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Hypochlorous acid inhibits glutathione *S*-conjugate export from human erythrocytes

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Abstract

It was found that the hypochlorous acid (HOCl) inhibits the active efflux of glutathione *S*-conjugates, 2,4-dinitrophenyl-*S*-glutathione (DNP-SG, $c_{50\%} = 258 \pm 24$ μM HOCl) and bimane-*S*-glutathione (B-SG, $c_{50\%} = 125 \pm 16$ μM HOCl) from human erythrocytes, oxidises intracellular reduced glutathione (the ratio $[HOCl]/[GSH]_{oxidized} = 4$) and inhibits basal as well as 2,4-dinitrophenol- (DNP) and 2,4-dinitrophenyl-*S*-glutathione (DNP-SG)-stimulated Mg^{2+}-ATPase activities of erythrocyte membranes. Multidrug resistance-associated protein (MRP1) mediates the active export of glutathione *S*-conjugates in mammalian cells, including human erythrocytes. A direct impairment of erythrocyte membrane MRP by hypochlorous acid was shown by electrophoresis and immunoblotting ($c_{50\%} = 478 \pm 36$ μM HOCl). The stoichiometry of the MRP/HOCl reaction was 1:1. These results demonstrate that MRP can be one of the cellular targets for the inflammatory mediator hypochlorous acid.

Keywords: Hypochlorous acid; Multidrug resistance-associated protein; Erythrocyte membrane; Glutathione; Glutathione *S*-conjugate transport

1. Introduction

Hypochlorous acid (HOCl) is a highly reactive biological oxidant and is thought to play an important role in both microbial killing (bactericidal action of phagocytic cells) and inflammatory tissue injury by neutrophils [1,2]. Stimulated neutrophils and monocytes, cells present under inflammatory conditions, produce HOCl in substantial amounts via the myeloperoxidase-catalysed reaction of H_2O_2 with Cl^-. $HOCl/OCl^-$ can be found at concentrations up to 200 μM in some tissues under pathological conditions [3]. HOCl reacts with a wide range of biological target molecules including lipids, proteins and DNA, to form long-lived chloramines on reaction with amine groups. Chloramines are powerful oxidising agents and are thus extremely toxic to mammalian cells [4–6]. HOCl has been implicated in the injury associated with neutrophil accumulation in myocardial reperfusion injury, inflammatory disease and rheumatoid arthritis [1,2,7]. Hypochlorous acid oxidation modifies lipoproteins and this effect is believed to participate in the development of atherosclerotic lesions [8].

Toxic effects of HOCl have been studied using different types of cells [4,6–8]. HOCl can enter cells and the loss of intracellular glutathione is an early event of cell exposure to

Abbreviations: ATP, adenosine-5-triphosphate; B-SG, bimane-*S*-glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenol; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetatic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; GSH, reduced glutathione; GSSG, oxidised glutathione; mBCl, monochlorobimane; MRP, multidrug resistance-associated protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RBC, human red blood cell; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TCA, trichloroacetic acid

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this oxidant as was shown for human erythrocytes [6] and endothelial cells [7]. It has been demonstrated that low sublethal doses of HOCl react with cellular thiols (glutathione and protein thiols) and could modulate specific cell processes in a manner similar to that seen with H_2O_2 or peroxynitrite [7,9]. The plasma membrane has been envisaged as one of the main targets of HOCl in the cell [10,11]. Exposure of human vein endothelial cells to HOCl causes a decrease in cell viability, concentrations of <25 μM HOCl being sublethal [7]. Whereas higher concentrations of hypochlorous acid cause rapid necrosis, lower amounts of this oxidant (20–40 nmol per 1.25×10^5 cells) initiate apoptosis, as demonstrated by Vissers et al. [9] for human umbilical-vein endothelial cells. HOCl induces caspase activity and can modify cellular responses that are dependent on various signal transduction pathways [9].

Glutathione conjugation and active transport from a cell of glutathione *S*-conjugates formed (detoxification phases II and III, respectively) are important steps of the detoxification pathway of many xenobiotics in different cell types [9,12]. Similarly, active export of GSSG is the way of elimination of this potentially deleterious compound [12,13]. It was suggested that the glutathione *S*-conjugates in human erythrocytes are exported via the multidrug resistance-associated protein (MRP1) gene product [14]. Erythrocytes, due to their simple structure and the presence of MRP, are a good model system for investigation of such processes.

It has been demonstrated that peroxynitrite, formed in the reaction of nitric oxide with superoxide and believed to be the main agent responsible for nitric oxide toxicity, damages MRP in the human erythrocytes, effectively inhibits the active efflux of glutathione *S*-conjugates from these cells and inhibits the erythrocyte membrane ATPase activity stimulated by glutathione *S*-conjugates [15].

The aim of the present work was to examine the effect of another highly reactive biological oxidant, hypochlorous acid, on this important cellular transport process, viz. the active export of glutathione-*S*-conjugates, from human red blood cells (RBCs). We used erythrocytes not only as a model system in which some of the effects of hypochlorous acid have been characterized [4,6,10,11,16] (one can suggest similar cellular events produced by HOCl for different kind of cells), but also because these cells may be one of the main targets for neutrophil-derived oxidants, including HOCl, during inflammation. Hypochlorous acid-produced impairments of erythrocytes due to direct interaction between neutrophils and RBCs in circulation can play an important role in the development of the long-term vascular complications in inflammation.

The time course and stoichiometry of intracellular GSH oxidation by HOCl, inhibition of active export of two glutathione *S*-conjugates, changes in the membrane ATPase activity linked to the process of glutathione *S*-conjugate transport, and modification of erythrocyte MRP have been studied.

2. Materials and methods

2.1. Materials

2,4-Dinitrophenol (DNP), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman reagent; DTNB), ouabain, adenosine-5-triphosphate (ATP), ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetatic acid (EDTA), reduced glutathione (GSH), Malachite Green, sodium hypochlorite, phenylmethylsulfonyl fluoride (PMSF) and anti-rat IgG antibodies conjugated with peroxidase were obtained from Sigma (Deisenhofen, Germany). Monochlorobimane (mBCl) was from Molecular Probes (Eugene, USA). 2,4-Dinitrophenyl-*S*-glutathione (DNP-SG) was synthesised from CDNB and glutathione nonenzymatically [17]. Bimane-*S*-glutathione (B-SG) was obtained by nonenzymatic conjugation of mBCl to glutathione [18]. HBS cellulose was from Serva (Heidelberg, Germany). Immobilon-P was from Millipore. Anti-human MRP rat monoclonal antibodies (clone MRP r1) were from Kamiya Biomedical Company. All other chemicals were from POCh (Poland) and were of analytical grade. All solutions were made with water purified by the Milli-Q system (Millipore) having a resistivity of 18.4 MΩ cm^{-2}.

Human erythrocytes were obtained from fresh blood of healthy donors anticoagulated with citrate. Erythrocytes were isolated by centrifugation at 4 °C, $2000 \times g$ and purified by three cycles of resuspension and washing with 20 volumes of PBS (150 mM NaCl + 10 mM phosphate buffer, pH 7.4) containing 1 mM EDTA and 0.5 mM PMSF. After careful removal of the buffy coat, residual leukocytes were removed by passing erythrocyte suspensions through a column of HBS cellulose. Erythrocyte ghosts for the determination of ATPase activity were prepared from washed cells according to a modification of the method of Dodge et al. [19] consisting the borate buffer instead of the phosphate buffer (20 mM, pH 7.4) and containing 1 mM EDTA and 0.5 mM PMSF as proteolytic inhibitors during haemolysis process.

2.2. Exposure of erythrocytes and erythrocyte ghosts to hypochlorite

Stock solution of sodium hypochlorite was diluted three times with PBS (or with 100 mM borate buffer, pH 7.4, for determination of inhibition of ATPase activity). The final concentration of hypochlorite (OCl^-) was about 30–35 mM and before each experiment was estimated spectrophotometrically using an absorbance coefficient of $\varepsilon = 350\ M^{-1}\ cm^{-1}$ at 292 nm in 0.1 M NaOH [20]. Erythrocytes obtained as described above (preincubated with CDNB or monochlorobimane if for transport experiments) were and resuspended in appropriate buffer at haematocrits of 5%, 10%, or 20% were added with different concentrations of hypochlorite and examined for glutathione *S*-conjugate

efflux or for GSH content. For the estimation of GSH content, RBCs after incubation with hypochlorite were washed by excess of cold PBS.

For the assay of glutathione *S*-conjugate-stimulated ATPase activity, erythrocyte ghosts (protein concentration of 3 mg/ml) were added with different concentrations of hypochlorite in 5 mM borate buffer, pH 7.4, for 10 min and enzymatic activity was estimated as described below. For analysis of MRP content, erythrocyte ghosts (protein concentration of 3 mg/ml) were added with different concentrations of hypochlorite for 10 min.

2.3. Measurement of the transport of glutathione S-conjugates

Transport of 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) was measured according to the procedure of Board [21]. Briefly, washed erythrocytes were resuspended in phosphate-buffered saline (PBS) and incubated with 1 mM CDNB for 15 min at 37 °C to form intracellular 2,4-dinitrophenyl-*S*-glutathione. Then, the cells were washed for the removal of the excess of CDNB at 0 °C and suspended at a haematocrit of 20% in the transport buffer containing 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 1 mg/ml glucose, pH 7.4. Initial concentration of DNP-SG in so treated cells was 1.65 ± 0.16 mmol/l cells (mean ± S.D.; $n = 5$). The cell suspensions were then incubated at 37 °C, treated with hypochlorite and the export of DNP-SG was quantified by withdrawal of aliquots of the cell suspensions after "zero-time" (about 10 s), 10, 20 and 30 min, centrifugation and estimation of absorbance of the conjugate at 340 nm in the supernatants [21]. Haemolysis was negligible; however, in order to exclude interference from absorbance by the released haemoglobin, the supernatants were added with an equal amount of 10% trichloroacetic acid (TCA), centrifuged and absorbance of DNP-SG was read in so obtained extracts. In order to make a correction for DNP-SG released from cells, which haemolysed during the experiment, absorbance of the supernatants at 540 nm was read to estimate percent of haemolysis and the amount of DNP-SG liberated with haemoglobin was calculated on the basis of haemoglobin and DNP-SG content of cells at time zero. The correction did not exceed 5% for the lower concentrations of hypochlorite, and about 10% for 750 μM HOCl.

Transport of B-SG through erythrocyte membranes was made as described previously [18] with some modifications. Briefly, human erythrocytes at the haematocrit of 50% in the transport buffer were incubated for 5 min with 10 μM mBCl (final concentration, mBCl was added from a stock solution of 5 mM in ethanol) at room temperature. Then they were suspended at a haematocrit of 5% (for some experiments of 10%, 15% and 20%) in the transport buffer, incubated at 37 °C and treated with HOCl. Initial intracellular concentration of B-SG was 14.7 ± 2.8 μmol/l cells. Aliquots of the suspensions were withdrawn after zero-time, 3, 6, 9, 12 and 15 min, centrifuged and the supernatant was added with an equal volume of ice-cold 10% TCA for haemoglobin removal. Haemolysis was checked and it was always below 5%. Nevertheless, a correction of each fluorescence value for B-SG released by haemolysis (based on the determination of the amount of released haemoglobin from absorbance at 540 nm) was made. Fluorescence of the supernatants was measured in a Perkin-Elmer LS-5B fluorescence spectrophotometer at excitation wavelength of 386 nm and emission wavelength of 476 nm [18].

2.4. Determination of the glutathione S-conjugate-stimulated ATPase activity

ATPase activity was assayed as described elsewhere [22]. Briefly, erythrocyte membranes were incubated in the assay medium consisting of 100 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$, 2 mM ATP, 1 mM EGTA (to inhibit Ca^{2+}-ATPase) and 0.1 mM ouabain (to inhibit Na^+, K^+-ATPase) for 30 min at 37 °C in the absence of other additives or in the presence of 4 mM DNP-SG or 1 mM DNP. DNP was demonstrated to stimulate the activity of erythrocyte membrane Mg^{2+}-ATPase even better then DNP-SG [22,23]. The differences in activities in the presence and in the absence of the stimulants were referred to as the DNP-SG-dependent and DNP-dependent ATPase activities, respectively. The inorganic phosphate liberated was quantified with Malachite Green [24]. Protein was measured according to the method of Lowry et al. [25].

2.5. GSH content

The concentration of GSH was determined by the method of Ellman [26], using the absorption coefficient 13.6 mM^{-1} cm^{-1}.

2.6. Electrophoresis and immunoblotting

For electrophoresis, the erythrocyte ghosts (control and treated with hypochlorite) were solubilized with 3% SDS, 50 mM Tris–HCl buffer (pH 6.8) and 200 mM dithiothreitol (final concentration). After incubation at 37 °C for 30 min, an aliquot of the mixture containing 25 μg of protein was subjected to one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system of Laemmli [27]. The proteins bands from SDS-PAGE gels were transferred to an Immobilon P membrane according to the method of Towbin et al. [28]. After blocking with 5% nonfat milk, the rat anti-MRP antibodies (at a dilution of 1:2000), and the second anti-rat IgG (whole molecule)-peroxidase conjugated antibody at a dilution of 1:2000 were used. The immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) technique, according to the instructions of the manufacturer (Amersham). Densitometric analysis of the MRP was

performed with a computer-assisted Desaga CD-60 densitometer (Heidelberg, Germany).

All given values are means (± S.D.) of four to six independent experiments.

3. Results

3.1. Oxidation of cellular glutathione

We have studied the kinetic parameters of glutathione oxidation at the haematocrit used in the glutathione *S*-conjugate transport experiments. Exposure of RBCs to HOCl resulted in effective dose- and time-dependent oxidation of intracellular reduced glutathione (Fig. 1). At high (more than 0.4 mM) HOCl concentrations, the reaction required less then 1 min for reaching the equilibrium. At 0.8 mM HOCl in the case of 20% haematocrit, complete GSH oxidation took place. At this HOCl concentration, some recovery of GSH at longer time of cell incubation was observed.

3.2. Inhibition of glutathione S-conjugate transport

Treatment of RBCs with hypochlorous acid significantly decreased the rate of extrusion of DNP-SG (Fig. 2) and B-SG (Fig. 3) from the cells in a concentration-dependent manner. Figs. 2 and 3 show the time course of DNP-SG and B-SG efflux for native and HOCl-treated RBCs. Initial transport rates during first 3 min after HOCl addition to the RBCs were compared because nonlinearity of the time course of B-SG efflux, in contrast to DNP-SG efflux (Figs. 2 and 3) was observed, in agreement with previous observations [15]. Hypochlorous acid concentration needed for 50% inhibition of DNP-SG efflux was 258 ± 24 μM while

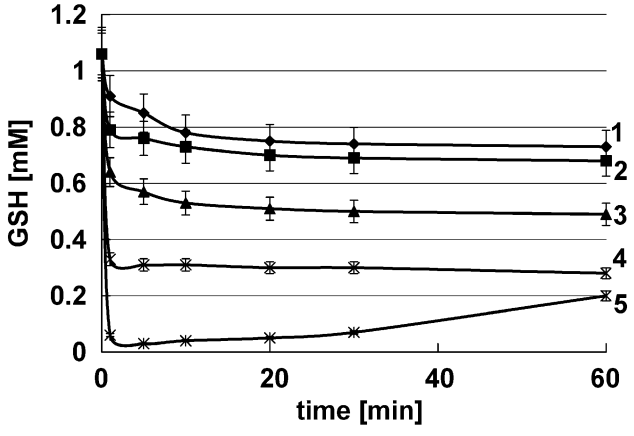

Fig. 1. Time course of intracellular GSH oxidation during RBC exposure to 100 μM (1), 200 μM (2), 400 μM (3), 600 μM (4) and 800 μM (5) hypochlorite. Cells at a haematocrit of 20% were suspended in PBS, pH 7.4, at 37 °C and different HOCl concentrations were added. Data presented as mean ± S.D. (n = 3–6); in some cases, S.D. is lower than the data points.

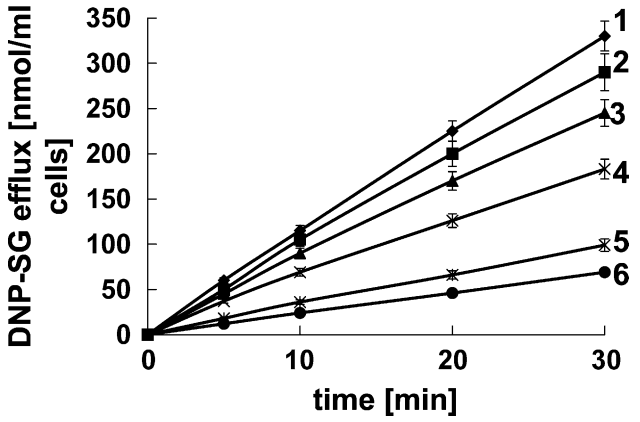

Fig. 2. DNP-SG efflux from human erythrocytes in the absence (1) and in the presence of 50 μM (2), 100 μM (3), 200 μM (4), 500 μM (5) and 750 μM (6) hypochlorite. Erythrocytes (20% haematocrit), preloaded with CDNB were added with hypochlorite and incubated at 37 °C. Data presented as mean ± S.D. (n = 3–6); in some cases, S.D. is lower than the data points.

that for 50% inhibition of B-SG efflux was 125 ± 16 μM. (For studies of DNP-SG transport, RBCs were exposed to HOCl at 20% haematocrit and for studies of B-SG transport, at 5% haematocrit so the sensitivities of the transport of the two conjugates cannot be directly compared). In the case of B-SG transport, we observed some recovery-type increase of transport rate during longer cell incubation after treatment with higher doses of HOCl (Fig. 3). A similar phenomenon has been previously observed after exposure of erythrocytes to peroxynitrite [15].

3.3. Inhibition of stimulated Mg^{2+}-ATPase activities of the erythrocyte membranes

The activity of the glutathione *S*-conjugate transporter in the erythrocyte membrane is reflected by DNP- and DNP-SG- stimulated ATPase activities of the membrane. RBC

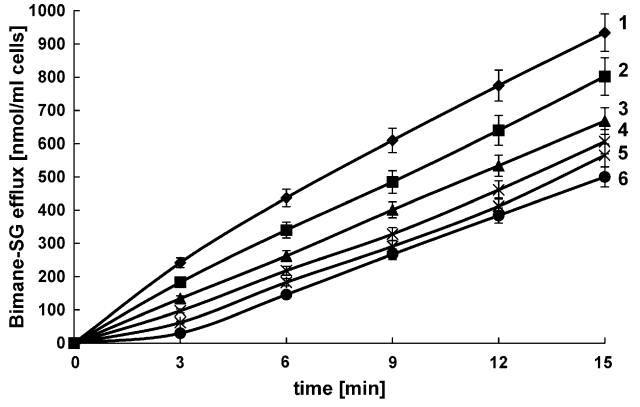

Fig. 3. B-SG efflux from human erythrocytes in the absence (1) and in the presence of 50 μM (2), 100 μM (3), 200 μM (4), 500 μM (5) and 750 μM (6) hypochlorite. Erythrocytes, preincubated at 50% haematocrit with monochlorobimane, were suspended at a haematocrit of 5%, treated with hypochlorite and incubated at 37 °C. Mean ± S.D. (n = 3–6).

Table 1
Effect of hypochlorite on the basal Mg^{2+}-ATPase and ATPase activity stimulated by DNP-SG and DNP, and on the RBC membrane potential

Hypochlorite concentration	0 (control)	50 μM	100 μM	200 μM	500 μM	750 μM
Mg^{2+}-ATPase	187 ± 13 (=100%)	159 ± 7 (=85%)	130 ± 18 (=70%)	111 ± 8 (=59%)	52 ± 4 (=28%)	36 ± 11 (=19%)
DNP-SG-stimulated activity	71 ± 19 (=100%)	61 ± 12 (=86%)	35 ± 11 (=49%)	10 ± 5 (=14%)	11 ± 5 (=15%)	5 ± 3 (=7%)
DNP-stimulated activity	178 ± 13 (=100%)	150 ± 11 (=84%)	144 ± 16 (=81%)	113 ± 8 (=63%)	54 ± 12 (=30%)	34 ± 8 (=19%)
RBC membrane potential	− 13.1 ± 0.6	− 13.2 ± 0.9	− 13.2 ± 0.8	− 12.6 ± 0.8	− 12.1 ± 0.7	n.d.

ATPase activity expressed in nmol P_i/(mg protein × h); erythrocyte ghosts (3 mg protein/ml) were incubated with hypochlorite. DNP (DNP-SG)-stimulated ATPase activity = activity measured in the presence of DNP (DNP-SG) minus activity measured without the stimulant. RBC membrane potential expressed in mV; RBCs (10% haematocrit) in PBS, pH 7.4 were treated with different HOCl concentrations for 10 min at 22 °C and washed before measurement. Mean ± S.D., $n=3$.

membrane exposure to HOCl resulted in a dose-dependent inhibition of the basal Mg^{2+}-ATPase activity as well as DNP-SG- and DNP-stimulated Mg^{2+}-ATPase activities. Hypochlorous acid concentration needed for the 50% inhibition of the basal Mg^{2+}-ATPase activity, the DNP-SG-stimulated Mg^{2+}-ATPase activity and the DNP-stimulated activity were 245 ± 26, 172 ± 14 and 255 ± 28 μM, respectively. Table 1 shows the effect of the hypochlorite on the basal Mg^{2+}-ATPase activity and the DNP-SG- and DNP-stimulated activities.

3.4. Changes of erythrocyte membrane potential

Because of the anionic nature of the conjugates, we have studied the changes of RBC membrane potential as a possible mechanism of HOCl influence on the glutathione *S*-conjugate transport. Even at high oxidant concentrations inducing cell haemolysis, we did not observed any significant changes of the membrane potential (Table 1).

3.5. Immunological identification of MRP1 in hypochlorous acid-treated RBC membranes

Using the method of immunoblotting with rat anti-MRP1 serum, MRP1 was detected in electrophoregrams of RBC membranes in the 200 kDa molecular weight range before

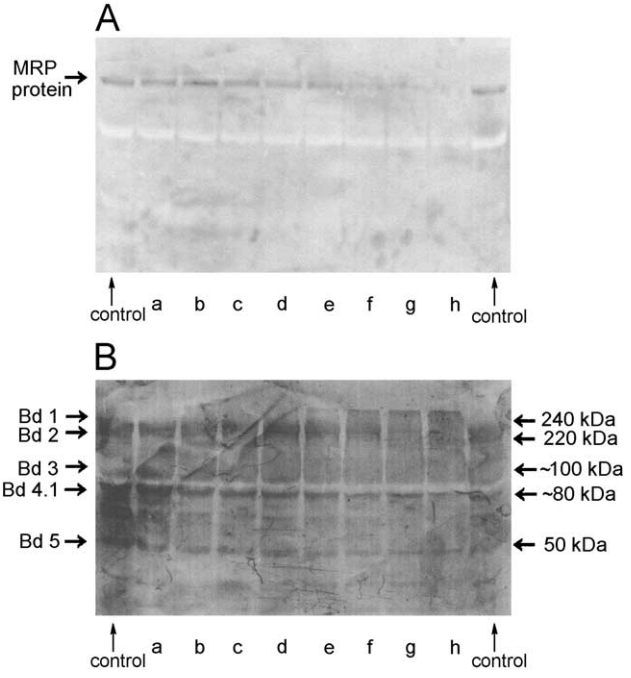

Fig. 4. Analysis of MRP content in the erythrocyte membranes treated with hypochlorite: (A) Immunoblotting of the control RBS membranes and membranes treated with 50 μM (a), 100 μM (b), 200 μM (c), 300 μM (d), 500 μM (e), 750 μM (f), 1000 μM (g) and 1500 μM hypochlorite (h) using anti-MRP monoclonal antibodies. (B) The same Immobilon sheet was stained for protein identification with Sudan Black. The main RBC membrane proteins and their molecular masses are indicated.

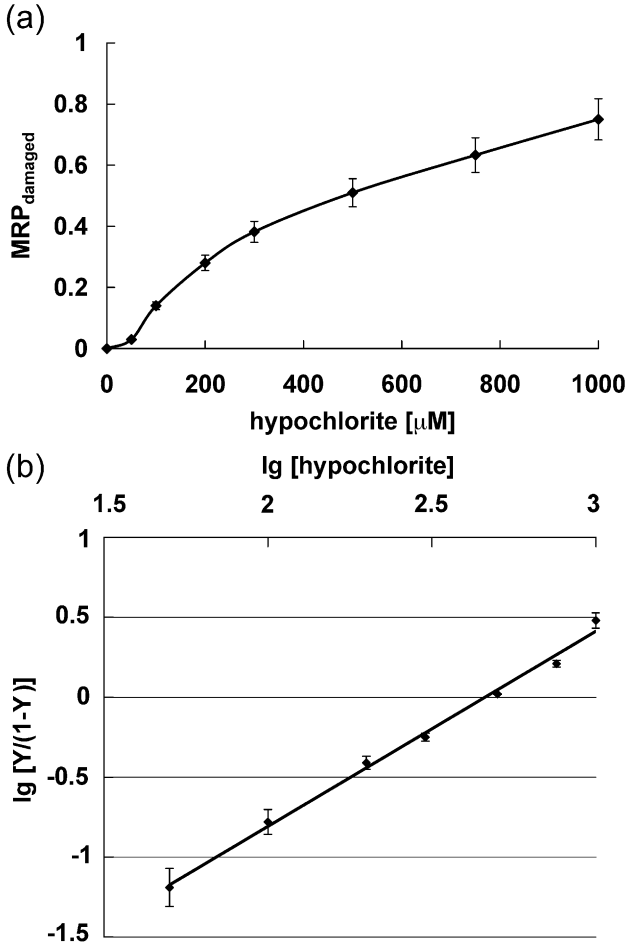

Fig. 5. Decrease of the content of immunoreactive MRP after RBC membrane treatment with hypochlorite: (a) Dependence of immunoreactive MRP band area on HOCl concentration. (b) The same dependence, linearised according to the Hill equation.

and after membrane exposure to HOCl (Fig. 4A). Simultaneously, the main erythrocyte membrane proteins were identified on the immunoblots (Fig. 4B). Cell membrane treatment with hypochlorite resulted in a significant disappearance of the immunoreactive MRP1 (Figs. 4A and 5). Fig. 5a shows densitometric analysis of the MRP1 content in the RBC membranes after exposure to hypochlorite. At the same time, disappearance of the main membrane proteins was also observed (Fig. 4B).

4. Discussion

ATP-dependent glutathione *S*-conjugate export from human erythrocytes has two kinetic components, one of high affinity ($K_m = 7.4 \pm 0.2$ μmol/ml cells, in the case of B-SG transport) and low capacity and another of low affinity ($K_m = 242 \pm 8$ μmol/ml cells for B-SG) and high capacity [18]. Recently, the MRP1 gene product was identified as an ATP-dependent export pump for glutathione *S*-conjugates in human erythrocytes as well as in the other cell types [14,29–32]. MRP1 is a membrane glycoprotein of apparent molecular mass of 190 kDa, belonging to the ATP-binding cassette (ABC) protein family [29,31,33].

In the present study, we investigated whether modification of erythrocyte membrane MRP by hypochlorous acid may lead to inhibition of glutathione *S*-conjugate transport across the membrane. The first step of our work was the investigation of GSH oxidation (GSH is the substrate for the conjugation reaction and GSSG formed by GSH oxidation is also the substrate for the transporter). It is known that HOCl easily penetrates the red cell membrane and highly selectively oxidizes intracellular GSH [6]. In agreement with the observation of Vissers and Winterbourn [6], we found that the loss of GSH is a very fast process and requires less than 1 min for attaining equilibrium (Fig. 1). We observed some recovery of GSH after cell exposure to oxidant (Fig. 1, curve 5) due to activity of cell GSSG reductase. At high concentrations of HOCl (higher than 0.2 mM), the ratio $[HOCl]/[GSH]_{oxidised} = 4$, i.e. four molecules of HOCl were required for oxidation of one molecule of intracellular GSH under these conditions. At lower oxidant concentrations, this ratio was lower (two molecules of HOCl per molecule of GSH at 0.1 mM HOCl). Earlier, it was calculated that oxidation of intracellular GSH required approximately 2.5 mol of HOCl per mol of GSH lost [6]. Thus, the ratio $[hypochlorite]/[GSH]_{oxidised}$ increases with the increase of HOCl concentration. It was suggested by Prutz [34] that one molecule of HOCl reacts with the terminal α-amino-group of GSH and three molecules of HOCl react with the SH-group.

Different GSH-oxidizing agents (H_2O_2, *t*-butyl hydroperoxide, phenazine methosulfate and nitrite), have been shown to stimulate GSSG export from human erythrocytes [13]. No relation between the ability of these agents to stimulate GSH oxidation and GSSG export was observed, and the sensitivity of the transporter itself to the action of the oxidants (thiol-modifying agents) was suggested [13]. Treatment of RBCs with HOCl brings about different interconnected processes: effective intracellular GSH oxidation, stimulation of GSSG export (observed previously for other oxidative agents [13]), as well as modification and inhibition of the conjugate export pump, i.e. MRP1.

We have shown that hypochlorite treatment of RBCs did not alter the membrane potential (Table 1) and thus cannot influence the transport of anionic conjugates by changing charge distribution at the membrane. The lack of detectable changes in membrane potential can be explained by the compensation of potassium leakage through the damaged membrane by sodium influx. Such a situation may take place if membrane pores formed by HOCl treatment are not cation selective. Previously, we have shown that RBC treatment by another oxidative agent, organic hydroperoxide, resulted in considerable membrane hyperpolarization due to intensive potassium leakage [35].

The transport of both substrates studied, DNP-SG and B-SG, was inhibited by HOCl, that of B-SG being more prone to inhibition. Measurements of the DNP-SG efflux from erythrocytes given millimolar concentrations of CDNB allow for characterisation of the low-affinity, high-capacity transport component while monitoring the B-SG efflux from cells treated with micromolar concentrations of monochlorobimane provides an insight into the activity of the high-affinity, low-capacity component. Like in the case of inhibition of glutathione *S*-conjugate transport by peroxynitrite [15], one can propose that hypochlorous acid: (i) modulates the glutathione *S*-conjugate pump (i.e. MRP1) itself, (ii) causes GSH oxidation, and oxidised glutathione inhibits glutathione *S*-conjugate pump as a competitive substrate. We did not observe any significant lipid peroxidation produced by HOCl treatment (data not shown). However, HOCl can produce lipid chlorohydrins [4] and in this way disturb membrane lipid bilayer and affect in turn the glutathione *S*-conjugate pump in the membrane.

Some organic anions, notably DNP, were shown to stimulate membrane ATPase activity, ascribed by some authors to the glutathione *S*-conjugate transporter [23]. Mg^{2+}-ATPase activity stimulated by DNP-SG is more sensitive to hypochlorous acid in comparison to basal or DNP-stimulated ATPase activities. This is in agreement with our previous results on different sensitivity of both these activities to radiation inactivation [37]. Glutathione *S*-conjugate pump is known to have sulfhydryl group(s) essential for its activity [18,30,33,36]. Thus, modification of vital sulfhydryl group(s) of MRP by HOCl may inhibit active transport of glutathione *S*-conjugates. We observed differences in the sensitivity of B-SG and DNP-SG transport to the action of HOCl. Earlier, we have shown that RBC membrane treatment with HOCl resulted in a considerable inhibition of Mg^{2+}- and Na^+-, K^+-ATPase activities (responsible for other very important cell transport process), apparently due to protein thiol group modification [16].

It was shown by the method of radiation inactivation that two components of 2,4-dinitrophenyl-*S*-glutathione transport across the erythrocyte membrane (high and low affinity), as well as DNP-SG-stimulated ATPase activity, correspond to the MRP protein(s), and suggested that MRP1 protein exist in erythrocyte membrane as a dimer (the molecular masses of functional units were 437 ± 69 kDa for high-affinity component and 466 ± 67 kDa for low-affinity component) [37]. It was suggested that the DNP-stimulated activity might be linked to a different protein, perhaps aminophospholipid translocase [37]. Electrophoresis and immunoblotting of MRP1 in RBC membrane after exposure to hypochlorite by the immunoprecipitation showed a considerable impairment of this protein by HOCl (Figs. 4A and 5a,b). The decrease of the amount of immunoreactive MRP1 (the apparent molecular mass is in the range 200 kDa) in erythrocyte membranes paralleled the decrease of the content of main RBC membrane proteins (Fig. 4A,B). Earlier, Vissers et al. [11] showed irreversible cross-linking of band 3 protein as well as all other major membrane proteins by RBC membrane exposure to HOCl using SDS/PAGE.

The curve describing destruction of the MRP molecules in the membrane on the HOCl concentration has a sigmoidal form (Fig. 5a). The dependence of MRP degradation on oxidant concentration was presented as a Hill plot of $\log [Y/(1-Y)]$ versus $\log$ [HOCl], where Y is the fraction of the destroyed MRP (i.e., the degree of saturation of HOCl-binding sites on the MRP molecule) (Fig. 5b). The slope of the Hill plot (the Hill coefficient n) characterizes the cooperativity of erythrocyte membrane MRP modification by HOCl. If the cooperativity is full, the Hill coefficient equals to the number of ligand-binding sites. The Hill plot intersects the abscissa axis at the half-saturating concentration of the oxidant (ligand), which reflects the affinity of the protein for the modifier. The Hill coefficient calculated is equal to $n = 1.1$ (Fig. 5b). Thus, one molecule of hypochlorite modified one unit of MRP and the half-saturating concentration of hypochlorite (the concentration of hypochlorite, needed for 50% modification of MRP) was $c_{50\%} = 478 \pm 45$ μM. This value is higher than that needed for 50% inhibition of the stimulated ATPase activities, which may be due to the fact that the disappearance of MRP is due to its crosslinking to other membrane proteins rather than specific alteration of the epitope by HOCl. This value is also higher than that required for inhibition of glutathione *S*-conjugate efflux but these values cannot be directly compared as the conditions of exposure were different (isolated membranes vs. whole cells).

Our results demonstrate therefore that the glutathione *S*-conjugate pump can be one of the cellular targets for hypochlorous acid and modification of MRP in the erythrocyte membrane can inhibit the transport of such conjugates and GSSG, perpetuating the oxidative stress induced by this oxidant.

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