Comparative characteristics of the leaving of glutathione from cells of different types.

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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*

1. 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, ɣ-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].

2. 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 KH_2PO_4, 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.

3. 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 H_2O), 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).
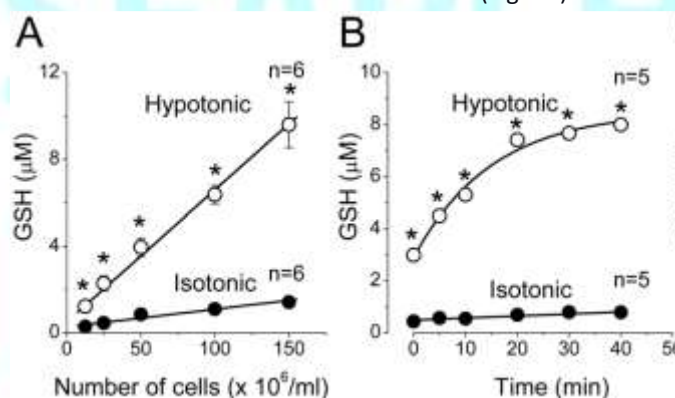

Fig. 1.Glutathione release in normal isotonic medium (light symbols) and under conditions of hypoosmotic stress (147 mOsm/kg H_2O; dark symbols) depending on the concentration of cells in suspension (A: incubation time 10 min) and time (B: cell concentration 100 mln/ml). The experiments were carried 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).
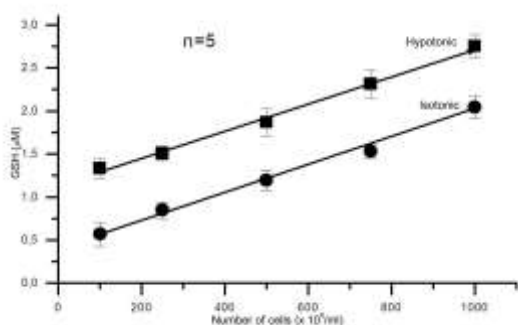
plasma. The high rate of glutathione exit from thymocytes may indicate the important role of this molecule in physiological processes occurring in the extracellular environment of the thymus. At the next stage of the experiments, we investigated the yield of glutathione from cancer cells of melanoma 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.
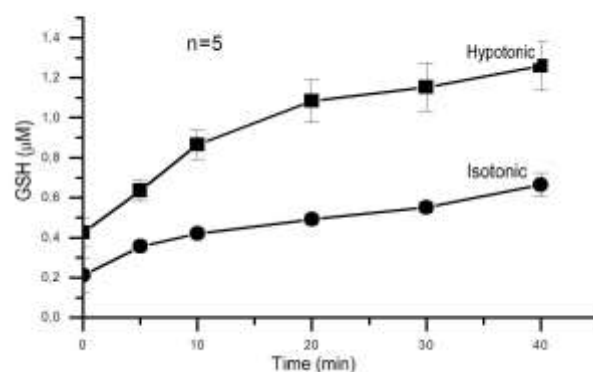

Fig. 2. The output of glutathione from human red blood cells in normal conditions (290 mOsm / kg H_2O) and under hypoosmotic stress (147 mOsm / kg H_2O). Asterisks indicate a statistically significant difference from control at a level of $P < 0.05$.

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

Fig. 3. The output of glutathione from the culture of cancer cells of melanoma in the normal state (290 mOsm / kg H_2O) and under hypoosmotic stress (147 mOsm/kg H_2O). Asterisks indicate a statistically significant difference from control at a level of $P < 0.05$.

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