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Peroxynitrite inhibits glutathione *S*-conjugate transport

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Abstract

Peroxynitrite was demonstrated to inhibit the active efflux of glutathione *S*-conjugates (2,4-dinitrophenyl-*S*-glutathione and bimane-*S*-glutathione) from human erythrocytes and the erythrocyte membrane ATPase activity stimulated by glutathione *S*-conjugates. As the multidrug resistance-associated protein (MRP) is responsible for the transport of glutathione *S*-conjugates in mammalian cells, these results point to the possibility of the effect of peroxynitrite on the MRP function.

Keywords: Erythrocyte; Membrane; Transport; Glutathione *S*-conjugate; Oxidative stress; Peroxynitrite; Multidrug resistance-associated protein

1. Introduction

Peroxynitrite (oxoperoxonitrate(-1)), formed in the reaction between nitric oxide and superoxide is thought to be the main agent responsible for nitric oxide toxicity. It is believed to contribute to the bactericidal action of the phagocytes [1,2] and be the major compound responsible for the ischaemia-reperfusion injury [3–5] and tissue damage by inflammation [6–8]. Peroxynitrite is known to damage nucleic acids [9,10] and proteins [11–13], and to induce lipid peroxidation [14,15]. Nitration of tyrosine residues by peroxynitrite may disturb signal transduction pathways, especially those involving tyrosine kinases [16,17]. Its low concentrations have apoptotic while higher concentrations a necrotic action on mammalian cells [18,19]. We found that the action of peroxynitrite on the erythrocyte causes oxidation of intracellular glutathione, peroxidation of membrane lipids, aggregation and nitration of membrane proteins and inactivation of acetylcholinsterase though not a significant hemolysis [20].

Peroxynitrite affects also cellular transport processes. This compound decreases the Na^+ transport in rabbit alveolar type II cells by damaging apically located amiloride-sensitive Na^+ channels [21] and inhibits erythrocyte membrane Ca^{2+}-ATPase, Mg^{2+}-ATPase and Na^+/K^+-ATPases [20] and high affinity glutamate transporters from rat brain reconstituted in liposomes [22]. Peroxynitrite inhibits mitochondrial respiration [23] strongly depressing complex I- and complex II-dependent mitochondrial oxygen consumption and activities of succinate dehydrogenase and mitochondrial ATPase [24]. Exposure of mitochondria to higher doses of peroxynitrite induces

Abbreviations: ATPase, adenosine triphosphatase; B-SG, bimane-*S*-glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N,N,N′,N′*-tetraacetic acid; GSH, glutathione; mBCl, monochlorobimane; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid

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depolarisation and cyclosporin-A-sensitive calcium efflux [25].

The aim of the present paper was to examine the effect of peroxynitrite on another important transport process, extensively studied recently viz. the active transport of glutathione *S*-conjugates.

2. Materials and methods

2.1. Materials

2,4-Dinitrophenol (DNP), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), ouabain, adenosine-5-triphosphate (ATP), ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetatic acid (EDTA), reduced glutathione (GSH), Malachite Green and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (Deisenhofen, Germany). Monochlorobimane (mBCl) was from Molecular Probes (Eugene, USA). 2,4-Dinitrophenyl-*S*-glutathione (DNP-SG) was synthesized from CDNB and glutathione non-enzymatically [26]. HBS cellulose was from Serva (Heidelberg, Germany). 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}.

Fresh human blood were obtained from blood of healthy donors anticoagulated with citrate. Erythrocytes were isolated by centrifugation at 4°C, 2000 × *g* and purified by three cycles of resuspension and washing with 20 volumes of phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetate (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (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 determination of ATPase activity were prepared from washed cells according to a modification of the method of Dodge et al. [27]. Briefly, erythrocytes were hemolysed on ice with 20 volumes of 20 mM borate buffer (pH 7.6), containing 1 mM EDTA and 0.5 mM PMSF as proteolytic inhibitors, and centrifuged for 20 min at 4°C at 20 000 × *g*. The ghosts were resuspended in ice-cold 5 mM borate buffer (pH 7.6) and 0.1 mM EDTA, and this process was continued until the ghosts were free from residual hemoglobin.

2.2. Exposure of erythrocytes and erythrocyte ghosts to peroxynitrite

Peroxynitrite was synthesized according to the method of Pryor et al. [28]. This method provides peroxynitrite of low ionic strength that does not contain hydrogen peroxide as an impurity. Briefly, an ozone stream from an ozonator (75 μg/ml in oxygen, 100 ml/min) was bubbled through a glass-frit into 100 ml of 0.1 M sodium azide in water (pH adjusted previously to 12 with 1 N NaOH) chilled to 0°C in an ice-water mixture for about 60 min. An ATO-3 medical ozonator (Metrium-Krio, Warsaw, Poland) was used. Peroxynitrite formation was monitored spectrophotometrically during the synthesis and the main sample was collected about 5 min after obtaining the maximum concentration. So obtained peroxynitrite solution contains about 1 mM sodium azide [28]. The final concentration of peroxynitrite was about 30–35 mM. Stock solutions were stored at −25°C and used within 3–4 weeks after synthesis. Before each experiment the concentration of peroxynitrite was estimated spectrophotometrically at 302 nm in 0.1 M NaOH ($\epsilon_M = 1670$). Erythrocytes obtained as above at the hematocrits of 5, 10, 15 or 20% resuspended in appropriate buffer (or preincubated with CDNB or monochlorobimane) were treated with peroxynitrite and examined for glutathione-*S*-conjugate efflux or for GSH content. The highest dose of peroxynitrite used did not affect the DNP-SG absorbance and diminished the fluorescence of B-SG by less than 10%. For the assay of glutathione *S*-conjugate-stimulated ATPase activity erythrocyte ghosts (protein concentration of 4 mg/ml) were treated with peroxynitrite and enzymatic activity was estimated as described below. To check for the potential effect of the products of peroxynitrite decomposition, peroxynitrite was allowed to decompose for 30 min in the phosphate buffer (pH 7.4) before the addition of erythrocytes.

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 et al. [29]. Washed erythrocytes were resuspended in phosphate-buffered saline (PBS) and incubated with 1 mM CDNB for 15 min at 37°C to form 2,4-dinitrophenyl-*S*-glutathione. Then the cells were washed of excess CDNB at 0°C and suspended at a hematocrit 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 peroxynitrite 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 [29]. Hemolysis was negligible; however, in order to exclude interference from absorbance by the released hemoglobin, the supernatants were added with an equal amount of 10% trichloroacetic acid (TCA), centrifuged and absorbance of DNP-SG read in so obtained extracts. In order to make a correction for DNP-SG released from cells which hemolysed during the experiment, absorbance of the supernatants at 540 nm was read to estimate per cent of hemolysis and the amount of DNP-SG liberated with hemoglobin was calculated on the basis of hemoglobin and DNP-SG content of cells at time zero. The correction did not exceed 2%.

Transport of bimane-*S*-glutathione through erythrocyte membranes was made as described previously [30] with some modifications. Briefly, human erythrocytes at the hematocrit 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 hematocrit of 5% (for some experiment of 10, 15 and 20%) in the transport buffer and incubated at 37°C. 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 supernatant was added with an equal volume of ice-cold 10% TCA for hemoglobin removal. Hemolysis was checked and it was always below 1%. Nevertheless, a correction of each fluorescence value for BSG released by hemolysis (based on the determination of the amount of released hemoglobin from absorbance at 540 nm) was made. Fluorescence of the supernatants were measured in a Perkin-Elmer LS-5B fluorescence spectrophotometer at excitation wavelength of 386 nm and emission wavelength of 476 nm [30].

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

ATPase activity was assayed as described elsewhere [31]. 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 glutathione *S*-conjugate pump even better then DNP-SG [31,32]. The differences in activities in the presence and in the absence of the stimulators was referred to as the DNP-SG-dependent and DNP-dependent ATPase activities, respectively. The inorganic phosphate liberated was quantitated with Malachite Green [33]. Protein was measured according to Lowry et al. [34].

2.5. GSH content

The concentration of GSH was determined by the method of Ellman [35]. Briefly, 2-ml aliquots of control and peroxynitrite-treated red cell suspensions were added with 0.2 ml of 50% TCA and centrifuged. To 1 ml of the supernatants 1 ml of 1 M phosphate buffer (pH 7.4) and 0.1 ml of the Ellman reagent (1 mM) were added. Concentration of GSH was determined spectrophotometrically at 412 nm using the absorption coefficient 13.6 mM^{-1} cm^{-1}.

3. Results

Exposure of erythrocytes to peroxynitrite decreased the rate of extrusion of DNP-SG (Fig. 1, Table 1) and B-SG (Fig. 2, Table 2). However, while the time course of DNP-SG efflux showed a considerable linearity (Fig. 1), that of B-SG efflux was not linear. In control cells, the transport rate decreased in time. Initial transport rate decreased in peroxynitrite-

Table 1
Rate of DNP-SG efflux (nmol/(ml · h)) from erythrocytes (20% hematocrit) treated with peroxynitrite

Peroxynitrite concentration	Control	300 μM	500 μM	700 μM	1 mM	1.5 mM
DNP-SG efflux (nmol/(ml cells · h))	578.5 ± 23.2	508.3 ± 30.0	452.5 ± 27.9	404.5 ± 23.5	358.8 ± 21.5	265.0 ± 12.2

Mean ± S.D., $n = 4$.

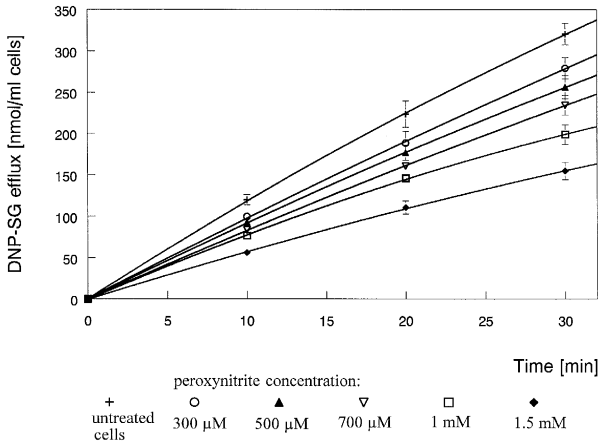

Fig. 1. DNP-SG efflux from human erythrocytes in the absence and in the presence of peroxynitrite (300 μM–1.5 mM). Erythrocytes (20% hematocrit), preloaded with CDNB were added with peroxynitrite and incubated at 37°C. Data presented as mean ± S.D. ($n = 4$).

treated cells as a function of peroxynitrite concentration but the time-dependent decrease became progressively smaller in cells treated with increasing doses of peroxynitrite until in cell treated with the highest doses there was a recovery-type increase in the transport rate during incubation (Fig. 2, Table 2). Therefore, initial transport rates measured during first 3 min of incubation were compared.

Peroxynitrite concentration needed for 50% inhibition of the rate of DNP-SG efflux was 1.31 ± 0.22 mM while that for 50% inhibition of B-SG efflux was 0.39 ± 0.09 mM (mean ± S.D.). It cannot be concluded on these basis that the B-SG transport is more sensitive to peroxynitrite than the DNP-SG transport since the transport of both substrates was studied in cells exposed to peroxynitrite at different hematocrits (20% and 5%, respectively). However, comparison of $c_{50\%}$ values for both compounds at the same hematocrit values demonstrates that it is indeed the case (Table 3).

We have demonstrated previously that peroxynitrite brings about a transient oxidation of erythrocyte glutathione [20]. In this study we confirmed this effect under conditions of measurement of B-SG transport (Table 4). The recovery of reduced glutathione observed upon incubation of peroxynitrite-treated cells seemingly coincides with the time-dependent increase of the B-SG extrusion rate (Fig. 2) and apparently contributes to this effect.

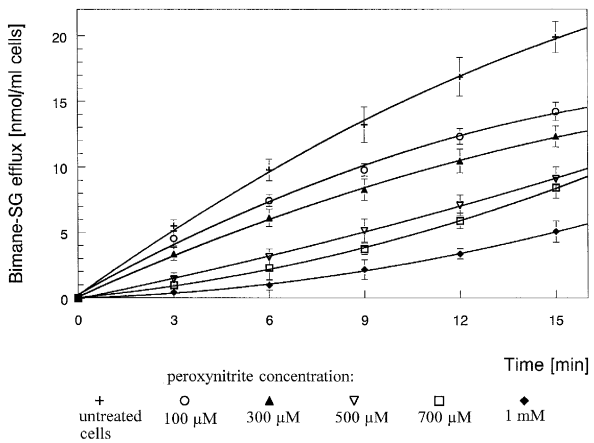

Fig. 2. Bimane-*S*-glutathione efflux from human erythrocytes in the absence and in the presence of peroxynitrite (300 μM–1.5 mM). Erythrocytes, preincubated at 50% hematocrit with monochlorobimane were suspended at a hematocrit of 5%, treated with peroxynitrite and incubated at 37°C. Mean ± S.D. ($n = 3$–8).

Table 2
Initial rate of bimane-*S*-glutathione efflux (nmol/(ml · h)) from erythrocytes (5% hematocrit) treated with peroxynitrite

Peroxynitrite concentration	Control	100 μM	300 μM	500 μM	700 μM	1 mM
B-SG efflux (nmol/(ml cells · h))	110.0 ± 10.3	91.0 ± 12.0	61.0 ± 5.6	30.4 ± 5.2	25.0 ± 6.3	9.3 ± 1.8

Mean ± S.D., $n = 3$–8.

Table 3
Concentration of peroxynitrite inducing 50% inhibition of the rate of DNP-SG and B-SG efflux from erythrocytes exposed at various hematocrits

Hematocrit (%)	$c_{50\%}$ (mM)	
	DNP-SG	B-SG
5	0.93 ± 0.11	0.39 ± 0.09
10	1.18 ± 0.21	0.44 ± 0.08
15	1.26 ± 0.20	0.43 ± 0.12
20	1.31 ± 0.22	0.45 ± 0.10

Mean ± S.D., $n = 3$.

The transport inhibition observed was due to the action of peroxynitrite and not to impurities or decomposition products contained in the peroxynitrite preparations as exposure of erythrocytes to peroxynitrite solutions allowed to decompose for 15 min at pH 7.4 did not bring about changes in the glutathione *S*-conjugate transport higher than 10% of those seen with solutions containing peroxynitrite (not shown).

The activity of the high-affinity component of the glutathione *S*-conjugate transport can also be monitored in erythrocyte membrane by measurement of the DNP-SG-stimulated Mg^{2+}-ATPase activity of the membranes [31,36]. This activity was inhibited in a dose-dependent manner upon treatment of erythrocyte membranes with peroxynitrite (Table 5).

Table 4
Time course of GSH content of peroxynitrite-treated erythrocytes

Peroxynitrite (μM)	GSH content (%)			
	100	300	500	700
'Zero-time'	73.0 ± 11.5	28.7 ± 7.6	12.3 ± 4.3	10.3 ± 3.6
5 min	71.7 ± 6.6	18.7 ± 6.8	9.7 ± 2.4	9.0 ± 2.9
10 min	69.7 ± 7.1	18.8 ± 5.3	10.7 ± 2.8	8.7 ± 2.6
20 min	69.9 ± 8.3	22.3 ± 6.0	10.7 ± 3.1	8.6 ± 2.1
30 min	71.4 ± 6.3	23.3 ± 5.3	12.3 ± 3.3	11.7 ± 2.8
40 min	73.3 ± 10.2	29.3 ± 5.9	13.7 ± 1.4	14.7 ± 2.6

Hematocrit of the suspension: 5% GSH content of control cells (1.72 ± 0.14 μmol/ml cells) assumed to be 100%. Mean ± S.D., $n = 3$.

4. Discussion

Transport of glutathione *S*-conjugates is an important element of xenobiotic detoxification, often referred to as detoxification phase III [37]. The 'glutathione *S*-conjugate pump' plays apparently a significant role in cellular defense under oxidative stress conditions since (i) is able to transport glutathione conjugates of lipid peroxidation products and (ii) extrudes oxidized glutathione [37,38] helping in maintaining and restoration the proper redox state of the cells.

Studies on the glutathione *S*-conjugate transport across the human erythrocyte membrane pointed to the existence of two kinetic components of the transport [38–41]. The nature of this kinetic heterogeneity of the transport is unclear and its elucidation requires structural studies [42]. Anyhow, measurements of the DNP-SG efflux from erythrocytes given millimolar concentrations of CDNB allow for characterization 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.

The transport of both substrates studied was inhibited by peroxynitrite, that of B-SG being more prone to inhibition. As peroxynitrite affects various constituents of the erythrocyte, several possibilities of explanation of the effects observed should be considered: (i) peroxynitrite modulates the glutathione *S*-conjugate pump itself, (ii) peroxynitrite induces lipid peroxidation and this influences the pump activity,

Table 5
Effect of peroxynitrite on the basal Mg^{2+}-ATPase and ATPase activity stimulated by DNP-SG and DNP

Peroxynitrite	ATPase activity (nmol P_i/(mg protein · h))				
	Control	100 μM	300 μM	500 μM	1 mM
Mg^{2+}-ATPase	239 ± 34 (= 100%)	172 ± 8 (72%)	154 ± 17 (64%)	144 ± 23 (60%)	136 ± 21 (57%)
DNP-SG-stimulatory activity	101 ± 35 (= 100%)	86 ± 19 (85%)	58 ± 22 (57%)	67 ± 26 (66%)	56 ± 23 (32%)
DNP-stimulatory activity	409 ± 55 (= 100%)	345 ± 26 (84%)	290 ± 37 (71%)	206 ± 37 (50%)	122 ± 25 (33%)

Erythrocyte ghosts (4 mg protein per ml) were incubated with peroxynitrite. Mean ± S.D., $n = 3$.

(iii) peroxynitrite causes oxidation of glutathione and oxidized glutathione inhibits the glutathione *S*-conjugate pump, and (iv) peroxynitrite causes formation of *S*-nitrosoglutathione which inhibits the pump.

Inhibition by peroxynitrite of the ATPase activity stimulable by glutathione *S*-conjugates in isolated membranes can be explained only by (i) or (ii). These two possibilities cannot be easily distinguished since peroxynitrite induces lipid peroxidation in erythrocyte membranes [20]. However, we have observed inhibition of erythrocyte glutathione *S*-conjugate pump by organic peroxides [43] under conditions of no detectable peroxidation of red cell membrane lipids [44] which demonstrates that these two events can proceed independently. (iii) Oxidized glutathione inhibits competitively the high-affinity kinetic component of the glutathione *S*-conjugate pump [38] but has no effect on the low-affinity component of the pump [38,45]. Therefore, glutathione oxidation by peroxynitrite could contribute to the higher inhibition of the B-SG transport as compared with DNP-SG transport (Table 3) and to the partial reversibility of the inhibition of B-SG transport (Fig. 2). (iv) Inhibition of the glutathione *S*-conjugate pump by *S*-nitrosoglutathione formed by peroxynitrite does not seem to be a significant factor as the main product of the interaction of peroxynitrite with glutathione is oxidized glutathione, *S*-nitrosoglutathione being formed in very low amounts (corresponding to about 1% of glutathione in endothelial cells) [46]. Moreover, although *S*-nitrosoglutathione inhibits glutathione reductase [47], it seems to be a substrate for the glutathione *S*-conjugate pump (Rychlik et al., unpublished).

The concentrations of peroxynitrite used in this study may appear unphysiologically high though the highest local rates of peroxynitrite production were estimated to be of an order of 1 mM/min [48]. However, it should be considered that, due to the high decomposition rate of peroxynitrite at neutral pH, exposure to a single dose of peroxynitrite is equivalent to a prolonged exposure to a much lower concentration of this compound. For example, the exposure to a bolus of 250 μM peroxynitrite is equivalent to the exposure to a steady-state concentration of only 1 μM peroxynitrite for 7 min [49].

The data presented demonstrate that the 'glutathione *S*-conjugate pump' can be one of the cellular targets for peroxynitrite. The molecular identity of the 'glutathione *S*-conjugate pump' has been a matter of controversy. Recent findings identify it with the multidrug resistance-associated protein (MRP) [50–52], present also in the erythrocyte [53] though a related protein, canalicular MRP (cMRP) or canalicular multispecific organic anion transporter (cMOAT) has been found in the canalicular membranes of hepatocytes [54,55].

Therefore, the present data indicate that peroxynitrite can inhibit MRP, a transporter involved in the removal of xenobiotics and oxidized glutathione from various cells and in the removal of chemotherapeutics from malignant cells. This phenomenon may potentiate the cellular effects of oxidative stress but, on the other hand, can contribute to the role of nitric oxide in the host-tumor interactions.

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