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Mechanisms of glutathione disulfide efflux from erythrocytes

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ABSTRACT

Glutathione (GSH) plays numerous critical protective roles in the erythrocyte and GSH turnover is likely an important factor in regulating susceptibility to oxidative stress and toxins. Efflux of glutathione disulfide (GSSG) from erythrocytes is an important component in the regulation of GSH levels; however, little is known of the mechanisms involved. We hypothesize that multidrug resistance associated protein 1 (MRP1) is responsible, in part, for GSSG transport from erythrocytes. To test this, we determined the levels of MRP1 protein in erythrocyte membranes from healthy adults and compared them with intracellular levels of GSH. MRP1 levels varied substantially from person to person and were inversely correlated with levels of GSH ($r = -0.39$, $P < 0.05$). In contrast, activity levels of glutamyl cysteine ligase, the rate limiting GSH biosynthetic enzyme, were unrelated to GSH levels. To directly determine the role of MRP1 in GSSG transport, *in vitro* studies were conducted examining the effects of MRP1 inhibitors MK571 and verapamil on GSSG efflux. Both compounds resulted in significant but not complete inhibition (20–53%) of GSSG efflux from cells. Overall, these findings support a role for MPR1 in the regulation of erythrocyte GSH levels through the transport and elimination of GSSG from cells.

1. Introduction

Glutathione (GSH) is the most abundant thiol and antioxidant present in animal tissues (1–15 mM) [1] and, together with its associated biosynthetic, redox and detoxification pathways represents the key defense system against oxidative stress and free radical damage in the cell [2–4]. In addition, GSH plays numerous protective roles including the detoxification of a variety of endogenous and exogenous compounds such as xenobiotics and carcinogens, preservation of protein structure and function, regulation of protein synthesis and degradation and modulation of immune function [5–7].

In erythrocytes, glutathione functions as the most abundant redox buffer, regulating the activity of redox-sensitive enzymes, limiting lipid peroxidation of the plasma membrane *via* glutathione peroxidases, and preventing oxidative denaturation of hemoglobin [8,9]. Erythrocyte glutathione also plays a key role in detoxification of endogenous and exogenous toxins in blood *via* the glutathione S-transferase family of enzymes.

Glutathione in erythrocytes from healthy individuals is normally found in high concentrations (~2 mM), but these levels can vary substantially from person to person [10,11]. Low GSH levels may be indicative of many diseases and disorders, including Diabetes, HIV infection, alcoholic liver disease and aging, and possibly play an instrumental role in their pathogenesis [12–14]. Low erythrocyte GSH levels are associated with increased oxidative stress [15] and consequences of severe erythrocyte GSH deficiency include hemolytic anemia, jaundice and central nervous disorders [16]. In conditions associated with GSH depletion, the restoration of normal erythrocyte GSH levels has been shown to have positive therapeutic effects [17,18].

In defending against oxidative stress, GSH is oxidized to glutathione disulfide (GSSG), which can in turn be reverted back to GSH by glutathione reductase. Alternatively, GSSG can be cleared from erythrocytes in an ATP-dependent manner [19–21]. Therefore, in addition to GSSG reduction by glutathione reductase, export of GSSG represents an important mechanism for preventing accumulation of GSSG in the erythrocyte and protecting against oxidative stress. Further, GSSG efflux is likely an important regulator of glutathione turnover in erythrocytes [22] since GSH itself is not transported from erythrocytes as it is in other cell types.

Abbreviations: MRP1, multidrug resistance associated protein 1; GSH, glutathione; GSSG, glutathione disulfide; GSX, glutathione S-conjugate; GCL, glutamine cysteine ligase; BSO, buthionine sulfoxamine; MK571, (E)-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]-thio]-propanoic acid; GSSP, protein bound glutathione; γGC, γ-glutamyl cysteine.

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While the mechanism of GSSG efflux from erythrocytes is not clear, both high and low affinity components ($K_{m\ GSSG}$ = 0.023–0.1 mM; $K_{m\ GSSG}$ = 5.0–7.3 mM) have been reported, similar to that observed for GSH S-conjugate (GSX) transport [23–25]. In addition, the transport of both GSSG and GSX are inhibited competitively by GSSG ($K_{i\ GSSG}$ = 0.088 mM) and GSX ($K_{i\ GSX}$ = 0.003 mM), respectively [23,25]. Thus, we hypothesize that multidrug resistance associated protein 1 (MRP1), which plays a major role in the transport of GSX is also responsible for GSSG efflux in the erythrocyte. Indeed, this ABC transporter has been identified as a GSSG pump in both HeLa and HL60 cells [26]. To test our hypothesis, we utilized inhibitors of MRP-1 (MK571 and Verapamil) and glutamate cysteine ligase, GCL, (buthionine sulfoximine, BSO) in incubation studies with erythrocytes and determined their effects on glutathione export and also examined relationships between erythrocyte membrane MRP-1 levels and intracellular GSH levels *in vivo*.

2. Materials and methods

2.1. Materials

Benzenacetonitrile (Verapamil) and (E)-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid (MK571) were obtained from Alexis Biochemicals (Axxora, LLC, San Diego, CA). MRP1-A23 polyclonal antibody was obtained from Alexis Biochemicals (Axxora, LLC, San Diego, CA). Goat anti-rabbit antibody was obtained from Santa Cruz (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Study subjects

Blood was obtained from healthy volunteers by venipuncture and collected into tubes containing EDTA as an anticoagulant. All subjects were employees of the Institute for Cancer Prevention and had signed a consent form before participating. All protocols and consent procedures were approved by the Institute for Cancer Prevention institutional Review Board for the protection of human subjects. Subjects were 22 healthy adult non-smokers, 5 males and 17 females, with a mean ± SD age of 36.8 ± 11.4 years. Prior to obtaining blood, subjects underwent an 8 h (overnight) fast.

2.3. Analysis of erythrocyte membrane MRP-1 levels

Erythrocytes were isolated by centrifugation (2100 × *g*) of whole blood for 15 min at 4 °C. Hemolysates (1:90, v/v) were prepared fresh in 2 mM HEPES/0.1 mM EDTA (pH 7.5) and centrifuged (28,000 × *g*) in polypropylene conical tubes for 20 min. Erythrocyte membrane-containing pellets were washed three times in HEPES. Washed pellets were resuspended in 10 mM Tris–HCl (pH 7.4). Protein concentration was determined using the Bio-Rad method (Bio-Rad Laboratories, Hercules, CA) and samples were frozen at −80 °C until use.

Erythrocyte membranes were suspended in 10 mM Tris–HCl and samples containing 25 μg of protein were loaded onto 7% SDS-Polyacrilimide gels and electrophoresis was conducted at 120 V. Proteins were transferred onto nitro-cellulose membranes using a semi-dry transfer blot system (Bio-Rad) at 20 V for 30 min. Membranes were incubated (4 °C) with the polyclonal antibody, MRP1-A23 (1:750), overnight, followed by incubation with goat anti-rabbit antibody (1:10,000) for 60 min. Visualization was achieved by enhanced chemiluminescence (Amersham Biosciences, GE Healthcare, Piscataway, NJ). Membranes were stained with Ponceau Solution to detect equal loading of lanes. Density of the bands was analyzed using ImageJ software. Mean optical density was normalized to a control sample (25 μg of erythrocyte hemolysate) run on each gel.

2.4. Erythrocyte incubations

Erythrocytes obtained from randomly selected study subjects described above were washed three times in 154 mM NaCl/8 mM glucose/10 mM NaH_2PO_4 (pH 7.4) and resuspended to 25% hematocrit in the buffer as above and incubated at 37 °C for 0–24 h in the presence or absence of 25–50 μM BSO, 25–50 μM MK571 or 100–250 μM verapamil. At various times thereafter, 200 μL aliquots were removed for GSH and GSSG measurement. Aliquots were centrifuged (3000 × *g*) for 10 min to yield erythrocyte and buffer fractions. Erythrocyte fractions were treated with 5% MPA (1:5, v/v) for protein precipitation. Acid soluble supernatants and acid insoluble pellets were isolated by centrifugation at 14,000 × *g* for 3 min). The resulting buffer fraction was treated with MPA as above.

2.5. Analysis of glutathione and its metabolites

Intracellular levels of GSH and GSSG were analyzed in MPA extracts of erythrocytes by HPLC with electrochemical detection as described previously [27]. Levels of protein bound GSH (GSSP) were analyzed in potassium borohydride-reduced MPA-insoluble pellets as described previously [28]. GSSG efflux was determined by measuring GSSG levels in MPA treated buffer fractions after erythrocyte incubations by a previously described enzymatic recycling method [10].

2.6. Analysis of GCL activity

On the day of analysis, hemolysates in dH_2O (1:10, v/v) were prepared and exposed to three cycles of freeze/thawing and centrifugation at 10,000 × g for 20 min. Proteins were concentrated ~10-fold by centrifugal ultrafiltration in microcon-10 tubes (Millipore, Bedford, MA) to remove small molecules. Protein concentration was measured using the Bradford method and samples were diluted 20-fold in 100 mM Tris buffer (150 mM KCl, 20 mM $MgCl_2$, 2 mM EGTA; pH 8.2) containing substrates Glu (20 mM), Cys (5 mM) and ATP (10 mM). Following incubation (15–30 min, 37 °c), reactions were stopped by addition of 1 volume of 5% MPA. Precipitated proteins were removed by centrifugation and, after passing through 0.2 μm filters, γ-glutamyl cysteine (γGC) levels were measured using a PerkinElmer Liquid Chromatograph equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA) [29]. A Bio-Sil ODS-5S 5-μm, 4.0 × 250 mm, C18 column (Bio-Rad, Life Science Research Group, Hercules, CA) was used with a mobile phase consisting of 50 mM NaH_2PO_4, 0.05 mM octane sulfonic acid, 1% (v/v) acetonitrile and 0.5% *N*,*N* dimethylformamide (v/v) (pH 2.52) at a flow rate of 1 mL/min and a sample volume of 5 μL. The 8-channel CoulArray detector channels were set at potentials of 250, 400, 450, 500, 550, 600, 650, and 700 mV, respectively. Concentrations were obtained from standard curves based on peak areas.

2.7. Biostatistics

Data represent the mean of three separate experiments. Data were analyzed using NCSS statistical software (Kaysville, UT). Analysis of variance (ANOVA) with Bonferroni post hoc test was utilized to determine if the effects of BSO, MK571 and verapamil were significantly different from untreated cells and from each other. Tests were determined to be significant at $P < 0.05$.