Available online at www.sciencedirect.com
ScienceDirect
Blood Cells, Molecules, and Diseases 37 (2006) 180–187
BLOOD CELLS, MOLECULES, & DISEASES
www.elsevier.com/locate/ybcmd

Membrane skeletal protein *S*-glutathionylation and hemolysis in human red blood cells

Ranieri Rossi [a], Daniela Giustarini [a], Aldo Milzani [b], Isabella Dalle-Donne [b,*]

[a] *Department of Neuroscience, University of Siena, I-53100 Siena, Italy*
[b] *Department of Biology, University of Milan, via Celoria 26, I-20133 Milan, Italy*

Submitted 1 September 2006; revised 20 September 2006
Available online 23 October 2006
(Communicated by E. Beutler, M.D., 20 September 2006)

Abstract

In this work, protein–glutathione mixed disulfide formation in human red blood cells (RBCs) was evaluated in vitro by using the thiol-specific reagent diamide. We investigated what mechanism could lead to *S*-glutathionylation of membrane skeletal proteins, what are the main target proteins, and the correlation between protein *S*-glutathionylation and RBC hemolysis. Diamide caused a decrease in the reduced form of glutathione (GSH), which was accompanied by an increase in the basal level of glutathione disulfide (GSSG) and in *S*-glutathionylation of protein 4.2 and spectrin. The increase in membrane skeletal protein *S*-glutathionylation was correlated with a lower susceptibility of RBCs to osmotic hemolysis, suggesting that *S*-glutathionylation of protein 4.2 and spectrin could contribute to regulate RBC membrane stability.

Keywords: Erythrocytes; Cytoskeletal proteins; Diamide; Osmotic fragility; Protein–glutathione mixed disulfides

Introduction

The maintenance of normal membrane deformability and membrane structural integrity is critical for human red blood cells (RBCs) to undergo extensive deformations in the microvasculature, which allows RBCs to pass through capillaries that are about half their diameter without any damage during their 120-day life span. The plasticity of RBCs relies on a specific membrane skeleton, organized as a polygonal network formed by spectrin tetramers (α- and β-spectrin) linked to short actin filaments, which is closely associated with the inner face of the membrane, primarily by association of spectrin with ankyrin, which in turn is bound to the cytoplasmic domain of band 3 (anion exchanger 1, AE1) and is responsible for maintaining the biconcave disc shape of the RBC, for its deformability, and for membrane structural integrity [1]. Erythrocytes with decreased membrane deformability were observed in diabetic children [2], in cardiogenic shock [3], hemorrhagic shock, and sepsis [4]. Intravital video microscopy studies performed mostly in septic shock models demonstrate capillary occlusion by RBCs with decreased membrane deformability [5].

Macrophage- and neutrophil-derived reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been shown to cause membrane oxidation and decreased RBC membrane deformability during sepsis and different forms of α-thalassemia [5–8]. Membrane oxidative damage has been reported in vivo in α- and β-thalassemia [9]. In particular, oxidant injury to circulating RBCs was indicated of critical importance for hemolysis in β-thalassemia, with evidence of oxidant damage to membrane skeletal proteins protein 4.1 and band 3 [10]. Membrane oxidative damage has also been reported in hemolytic anemia associated with a number of

Abbreviations: Diamide, azodicarboxylic acid-bis-dimethylamide; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, glutathione disulfide; H_{50}, 50% hemolysis; Hb, hemoglobin; mBrB, monobromobimane; NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline; PSH, protein thiol groups; PSSG, protein–glutathione mixed disulfides; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCA, trichloroacetic acid.

* Corresponding author. Fax: +39 02 50314781.
E-mail address: quack@unimi.it (I. Dalle-Donne).

1079-9796/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bcmd.2006.09.003

drugs, including ribavirin [11,12]. Erythrocytes with low protein sulfhydryl group and thioredoxin levels, high protein mixed disulfide and GSSG levels, and changes in membrane skeletal proteins are a risk factor for severe ribavirin-induced anemia [13].

Under oxidative stress, a significant amount of glutathione may be reversibly bound to proteins, by a mechanism called *S*-glutathionylation, resulting in the formation of mixed disulfides between glutathione and protein sulfhydryl groups to form *S*-glutathionylated proteins (PSSG) [14–16]. Whereas several studies report the formation of mixed disulfides between glutathione and hemoglobin under conditions of oxidative stress, such as diabetes mellitus, hyperlipidemia, and smoking [17–22], there are little available data about membrane skeletal protein *S*-glutathionylation in human RBCs [20,23,24].

In this study, we investigated the *S*-glutathionylation of membrane skeletal proteins in human RBCs exposed to the thiol-specific reagent diamide, which has been shown to cause a decreased membrane deformability in RBCs [25,26], and the formation of PSSG in human RBCs [19], human platelets [27] and human T lymphocytes [28]. The correlation between osmotic hemolysis and the *S*-glutathionylation of membrane skeletal proteins was also evaluated.

Materials and methods

Chemicals

All reagents used in this study were of analytical grade from Sigma-Aldrich (Milan, Italy) unless mentioned otherwise. Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA) and HPLC grade reagents from BDH (Poole, England). HPLC column Sephasil C18 (250×4 mm) was purchased from Pharmacia (Uppsala, Sweden). Mouse monoclonal anti-GSH antibody (101-A) was obtained from Virogen (Watertown, MA, USA). Sheep anti-mouse IgG, horseradish peroxidase conjugate was obtained from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, England). The Opti-4CN Substrate Kit and Precision plus protein standards molecular weight, ranging from 10 kDa to 250 kDa, were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Human RBC purification

Blood samples were drawn from healthy volunteers (age 30–45), after informed consent, with K_3EDTA as an anticoagulant. RBCs were prepared by centrifugation at 10,000×*g* for 20 s and washed three times with phosphate buffered saline (PBS), pH 7.4, containing 10 mM glucose. The washed RBCs were suspended in PBS containing 10 mM glucose to a hematocrit value of 50% and were incubated with 1.5 mM (final concentration) diamide. Aliquots were taken before and after 5, 20, 60, and 100 min from diamide treatment, unless otherwise indicated. To terminate the diamide reaction with thiol groups, 200 μl of RBCs was diluted with 1.3 ml PBS containing 20 mM *N*-ethylmaleimide (NEM); after 1 min, RBCs were packed by a 20 s centrifugation at 10,000×*g* and PBS removed.

RBC morphology

Erythrocyte morphology was examined using a scanning electron microscope (SEM, model S-800, Hitachi) after fixation with 1% glutaraldehyde in PBS (isotonicity of the fixative was adjusted with NaCl) and then with 1% OsO_4.

Osmotic fragility assay

Osmotic fragility assays were performed in a series of dilutions of a buffer containing 90 g/l NaCl, 13.65 g/l Na_2HPO_4 and 1.87 g/l NaH_2PO_4, pH 7.4. This solution is osmotically equivalent to 10% NaCl and was diluted with deionized water to yield solutions of 0–0.9% (w/v) NaCl equivalents. Ten microliters of RBC (50% hematocrit) aliquots was diluted with 1 ml of each solution and gently shaken for 30 s. Samples were then centrifuged at 2500×*g* for 2 min to pellet intact RBCs. Hemoglobin concentration in the supernatants was measured as below indicated. Hemolysis in each sample was expressed as a percentage, taking as 100% the maximum value of absorbance obtained in distilled water. A fragility curve was generated by plotting varying salt concentrations vs. hemolysis [29]. The half-maximal hemolysis values (H_{50}) were calculated from fitting data to a four-parameter sigmoidal curve by using the Sigmaplot software.

Protein concentration measurement

Hemoglobin (Hb) concentration was measured by means of a standard kit, based on the Drabkin method, according to the manufacturer's instructions in samples hemolysed by 5 mM Na^+/K^+ phosphate buffer, pH 7.4. Membrane protein concentration was measured by the Bradford assay [30].

GSH, GSSG and PSSG determination by HPLC

GSH and GSSG were titrated by HPLC [31], on NEM-treated and packed RBCs, obtained as above described. Briefly, 200 μl of diamide-treated or control RBCs was mixed (after treatment with NEM and PBS removal by centrifugation), at specified times, with 40 μl of 60% (w/v) trichloroacetic acid (TCA) and proteins discarded by centrifugation at 15,000×*g* for 2 min. NEM excess was extracted from acidified supernatants with 10 vol of dichloromethane, and samples were alkalized by addition of solid $NaHCO_3$. GSH and GSSG were reacted with an equal volume of 1-fluoro-2,4-dinitrobenzene (1.5%, v/v, in ethanol) for 3 h at room temperature in the dark and detected by HPLC as previously described [31]. For PSSG determination, supernatants and membrane skeletal proteins were analyzed by HPLC after thiol derivatization with mBrB [19].

Membrane skeletal protein separation

The packed erythrocytes were hemolysed by addition of 1 ml of 5 mM phosphate buffer, pH 6.5, containing 2 mM NEM and centrifuged at 20,000×*g* for 15 min at 4°C. Supernatants were used for cytosolic analyses. For membrane skeletal protein