Chemical and Pathological Oxidative Influences on Band 3 Protein Anion-exchanger

Diana Teti^a, Maria Crupi^a, Maria Busà^a, Andrea Valenti^a, Saverio Loddo^a, Maurizio Mondello^b and Leonardo Romano^c

^aDepartment of Experimental Pathology and Microbiology, Section of Experimental Pathology, Faculty of Medicine and Surgery, ^bDepartment of Statistic, Faculty of Statistical Science, ^cDepartment of General Physiology and Pharmacology, Faculty of Science, Messina

Key Words

Erythrocytes • G6PDH-deficiency • Anion transport • Oxidative stress • K-Cl cotransport

Abstract

Background/aims: The erythrocyte is a cell exposed to a high level of oxygen pressure and to oxidative chemical agents. This stress involves SH-groups oxidation, cell shrinkage by activation of K-Cl cotransport (KCC) and elevation of the band 3 tyrosine phosphorylation level. The aim of our study was to test whether oxidative stress could influence band 3-mediated anion transport in human red blood cells. **Methods:** To evaluate this hypothesis, normal and pathological (glucose 6 phosphate dehydrogenase (G6PDH) deficient) erythrocytes were treated with known sulphhydryl-blocking or thiol-oxidizing agents, such as N-ethylmaleimide (NEM), azodicarboxylic acid bis[dimethylamide] (diamide), orthovanadate, Mg^{2+} and tested for sulphate (SO_4^-) uptake, K^+ efflux, G6PDH activity and glutathione (GSH) concentration. **Results:** In normal red blood cells, the rate constants of SO_4^- uptake decreased by about 28 % when cells were incubated with NEM, diamide and

orthovanadate. In G6PDH-deficient red blood cells, in which oxidative stress occurs naturally, the rate constant of sulphate uptake was decreased by about 40% that of normal red cells. Addition of oxidizing and phosphatase inhibitor agents to pathological erythrocytes further decreased anion transport. In contrast, G6PDH activity was increased under oxidative stress in normal as well as in pathological cells and was lower in the presence of exogenous Mg^{2+} in parallel to a significant increase in sulphate transport. In both cells, the oxidizing agents increased K^+ efflux with depletion of GSH. **Conclusion:** The data are discussed in light of the possible opposite effects exerted by oxidative agents and Mg^{2+} on KCC and on the protein tyrosine kinase (PTK)-protein tyrosine phosphatase (PTP) equilibrium. The decreased sulphate uptake observed in the experimental and pathological conditions could be due to band 3 SH-groups oxidation or to oxidative stress-induced K-Cl symport-mediated cell shrinkage with concomitant band 3 tyrosine phosphorylation.

Copyright © 2005 S. Karger AG, Basel

Introduction

Band 3, an integral protein of about 100 kD in red blood cells (RBCs), mediates anion exchange which is the main function in blood CO₂ transport and pH equilibration across the RBC membrane. With more than 1 million copies per cell, it is the most abundant membrane protein in human RBCs. The protein consists of a membrane domain and a cytoplasmic domain that is anchored to the cytoskeleton. In addition, the N-terminal fragment of the cytoplasmic domain contains binding sites for glycolytic enzymes, haemoglobin, [1, 2] and phosphorylation sites for some tyrosine kinase proteins such as tyrosine 8 [3, 4]. In general, the level of tyrosine phosphorylation is determined by the balance between the activities of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) [4]. In physiological conditions the activity of PTP is higher than that of PTK, but many agents can influence the activities of these enzymes [4-7]. N-ethylmaleimide (NEM), diamide, orthovanadate, and osmotic stress induce cell shrinkage and alterations in the oxidation-reduction system with elevation of the band 3 tyrosine phosphorylation level by PTK activation or PTP inhibition [4, 8-11]. Furthermore, thiol-oxidizing agents are known to cross-link integral membrane proteins, in particular dimers and tetramers of band 3, and band 3 and haemoglobin, leading to the formation of high molecular weight aggregates [12-14]. Na⁺-K⁺-2Cl⁻ co-transport (NaKCC) and KCC play key roles in cell volume regulation [15-18]. Studies on red blood cells suggested that swelling-activated KCC and shrinkage-activated NaKCC ion transporters, in an isosmotic artificial medium, are almost inactive because they are co-ordinately controlled by a common mechanism termed "volume set point" [10, 19-21]. Depletion of cytoplasmic Mg²⁺ or treatments with oxidant agents reduce the set point and induce KCC- and Gardos K⁺ channel-dependent volume decrease [10-12, 15, 22-24]. Besides, it has been recently reported that free, ionized cytoplasmic Mg²⁺ determines the activity of transport processes that control the ionic composition and cellular volume of many cell types, including RBCs, [25-30]. In fact, in some diseases in which K-Cl co-transport is pathologically elevated, the dietary Mg²⁺ supplementation increases its content in erythrocytes and leads to inhibition of KCC [10, 31-33]. Mg²⁺ deficiency increases erythrocyte susceptibility to free radical injury and leads to significant reduction in erythrocyte glutathione (GSH) [10, 31-34].

The activity of G6PDH, the key enzyme which catalyses the first step of the pentose phosphate pathway,

represents the main line of defense of the erythrocyte against oxidative injuries by producing the β -nicotinamide-adenine-dinucleotide phosphate (NADPH) reduced equivalent. The major role of NADPH in erythrocytes is the regeneration of reduced glutathione, which in turn prevents heme oxidation and preserves the integrity of sulphhydryl groups of α and β chains to forestall Heinz bodies and the integrity of red blood cell membrane proteins [35]. G6PDH activity is also influenced by many chemical agents and drugs, like ampicillin sulphate, Mg²⁺, diazepam and ethanol [27, 36-38].

To investigate the influence of oxidative stress on band 3 efficiency, human RBCs were treated with known sulphhydryl-blocking or thiol-oxidizing agents, such as NEM and diamide, and tested for sulphate uptake, K⁺ efflux, G6PDH activity and GSH concentration. The same experiments were performed with G6PDH-deficient erythrocytes, characterized by low GSH production and greater sulphhydryl-group oxidation. Furthermore, to verify the influence of phosphorylation on band 3 function, both normal and G6PDH-deficient RBCs were incubated in the presence of the phosphatase inhibitor orthovanadate. In addition, other experiments were performed to test the influence of oxidative stress on erythrocyte membranes and sulphate uptake in the presence of Mg²⁺.

Materials and Methods

Erythrocyte preparation

After informed consent, human blood samples were obtained from 20 healthy volunteers and from 5 G6PDH-deficient volunteer patients (G6PDH: 3.8-5.1 U/g Hb), monitored at the Policlinico Universitario of Messina. Unless specified otherwise, the blood was collected in heparinized tubes and was divided in two tubes, washed in 166 mM NaCl (309 mOsm) unbuffered and centrifuged three times for 5 minutes at 3,000 rpm at 4°C. At each step the buffy coat was carefully removed. The cell suspensions were incubated for 1 hour at 37°C in the following isotonic medium (307 mOsm): 150 mM choline chloride, 20 mM N-2-hydroxy-ethylpiperazine N-2-ethanesulfonic (HEPES), 15 mM glucose, 0.1 mM ouabain at pH 7.4, (low Na⁺ to facilitate Mg²⁺ influx) and with ± 5 mM MgCl₂, ± 2 mM NEM, ± 1 mM diamide or with ± 0.1 mM sodium orthovanadate, for experiments where cells were treated under the same conditions. Some experiments were performed with erythrocytes pre-incubated with ± 2 mM NEM, ± 1 mM diamide, or ± 0.1 mM sodium orthovanadate before exposure to 5 mM MgCl₂. Other experiments were performed replacing 150 mM choline chloride by 150 mM KCl or 150 mM NaNO₃ ± 2 mM NEM.

After the last centrifugation, the erythrocytes were resuspended at hematocrit of 3% in the following isotonic medium (295 mOsm) in mM: 115 Na₂SO₄ or K₂SO₄, 20 HEPES, 15

glucose and 0.1 ouabain, with ± 2 NEM, ± 1 diamide, ± 0.1 sodium orthovanadate, or ± 5 MgCl_2 , and incubated at 25°C . At specified intervals, 5 ml samples of suspension were removed and added to a test tube containing $5\ \mu\text{M}$ of 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS) stopping medium and kept under ice. The presence of $5\ \mu\text{M}$ DIDS, a chemical agent that binds irreversibly and specifically to the exterior part of the integral band 3 protein [25, 39, 40], inhibited SO_4^- transport both in normal and G6PDH-deficient cells. After the last sample withdrawal, the erythrocytes were washed three times in a sulphate-free medium at 0°C to remove the sulphate trapped outside.

Sulphate transport measurement

To measure the intracellular SO_4^- content, cells were haemolysed with distilled water and trichloroacetic acid. The membranes were eliminated by centrifugation, and sulphate ions in the supernatant were precipitated by adding glycerol and distilled water solution (1:1), 4 mM NaCl and HCl (hydrochloric acid 37%) solution (12:1), and 124 mM $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ solution.

SO_4^- was measured by atomic absorption spectrophotometry at 425 nm wavelength. Using a calibrated standard curve obtained by precipitating known sulphate concentrations, we converted the absorption to mM of intracellular SO_4^- , to calculate the rate constant in min^{-1} , derived from the following equation:

$C(t) = C^\infty(1 - e^{-t}) + C_0$ where C_0 , C_t and C^∞ represent the intracellular sulphate concentrations measured at time 0, t and ∞ .

Erythrocyte cation analysis

The erythrocyte suspension was lysed by addition of 20 μl of saponine solution. The lysate was mixed well and 0.3 ml were added to 10 ml lithium nitrate diluent (10 mM) for Na^+ and K^+ determination. The standard cation solutions were diluted in the same manner and used to calibrate the flame photometer. Erythrocyte Na^+ and K^+ concentrations were expressed in mEq/l H_2O .

Cell Mg content ($[\text{Mg}^{2+}]_i$) was measured after the cells had been separated from the medium by rapid centrifugation through di-n-butylphthalate, following the method of Flatman [41]. The cell pellet was lysed in 1.5 ml distilled water. Total Mg^{2+} content was measured by atomic absorption spectroscopy in an aliquot of cellular suspension at zero time and after 1 hour.

K^+ efflux measurement

For K^+ efflux measurement, the blood, collected in heparinized tubes, was washed and centrifuged three times for 5 minutes at 3,000 rpm at 4°C in the following isotonic medium (307 mOsm): 150 mM choline chloride, 20 mM HEPES, 15 mM glucose, 0.1 mM ouabain at pH 7.4 or in 150 mM NaNO_3 , 20 mM HEPES, 15 mM glucose, 0.1 mM ouabain (309 mOsm) at pH 7.4. The K^+ efflux determinations are not at initial rates, in fact erythrocytes, at 20% hematocrit, were incubated for 1 hour at 37°C in the same medium with ± 2 mM NEM, ± 1 mM diamide, ± 0.1 mM sodium orthovanadate and ± 5 mM MgCl_2 .

The suspensions were spun at 4°C and 50 μl of the supernatants were placed in a LiNO_3 solution and the K^+ concentration was measured by a flame photometer. The total K^+ content of the flux media was corrected for the initial cell volume and K^+ efflux expressed in mEq/l RBC $\times$ h of the original cells.

Cellular Volume

Normal and G6PDH-deficient erythrocytes were washed and centrifuged three times for 5 minutes at 3,000 rpm at 4°C in the following isotonic medium (307 mOsm): 150 mM choline chloride, 20 mM HEPES, 15 mM glucose, 0.1 mM ouabain at pH 7.4. Erythrocytes, at 20% hematocrit, were incubated for 1 hour at 37°C in the same medium with ± 2 mM NEM, ± 1 mM diamide. The cellular volume, expressed in femtoliter (fl), was measured at time 0' and after 1h of incubation, by ABX PENTRA 120. The normal values are from 80 to 100 fl.

G6PDH activity measurement

The G6PDH activity was calculated by the CL-10 system, which is based on the variation of pH in the reaction of G6PDH oxidation by NADP^+ , in accordance with the method suggested by the W.H.O., with automatic subtraction of the catalyzed reaction of 6-P-gluconate dehydrogenase [42]. The activity of G6PDH was expressed as U/g Hb. The normal values are from 9.4 to 17.8 U/g Hb.

Glutathione (GSH) measurement

Normal and G6PDH-deficient erythrocytes were washed and resuspended at hematocrit of 20% in the following isotonic medium (307 mOsm): 150 mM choline chloride, 20 mM Hepes, 15 mM glucose, pH 7.4 with ± 1 mM diamide and ± 5 mM MgCl_2 for 1 hour at 37°C . The suspensions were spun and the erythrocytes were kept to measure the GSH. It was determined by Cayman's GSH assay kit that utilizes a carefully optimized enzymatic recycling method, using glutathione reductase. The sulphhydryl group of GSH reacts with 5,5-dithiobis-2-nitrobenzoic acid, Ellman's reagent (DTNB) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB by a spectrophotometer at 405 nm provides an accurate estimation of GSH in the sample.

All results were analyzed by a nonparametric permutation test with NPC statistical software. [43]. Appendix shows methods and results obtained.

Results

Table 1a shows that the basal intracellular concentrations of Na^+ , K^+ , Mg^{2+} and haemoglobin in normal and G6PDH-deficient RBCs are not significantly different. Both cells, after incubation with 5 mM MgCl_2 ,

Red blood cells	[Na ⁺] mEq/l H ₂ O	[K ⁺] mEq/l H ₂ O	Native [Mg ²⁺] _i mEq/l H ₂ O	+ 5 mM [Mg ²⁺] _o [Mg ²⁺] _i mEq/l H ₂ O	Hb g/100ml RBCs
Normal	11.1 ± 0.7	150.1 ± 4.2	2.84 ± 0.03 **	3.01 ± 0.02 **	14.4 ± 0.2 *
G6PDH-deficient	10.8 ± 0.9	152.2 ± 4.3	2.60 ± 0.02 **	2.82 ± 0.03 **	14.8 ± 0.2 *

Table 1a. Erythrocyte cation Na⁺, K⁺ and Mg²⁺ basal concentrations, in normal and G6PDH-deficient cells, are expressed in mEq/l H₂O. Hb content is expressed in g/100 ml of RBCs. Data are presented as means ± SD of 5 experiments. Significance * p< 0.05 and ** p< 0.0001 of nonparametric test by NPC test were performed between normal and G6PDH-deficient cells.

Table 1b. K⁺ efflux, in normal and G6PDH-deficient cells, in the presence of NEM, diamide, orthovanadate and Mg²⁺ after 1 h of incubation, 37°C, pH 7.4 in Cl⁻ and Cl-free media. K⁺ is expressed in mEq/ l RBC x h. Data are presented as means ± SD of 5 experiments. Significance * p< 0.05

		[K ⁺] mEq /l RBC x h				
Red blood cells	Control	NEM 2 mM	diamide 1 mM	orthovanadate 0.1 mM	[Mg ²⁺] 5 mM	
Normal	Cl ⁻ 6.0 ± 0.1**	38.8 ± 0.2**	37.7 ± 0.3**	35.8 ± 0.2**	4.9 ± 0.01*	
	NO ₃ ⁻ 4.0 ± 0.1**	8.5 ± 0.1*	7.9 ± 0.2**	5.9 ± 0.3**	3.1 ± 0.1*	
G6PDH-deficient	Cl ⁻ 7.0 ± 0.1**	39.7 ± 0.2**	37.7 ± 0.3**	36.7 ± 0.2**	5.0 ± 0.01*	
	NO ₃ ⁻ 5.0 ± 0.1**	9.0 ± 0.3*	8.8 ± 0.2**	8.7 ± 0.3**	3.2 ± 0.1*	

and ** p< 0.0001 of nonparametric test by NPC test were performed between normal and G6PDH-deficient cells. The statistical significance versus control showed, for Cl⁻, statistical differences for all comparison excepted for Mg²⁺; while, for NO₃⁻, there were not differences versus control.

showed a discrete increase in intracellular Mg²⁺ concentration that was similar in normal and G6PDH-deficient erythrocytes.

Table 1b shows the mEq/l RBC x h of K⁺ efflux measured in normal and G6PDH-deficient cells after treatment with NEM, diamide, orthovanadate and Mg²⁺. In both cells, the oxidizing agents increased chloride-dependent K⁺ efflux with respect to the control. A modest reduction was seen in presence of Mg²⁺. When the Cl⁻ was replaced by NO₃⁻ the K⁺ efflux was decreased in both cells.

Table 1c shows the initial RBC volume in normal and pathological cells and the decreased volume after treatment with NEM, diamide, and orthovanadate in both cells.

Fig. 1a shows the time course of SO₄⁻ uptake measured in normal RBCs both in the absence and presence of NEM, NEM+Mg²⁺, diamide, diamide+Mg²⁺, and DIDS. Experiments with the band 3 protein inhibitor DIDS were performed in parallel as a negative control, to exclude involvement of band 3. The SO₄⁻ uptake was lower in erythrocytes treated with thiol-oxidizing agents as compared with control cells. Sulphate uptake measured in RBCs pre-treated with NEM and diamide before exposure to 5 mM Mg²⁺ was increased compared to the uptake in RBCs treated with either NEM or diamide alone, although it did not reach the value of the control. Early

Red blood cells	Time	RBC volume (fl)		
		Control	NEM 2 mM	diamide 1 mM
Normal	0°	92 ± 0.2	92 ± 0,2	92 ± 0.2
	60°	90 ± 0.2**	59 ± 0.1**	57 ± 0.1 **
G6PDH-deficient	0°	89 ± 0.2	89 ± 0.2	89 ± 0.2
	60°	86 ± 0.2**	55 ± 0.2**	53± 0.2**

Table 1c. Cellular volume in normal and G6PDH-deficient erythrocytes, before and after 1 h of incubation in absence (control) and presence of NEM and diamide. Data are presented as means ± SD of 5 experiments. RBC volume is expressed in femtoliter (fl). Significance ** p< 0.0001 of nonparametric test by NPC test were performed between normal and G6PDH-deficient cells. The statistical significance versus control showed differences (p.value <0.05) in both cells.

DIDS treatment, nearly, abolished sulphate transport. SO₄⁻ uptake was tested in G6PDH-deficient RBCs under the same experimental conditions as above. G6PDH-deficient erythrocytes showed a significant decrease in the rate constant of anion transport compared with normal cells.

When these RBCs were treated with NEM and diamide they showed a behaviour similar to that of normal cells, the SO₄⁻ uptake was lower than in untreated cells (Fig. 1b). DIDS inhibited SO₄⁻ transport also in G6PDH-deficient cells.

Fig. 1. A, Time course of SO_4^{2-} uptake in normal red blood cells measured in control (O), in the presence of NEM (Δ), diamide ($\square$), NEM+ Mg^{2+} ($\blacktriangle$), diamide+ Mg^{2+} ($\blacksquare$), and with 5 μM DIDS as a negative control (x); pH 7.4, 25°C. Significance * $p < 0.05$ and ** $p < 0.0001$ by analysis of variance (ANOVA) as compared with control when the sulphate uptake reached the equilibrium. Bars represent mean $\pm$ SD. B, Time course of SO_4^{2-} uptake in G6PDH-deficient red blood cells measured in control (O) and in the presence of NEM (Δ), diamide ($\square$) and with 5 μM DIDS as a negative control (x); pH 7.4, 25°C. Significance ** $p < 0.0001$ by analysis of variance (ANOVA) as compared with control when the sulphate uptake reached the equilibrium. Bars represent mean $\pm$ SD.

Fig. 2a shows the time course of SO_4^{2-} uptake in normal RBCs in the absence and presence of orthovanadate, orthovanadate+ Mg^{2+} , Mg^{2+} alone, and DIDS. The SO_4^{2-} ionic equilibrium was lower in erythrocytes treated with orthovanadate compared to control cells and faster in cells incubated with Mg^{2+} which contained higher concentrations of the cation than control cells. Sulphate uptake measured in RBCs pre-treated with

Fig. 2. A, Time course of SO_4^{2-} uptake in normal red blood cells measured in control (O), in the presence of orthovanadate (Δ), orthovanadate+ Mg^{2+} ($\blacktriangle$), with added intracellular concentrations of magnesium ($\square$), and with 5 μM DIDS as a negative control (x); pH 7.4, 25°C. Significance * $p < 0.05$ and ** $p < 0.0001$ by analysis of variance (ANOVA) as compared with control when the sulphate uptake reached the equilibrium. Bars represent mean $\pm$ SD. B, Time course of SO_4^{2-} uptake in G6PDH-deficient red blood cells measured in control (O), in the presence of orthovanadate (Δ), with added intracellular concentrations of magnesium ($\square$), and with 5 μM DIDS as a negative control (x); pH 7.4, 25°C. Significance ** $p < 0.0001$ by analysis of variance (ANOVA) as compared with control when the sulphate uptake reached the equilibrium. Bars represent mean $\pm$ SD.

orthovanadate before exposure to 5 mM Mg^{2+} was increased compared with the uptake in orthovanadate alone, although it did not reach control levels.

Again, G6PDH-deficient erythrocytes exhibited a behaviour similar to that of normal cells in relation to the response to orthovanadate and Mg^{2+} effects (Fig. 2b), but the SO_4^{2-} concentration in all the tested conditions was lower than in normal erythrocytes. As for normal

Table 2. Rate constants x min⁻¹ of SO₄⁻ uptake in normal and G6PDH-deficient erythrocytes in the absence (control)

Red blood cells	Control	NEM 2 mM	diamide 1 mM	orthovanadate 0.1 mM	[Mg ²⁺] 5 mM
Normal	0.037 ± 0.001**	0.027 ± 0.002**	0.029 ± 0.002**	0.031 ± 0.001**	0.050 ± 0.001**
G6PDH-deficient	0.022 ± 0.001**	0.017 ± 0.003**	0.019 ± 0.003**	0.018 ± 0.002**	0.030 ± 0.002**

and presence of NEM, diamide, orthovanadate and Mg²⁺, at 25°C, pH 7.4. Data are presented as means ± SD of 5 experiments. Significance ** p< 0.0001 of nonparametric test by NPC test were performed between normal and G6PDH-deficient cells. The statistical significance versus control showed significant differences for all comparison (p.value <0.05).

Table 3. G6PDH activity (U/g Hb) in normal and G6PDH-deficient erythrocytes after 1 h of incubation,

Red blood cells	Control	NEM 2 mM	diamide 1 mM	orthovanadate 0.1 mM	[Mg ²⁺] 5 mM
Normal	14.2 ± 0.2**	16.5 ± 0.2**	16.1 ± 0.3**	17.1 ± 0.1**	12.3 ± 0.1**
G6PDH-deficient	4.8 ± 0.3**	6.8 ± 0.2**	7.0 ± 0.1**	6.7 ± 0.2**	3.1 ± 0.2**

37°C, pH 7.4, in the absence (control) and presence of NEM, diamide, orthovanadate and Mg²⁺. Data are presented as means ± SD of 5 experiments. Significance ** p< 0.0001 of nonparametric test by NPC test were performed between normal and G6PDH-deficient cells. The statistical significance versus control showed significant differences for all comparison (p.value <0.05).

RBCs, DIDS inhibited SO₄⁻ transport in G6PDH-deficient erythrocytes.

To test whether cell shrinkage mediated the observed effect of redox state on SO₄⁻ uptake, we repeated some of the uptake studies in Cl⁻ medium, high K⁺ medium (which prevents cell shrinkage) and Cl⁻ free medium. The results of the rate constants (x min⁻¹) of SO₄⁻ uptake measured in normal erythrocytes are reported in Fig. 3. In erythrocytes treated with high K⁺ and Cl⁻ free media, the rate constants relative to sulphate uptake showed a modest increase compared to erythrocytes in Cl⁻ medium, although they did not reach the value of the control cells.

Table 2 summarises the rate constants (x min⁻¹) relative to the measured sulphate uptake in normal and G6PDH-deficient erythrocytes treated with oxidizing agents, orthovanadate, and Mg²⁺. The basal rate constants in normal cells decreased by about 27 %, 21 % and 16 % in cells incubated with oxidizing agents and orthovanadate, respectively, and increased by about 26 % in Mg-treated erythrocytes. The rate constants in untreated pathological cells were lower by about 40 % compared with normal erythrocytes and decreased by about 22 %, 13 % and 18 % in cells treated with NEM, diamide and orthovanadate, respectively, but increased by 26.7 % in the presence of 5 mM MgCl₂.

The values of G6PDH activity measured in different experimental conditions are reported in Table 3. In normal RBCs, G6PDH activity increased with respect to the control (14.2 U/g Hb) by about 14 %, 12 % and 17 % in cells treated with NEM, diamide and orthovanadate, but

Fig. 3. Rate constants x min⁻¹ of SO₄⁻ uptake in normal erythrocytes in the absence (control) and presence of NEM, at 25°C, pH 7.4 in Cl⁻, Cl⁻ free and high K⁺ media. Data are presented as means ± SD of 5 experiments. Significance * p< 0.05 and ** p< 0.0001 by analysis of variance (ANOVA) was compared with control.

decreased by 13 % in cells incubated with 5 mM of MgCl₂. A similar pattern, although at a lower degree, was shown by G6PDH-deficient cells, whose G6PDH activity was 4.8 U/g Hb. In NEM- and diamide-induced oxidative stress conditions, the values increased by 29.4 % and 31.4 % respectively, and in the presence of the phosphatase inhibitor orthovanadate by about 30 %. In

Table 4. Reduced and oxidized glutathione (nmol/ml RBCs) and GSH/GSSG ratio in normal erythrocytes after 1 h of incubation, 37°C, pH 7.4, in the absence (control) and presence of diamide and Mg^{2+} . Data are presented as means $\pm$ SD of 5 experiments. Significance ** $p < 0.0001$ of nonparametric

test by NPC test were performed between normal and G6PDH-deficient cells. The statistical significance versus control showed significant differences for all comparison (p.value < 0.05).

Metabolites	Control	diamide 1 mM	[Mg^{2+}] 5 mM
GSH (nmol/ml RBCs)	1.911 $\pm$ 338**	1.025 $\pm$ 225**	2.187 $\pm$ 315**
GSSG (nmol/ml RBCs)	47.1 $\pm$ 11.5**	59 $\pm$ 10.7**	43 $\pm$ 13**
GSH/GSSG ratio	41 $\pm$ 6**	17 $\pm$ 2**	49 $\pm$ 15**

Table 5. Reduced and oxidized glutathione (nmol/ml RBCs) and GSH/GSSG ratio in G6PDH-deficient erythrocytes after 1 h of incubation, 37°C, pH 7.4, in the absence (control) and presence of diamide and Mg^{2+} . Data are presented as means $\pm$ SD of 5 experiments. Significance ** $p < 0.0001$ of nonparametric test by NPC test were

performed between normal and G6PDH-deficient cells. The statistical significance versus control showed significant differences for all comparison (p.value < 0.05).

Metabolites	Control	diamide 1 mM	[Mg^{2+}] 5 mM
GSH (nmol/ml RBCs)	1.705 $\pm$ 385**	988 $\pm$ 209**	1.898 $\pm$ 301**
GSSG (nmol/ml RBCs)	59.1 $\pm$ 15**	79 $\pm$ 22**	42 $\pm$ 26**
GSH/GSSG ratio	28.9 $\pm$ 3**	12.5 $\pm$ 2.8**	46 $\pm$ 31**

the presence of higher Mg^{2+} concentrations than native levels, a reduction of 35 % in G6PDH activity was observed with respect to the control cells.

Tables 4 and 5 show reduced and oxidized glutathione and GSH/GSSG ratio in normal (Table 4) and G6PDH-deficient (Table 5) erythrocytes. Both kinds of cells exhibited similar behaviour, even though at different levels. In normal RBCs, GSH concentration decreased by 46 % with respect to the control (1,911 nmol/ml RBCs) in cells treated with diamide, but increased by 12 % in cells incubated with 5 mM of $MgCl_2$. A similar pattern, although at a lower degree, was shown by G6PDH-deficient cells, whose GSH concentration was 1,705 nmol/ml RBCs. In diamide-induced oxidative stress conditions, the value was 42 % lower with respect to the control, while in the presence of higher Mg^{2+} concentrations than native levels, a 10 % increase was observed with respect to the control cells. During stress or naturally induced oxidation, GSH values decreased whereas GSSG values increased and a lower GSH/GSSG ratio was observed with respect to the control. On the contrary, in the presence of exogenous Mg^{2+} , lower GSSG values and higher GSH/GSSG ratios were observed with respect to the control cells.

Discussion

Oxidative stress frequently occurs in physiological and pathological states, and alterations in anion transport

may be responsible for the impairment of several functions observed under these conditions since SH-oxidizing agents are known to cause cross-linking of human erythrocyte band 3, and reduction in the content of the SH-groups of integral proteins of erythrocyte membranes [16, 44]. In fact, it has been previously demonstrated that treatments with thiol-oxidizing and alkylating agents, such as diamide, NEM, H_2O_2 and orthovanadate lead to alterations in the erythrocyte redox state and in the activation of the K-Cl co-transport which results in the cell shrinkage-induced inhibition of PTP activity and band 3 tyrosine phosphorylation [4, 8, 9, 11, 22, 23, 45]. To the best of our knowledge the role of band 3 protein in cell redox equilibrium changes has not been investigated, even though it has been shown that it affects several erythrocyte functions [2, 4]. The data presented here deals with changes in SO_4^- transport rate constants across erythrocyte membranes during induced conditions of oxidative stress in normal and G6PDH-deficient cells. They show that oxidative stress occurring in NEM and diamide-treated normal erythrocytes impaired the ability of band 3 to regulate anion transport (Fig. 1a). The decreased efficiency of anion transport may be due either to changes in the structural state of band 3 by SH-groups oxidation or to cell shrinkage (increased K^+ efflux) (Tab. 1b) with the concomitant increase in band 3 tyrosine phosphorylation [4, 11]. NEM and diamide are potent stimulators of KCC as well as target glutathione [45, 46], so the GSH concentration was tested. The lower GSH levels (Tab. 4) measured in normal cells treated with

diamide, confirm the presence of oxidative stress. Since oxidative damage occurs readily in cells possessing low levels of GSH, to verify the effect of naturally occurring oxidative stress on sulphate uptake, experiments using G6PDH-deficient red blood cells were carried out. In these cells characterized by lower GSH concentration with respect to the normal erythrocytes (Tab. 4-5), the rate constants of sulphate uptake was lower than in normal conditions (Tab. 2). Some basal parameters evaluated by us, such as haemoglobin, cations content K^+ , Na^+ , Mg^{2+} (Tab. 1a) and cellular volume (Tab. 1c) are similar to normal red cells. Since in G6PDH-deficient cells the tyrosine phosphorylation is pathologically elevated [47], because of the occurring oxidative stress, it may be conceivable that the decrease in sulphate uptake exhibited by G6PDH-deficient erythrocytes was a result either of alteration in SH-groups or of PTP/PTK disequilibrium. The inhibitory effect of oxidation on the PTP activity, in fact, has been considered as the mechanism more likely to be responsible for tyrosine phosphorylation [4, 9, 48]. The phenomenon was more pronounced when the G6PDH-deficient cells were exposed to the oxidizing agents NEM and diamide, since they exhibited a further decrease in SO_4^- transport (Fig. 1b). After treatments of G6PDH-deficient cells with NEM, diamide and orthovanadate, the K^+ efflux was similarly increased (Tab. 1b). It is conceivable that KCC activation induces the further decrease of SO_4^- . In addition to NEM and diamide, also orthovanadate has been demonstrated to induce Gardos K^+ channel activation and cell shrinkage [22, 23, 49]. The orthovanadate-induced K^+ efflux in normal as well as G6PDH-deficient cells (Tab. 1b), may be responsible for the decrease in sulphate uptake rate constants (Fig. 2a, 2b). In this case, as well we can say that K^+ efflux -induced cell shrinkage provoked phosphatase inhibition and, consequently, a decreased rate constant of sulphate uptake in both cells.

The cation Mg^{2+} plays a key role on the effects of oxidative stress-induced KCC-mediated cell shrinkage and tyrosine dephosphorylation on the band 3. Intracellular Mg^{2+} deficiency increases erythrocyte susceptibility to free radical injury and leads to depletion of GSH [10, 31-33]. In fact, dietary Mg^{2+} supplementation has been shown to cause significant increases in red cell Mg^{2+} and improvement of some diseases by inhibition of K-Cl co-transport which is excessively elevated in these pathologies [10, 24, 31-33, 50, 51]. It has recently been shown that the human erythrocyte membrane contains band 3-associated neutral PTP, named PTP1B [5, 6, 52, 53], which is responsible for the low level of tyrosine

phosphorylation in normal cells [5, 9, 52, 54] and may be important for band 3 function in the erythrocyte. This PTP1B is inhibited by orthovanadate and erythrocyte thiol oxidation, while it is activated by internal Mg^{2+} [4-6].

Since we incubated intact RBCs in a magnesium medium without adding an ionophore (Tab. 1), the increase in sulphate uptake observed in normal as well as G6PDH-deficient cells (Fig. 2a, 2b) may be due either to down regulation of K-Cl co-transport activity by Mg^{2+} (which prevents cell shrinkage) or to an increase in the dephosphorylation rate through the activation of neutral PTP1B associated with band 3. This role of Mg^{2+} is also preserved in normal cells pre-treated with oxidative and phosphatase inhibitor agents (Fig. 1a, 2a).

Our data confirm that RBC Mg^{2+} is a modulator of KCl co-transport and that a modest increase in cell Mg^{2+} (Tab. 1a) induces inhibition of this transport system (Tab. 1b). In addition, the free Mg^{2+} is able to increase the rate constant of sulphate uptake in every experimental condition tested (Fig. 1a-2a-2b) as well as GSH levels (Tab. 4-5). In fact, in normal as well as G6PDH-deficient erythrocytes, lower GSH, higher GSSG and a lower GSH/GSSG ratio have been found during stress or naturally induced oxidative state, which is opposite to the Mg^{2+} effects.

Besides, the measured G6PDH activity in normal and G6PDH-deficient cells (Tab. 3) subject to oxidative stress by NEM, diamide and orthovanadate showed an increase in G6PDH activity, even though it remained within the physiological and pathological ranges, respectively. This behaviour may be an effect of the adaptive response of cells to oxidative stress that is still efficient in G6PDH-deficient ones. On the contrary, the increase in intracellular Mg^{2+} induced a decrease in G6PDH activity, even though it remained, again, within physiological and pathological ranges. It is an unknown mechanism, but, it may be that magnesium affects the glycolytic pathway by Mg-ATP complex formation, as a substrate, as well as a modulator of hexokinase and phosphofructokinase enzymes in the glycolytic sequence [55, 56], with inhibition of the pentose phosphate pathway and G6PDH activity.

Taken together, the data presented here, correlate the altered oxidative processes occurring in G6PDH-deficient, NEM-, diamide- and orthovanadate-treated cells to modifications in anion transport. These results are consistent with the hypothesis that the decreased sulphate uptake could be consequent either to oxidative stress-induced K-Cl symport-mediated volume decrease, or to an impairment of band 3 activity through alteration in the

SH-groups of the transporter (Fig. 3). Cross-linking of band 3, in fact, induces conformational changes of ionic pumps which in turn could affect the sulphate uptake kinetics. Thus, the impairment of the ability of band 3 protein to regulate sulphate transport during oxidative stress may be of pathophysiological relevance, especially in relation to acid-base equilibrium and oxygen exchange, which are strictly dependent on anion transport efficiency and regulation.

Acknowledgements

A preliminary report of this work was presented as poster at the meeting of the Italian Society of Physiology in Chieti (September 2003, n 149, p. 77).

References

- Jensen FB, Jakobsen MH, Weber RE: Interaction between haemoglobin and synthetic peptides of the N-terminal cytoplasmic fragment of trout band 3 (AE1) protein. *J Exp Biol* 1998;201:2685-2690.
- Salhany JM: Erythrocyte band 3 protein. Boca Raton 1990;FL:CRC press.
- Yannoukakos D, Meyer HE, Vasseur C, Driancourt C, Wajcman H, Bursaux E: Three regions of erythrocyte band 3 protein are phosphorylated on tyrosines: characterization of the phosphorylation sites by solid phase sequencing combined capillary electrophoresis. *Biochim Biophys Acta* 1991;1066:70-76.
- Zipser Y, Piade A, Kosower NS: Erythrocyte thiol state regulates band 3 phosphotyrosine level via oxidation/reduction of band 3-associated phosphotyrosine phosphatase. *FEBS Letters* 1997;406:126-130.
- Zipser Y, Kosower NS: Phosphotyrosine phosphatase associated with band 3 protein in the human erythrocyte membrane. *Biochem J* 1996;15:881-887.
- Zipser Y, Piade A, Barbul A, Korenstein R, Kosower NS: Ca²⁺ promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. *Biochem J* 2002;15:137-144.
- Terra HT, Saad MJ, Carvalho CR, Vicentin DL, Costa FF, Saad ST: Increased tyrosine phosphorylation of band 3 in hemoglobinopathies. *Am J Hematol* 1998;58:224-230.
- Monteiro HP, Ivaschenko Y, Fischer R, Stern A: Inhibition of protein tyrosine phosphatase activity by Diamide is reversed by epidermal growth factor in fibroblasts. *FEBS Lett* 1991;295:146-148.
- Heffetz D, Bushkin I, Dror R, Zick Y: The insulinomimetic agents H₂O₂ and orthovanadate stimulate protein tyrosine phosphorylation in intact cells. *J Biol Chem* 1990;265:2896-2902.
- Lytle C, McManus T: Coordinate modulation of Na-K-2KCl co-transport and K-Cl co-transport by cell volume and chloride. *Am J Physiol Cell Physiol* 2002;283:C1422-31.
- Minetti G, Seppi C, Ciana A, Balduini C, Low PS, Brovelli A: Characterization of the hypertonically induced tyrosine phosphorylation of erythrocyte band 3. *Biochem J* 1998;335:305-11.
- Adragna NC, Lauf PK: Oxidative activation of K-Cl co-transport by Diamide in erythrocytes from humans with red cell disorders, and from several other mammalian species. *J Membr Biol* 1997;155:207-17.
- Giger U, Sticher B, Naef R, Burger R, Lutz HU: Naturally occurring human anti-band 3 autoantibodies accelerate clearance of erythrocytes in guinea pigs. *Blood* 1995;85:1920-1928.
- Turrini F, Mannu F, Cappadoro M, Ulliers D, Giribaldi G, Arese P: Binding of naturally occurring antibodies to oxidatively and nonoxidatively modified erythrocyte band 3. *Biochim Biophys Acta* 1994;1190:297-303.
- Kaji DK, Tsukitani Y: Role of protein phosphatase in activation of KCl co-transport in human erythrocytes. *Am J Physiol* 1991;260:C178-C182.
- Khan MT, Saleemijddin M: Unlike its human counterpart, band 3 anion exchange protein from goat erythrocyte membrane shows a lack of reactivity against various -SH oxidants and protease treatments. *Comp Biochem Physiol Biochem Molec Biol* 1995;110:399-343.
- Lauf PK, Adragna NC: A thermodynamic study of electroneutral K-Cl co-transport in pH- and volume-clamped low K sheep erythrocytes with normal and low internal magnesium. *J Gen Physiol* 1996;108:341-350.
- Lauf PK, Adragna NC: Proton (H⁺) modulation of K-Cl co-transport through both internal and external sites in DIDS-pH-clamped low magnesium LK sheep erythrocytes. *Biophys J* 1997;72:A408.
- Guizouarn H and Motais R: Swelling activation of transport pathways in erythrocytes: effects of Cl⁻, ionic strength, and volume changes. *Am J Physiol Cell Physiol* 1999;276:C210-220.
- Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, Häussinger D: Functional significance of cell volume regulatory mechanism. *Physiol rev* 1998;78:247-306.
- Parker JC, Dunham PB, Minton AP: Effects of ionic strength on the regulation of Na/H exchange and K-Cl co-transport in dog red blood. *J Gen Physiol* 1995;105:677-699.
- Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, Wieder T, Huber SM: Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 2003;10:249-56.
- Kaiserova K, Lakatos B, Peterajova E, Orlicky J, Varecka L: Investigation of properties of the Ca²⁺ influx and of the Ca²⁺-activated K⁺ efflux (Gardos effect) in orthovanadate-treated and ATP-depleted human red blood cells. *Gen Physiol Biophys* 2002;21:429-42.
- Bize I, Guvenc B, Buchbinder G, Brugnara C: Stimulation of human erythrocyte K-Cl co-transport and protein phosphatase type 2A by n-ethylmaleimide: role of intracellular Mg⁺⁺. *J Membr Biol* 2000;177:159-68.
- Teti D, Venza I, Crupi M, Busà M, Loddo S, Romano L: Anion transport in normal erythrocytes, sickle red cells, and ghosts in relation to hemoglobins and magnesium. *Arch Biochem Biophys* 2002;403:149-154.

- 26 Romano L, Peritore D, Simone E, Sidoti A, Trischitta F, Romano P: Chloride-sulphate exchange chemically measured in human erythrocyte ghosts. *Cell Mol Biol* 1998;44:351-355.
- 27 Ciftci M, Ozmen I, Buyukokuroglu ME, Pence S, Kufrevioglu OI: Effects of metamizol and magnesium sulphate on enzyme activity of glucose-6phosphate dehydrogenase from human erythrocyte in vitro and rat erythrocyte in vivo. *Clin Biochem* 2001;34:297-302.
- 28 Raftos JE, Lew VL, Flatman PW: Refinement and evaluation of Mg^{2+} buffering in human red cells. *Eur J Biochem* 1999;263:635-645.
- 29 Raftos JE, Flatman PW: Effect of intracellular magnesium on calcium extrusion by the plasma membrane calcium pump of intact human red cells. *J Physiol* 1995;489:63-72.
- 30 Flatman PW: The effects of magnesium on potassium transport in ferret red cells. *J Physiol* 1988;397:471-487.
- 31 De Franceschi L, Cappellini MD, Graziadei G, Manzato F, Olivieri O, Corrocher R, Fiorelli G, Beuzard Y, Brugnara C: The effect of dietary magnesium supplementation on the cellular G6PDH-deficientities of erythrocytes in patients with beta thalassemia intermedia. *Haematologica* 1998;83:118-25.
- 32 De Franceschi L, Villa-Moruzzi E, Fumagalli L, Brugnara C, Turrini F, Motta R, Veghini E, Corato C, Alper SL, Berton G: K-Cl cotransport modulation by Mg in erythrocytes from mice bred for low and high Mg levels. *Am J Physiol Cell Physiol* 2001;281:C1385-95.
- 33 Ortiz-Carranza O, Adragna NC, Lauf PK: Modulation of K-Cl co-transport in volume-clamped low-K sheep erythrocytes by pH, magnesium, and ATP. *Am J Physiol* 1996;271:C1049-58.
- 34 Fujise H, Higa K, Kanemaru T, Fukuda M, Adragna NC, Lauf PK: GSH depletion, K-Cl cotransport, and regulatory volume decrease in high-K/high-GSH dog red blood cells. *Am J Physiol Cell Physiol* 2001;281:C2003-9.
- 35 Ursini MV, Parrella A, Rosa G, Salzano S, Martini G: Enhanced expression of glucose-6phosphate dehydrogenase in human cells sustaining oxidative stress. *Biochem J* 1997;323:801-806.
- 36 Ciftci M, Kufrevioglu OI, Gundogdu M, Ozmen II: Effects of some antibiotics on enzyme activity of glucose-6phosphate dehydrogenase from human erythrocytes. *Pharmacol Res* 2000;41:107-111.
- 37 Altikat S, Ciftci M, Buyukokuroglu ME: In vitro effects of some anesthetic drugs on enzymatic activity of human red blood cell glucose-6phosphate dehydrogenase. *Pol J Pharmacol* 2002;54:67-71.
- 38 Buyukokuroglu ME, Altikat S, Ciftci M: The effects of ethanol on glucose-6phosphate dehydrogenase enzyme activity from human erythrocytes in vitro and rat erythrocytes in vivo. *Alcohol Alcohol* 2002;37:327-329.
- 39 Cabantchik ZI, Rothstein A: Membrane proteins related to anion permeability of human red blood cells. I localization of disulfonic stilbene binding sites in proteins involved in permeation. *J Membr Biol* 1974;15:207-226.
- 40 Lepke Lepke S, Fasold H, Pring M, Passow H: A study of the relationship between inhibition of anion exchange and binding to the red cell membrane of 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS) and of its dihydroderivative (H_2 DIDS). *J Membr Biol* 1976;29:147-177.
- 41 Flatman PW: The effect of buffer composition and deoxygenation on the concentration of ionized magnesium inside human red blood cells. *J Physiol* 1980;300:19-30.
- 42 Beutler E, Blume KG, Keplan C, Lohr W, Ramat B, Valentine WN: International committee for standardization in haematology. Recommended screening test for glucose-6-phosphatase dehydrogenase (G-6-PD). *Bri J of Haem* 1979;43:469-477.
- 43 Pesarin F: Multivariate Permutation Test. Wiley New York, 2001, pp 37-69.
- 44 Kozlova NM, Luk'ianenko LM, Antonovich AN, Kut'ko AG, Zubritskaia GP, Slobozhanina EI: Effect of diamide on protein oxidation and physico-chemical properties of lipids in erythrocyte membranes. *Biofizika* 2002;47:500-505.
- 45 Lauf PK, Adragna NC: K-Cl co-transport: properties and molecular mechanism. *Cell Physiol Biochem* 2000;10:341-54.
- 46 Adragna NC, Lauf PK: Role of nitrite, a nitric oxidative derivative, in K-Cl co-transport activation of low-potassium sheep red blood cells. *J Membrane Biol* 1998;166:157-167.
- 47 Yu G, Li J, Tian X, Lin H, Wang X: Changes of protein tyrosine phosphorylation in erythrocyte band 3 glucose-6phosphate dehydrogenase deficiency. *Zhonghua Xue Ye Xue Za Zhi* 2002;23:565-567.
- 48 Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T: Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 1995;270:296-299.
- 49 Brugnara C: Erythrocyte membrane transport physiology. *Curr Opin Hematol* 1997;4:122-7.
- 50 De Franceschi L, Bachir D, Galacteros F, Tchernia G, Cynober T, Alper SL, Platt O, Beuzard Y, Brugnara C: Oral magnesium supplements reduce erythrocyte dehydration in patients with sickle cells disease. *J Clin Invest* 1997;100:1847-52.
- 51 De Franceschi L, Brugnara C, Beuzard Y: Dietary magnesium supplementation ameliorates anemia in a mouse model of beta-thalassemia. *Blood* 1997;90:1283-90.
- 52 Brautigan DL: Great expectations: protein tyrosine phosphatases in cell regulation. *Biochim Biophys Acta* 1992;1114:63-77.
- 53 Mauro LJ, Dixon JE: 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address'. *Trends Biochem Sci* 1994;19:151-155.
- 54 Walton KM, Dixon JE: Protein tyrosine phosphatases. *Annu Rev Biochem* 1993;62:101-20.
- 55 Giardina B, Messina I, Scatena R, Castagnola M: The multiple functions of Hemoglobin. *Critical Reviews in Biochemistry and Molecular Biology* 1995;30:165-196.
- 56 Mota de Freitas D, Amari L, Srinivasan C, Rong Q, Ramasamy R, Abbraha A: Competition between Li^+ and Mg^{2+} for the phosphate groups in the human erythrocyte membrane and ATP: an NMR and fluorescence study. *CFGC. Geraldès, MK. Boyd, Biochemistry* 1994;33:4101-4110.