suspension of erythrocytes with a concentration of 100 million / ml is 0.57 ± 0.14 μM (n = 5) under isotonic conditions and 1.33 ± 0.11 μM (n = 5) in a hypoosmotic medium. The concentration of extracellular glutathione increased linearly with an increase in the number of cells in suspension in the range from 100 million / ml to 1 billion / ml (Fig. 2).

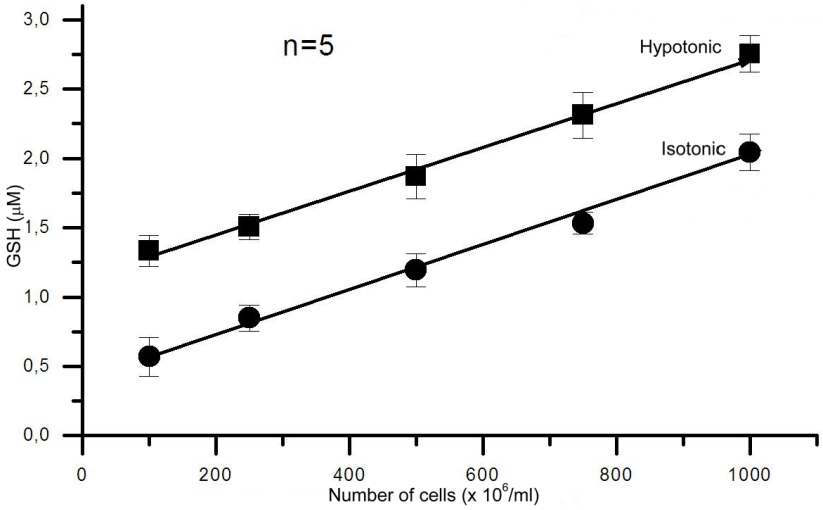

Fig. 2. The output of glutathione from human red blood cells in normal conditions (290 mOsm / kg H2O) and under hypoosmotic stress (147 mOsm / kg H2O). Asterisks indicate a statistically significant difference from control at a level of P <0.05.

Per cell, the rate of release of glutathione from erythrocytes was 0.09 × 10–15 g / min in a normal environment, and 0.2 × 10–15 g / min under hypoosmotic stress. To compare these values with the rate of release of glutathione from thymocytes, we recalculated the data shown in Figure 1 per cell. An analysis of the data showed that the rate of release of glutathione from thymocytes is 0.34 × 10–15 g / min and 1.96 × 10–15 g / min under normal and hypoosmotic conditions, respectively. Comparison of these values with data for red blood cells shows that the rate of release of glutathione from red blood cells is about 4 times slower than from thymocytes in a normal environment and almost 10 times slower under conditions of hypoosmotic stress. Although the rate of release of glutathione from erythrocytes was quite low compared to thymocytes, however, given the fact that red blood cells make up the bulk of blood cells, this speed is quite sufficient to ensure the physiological level of glutathione in plasma. The high rate of glutathione exit from thymocytes may indicate the important role of this molecule in physiological processes occurring in the extracellular environment of the thymus. At the next stage of the experiments,

we investigated the yield of glutathione from cancer cells of melanoma in culture (KML line). Cells were grown in glass Karelians at a temperature of 37 ° C until a confluent state (i.e., until the formation of a cell monolayer). In these experiments, the concentration of extracellular glutathione monotonically increased over time (Fig. 3) and after 40 minutes of incubation was 0.66 ± 0.06 µM (n = 5) under normal conditions and 1.26 ± 0.012 µM (n = 5) at hypoosmotic stress. The results show the presence of a glutathione release mechanism in the cancer cells of melanoma and its possible role in carcinogenesis.

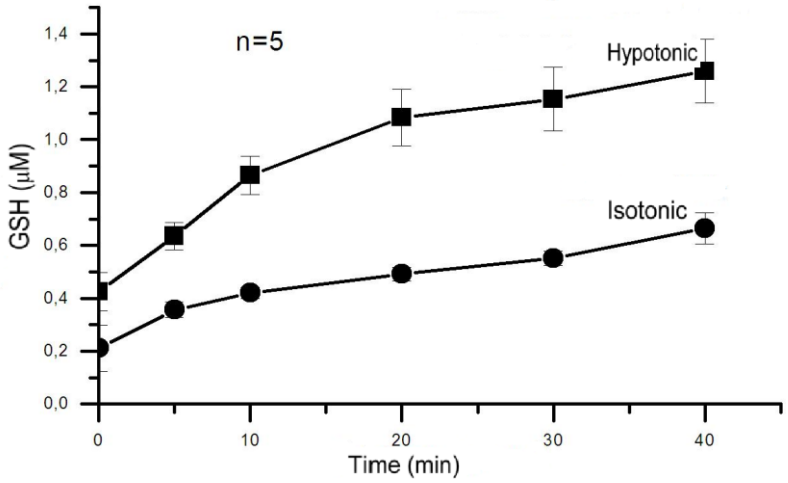

Fig. 3. The output of glutathione from the culture of cancer cells of melanoma in the normal state (290 mOsm / kg H2O) and under hypoosmotic stress (147 mOsm/kg H2O). Asterisks indicate a statistically significant difference from control at a level of P <0.05.

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