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Effect of pre-exposure of human erythrocytes to oxidants on the haemolytic activity of Sticholysin II. A comparison between peroxynitrite and hypochlorous acid

GLORIA CELEDÓN[1], GUSTAVO GONZÁLEZ[2], EDUARDO LISSI[3], TANIA CERDA[1,2], DENISSE BASCUÑANT[2], MARCIA LEPELEY[2], FABIOLA PAZOS[4], MARIA E. LANIO[4] & CARLOS ALVAREZ[4]

[1]*Departamento de Fisiología, Facultad de Ciencias, Universidad de Valparaíso, Chile,* [2]*Instituto de Química, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Chile,* [3]*Facultad de Biología y Química, Universidad de Santiago de Chile, Chile, and* [4]*Centro de Estudios de Proteínas, Facultad de Biología, Universidad de La Habana, Cuba*

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Abstract

Stichodactyla heliantus II (St II) is a haemolytic toxin whose activity depends of the characteristics of red blood cells (RBC). Among the factors that may tune the response of the RBC to the toxin activity stand the oxidative status of the cell. This study investigates how pre-oxidation of RBC modifies St II activity employing two oxidants, peroxynitrite and hypochlorous acid. Results show that peroxynitrite-treated RBC are more resistant to St II activity. On the other hand, hypochlorous acid-treated RBC become more susceptible to St II. This contrasting behaviour of both oxidants is related to the modifications elicited in RBC by both oxidant agents. Peroxynitrite does not modify RBC osmotic fragility but reduces anion transport through band 3 protein. This effect, together with an increase in K+ efflux, can explain the increased resistance to the toxin activity. On the other hand, results obtained with hypochlorous acid can be explained in terms of a disruption of the membrane organization without the compensating effect of a reduction in band 3-mediated anion transport. The present results, obtained employing the effect of a model haemolytic toxin on RBC, emphasize the specificity of the RBC response to different endogenous oxidative agents.

Keywords: *Sticholysin II, haemolysis, actinoporin, pre-oxidation, peroxynitrite, hypochlorous acid*

Introduction

Sticholysin II (St II) is one of two highly haemolytic isotoxins from the sea anemone *Stichodactyla helianthus* that form cation selective oligomeric pores of 2 nm diameter in natural and model lipid membranes [1–3]. The haemolytic activity of this pore-forming toxin takes place with a kinetics similar to that of the osmotic shock promoted by suspension of the red blood cells (RBC) in hypotonic media and is characterized by: (i) K^+ efflux prior to cell disruption [4], (ii) involvement of transporters and enzymes of the erythrocyte membrane [5,6] and (iii) a dependence of cell oxidation status and/or cell ageing [7]. In particular, RBC pre-incubation with a peroxyl radical source (AAPH), renders RBC populations more susceptible to haemolysis due to oxidation of membrane proteins and lipids without significant modification of intracellular components such as haemoglobin and glutathione [8].

Circulating erythrocytes can be oxidized by a variety of endogenous oxidants, such as hypochlorous acid and peroxynitrite. Peroxynitrite is produced by the reaction of superoxide (produced in haemoglobin oxidation) and nitric oxide [9], while hypochlorous acid originates in the activation of macrophages and neutrophiles [10]. These two oxidant agents may produce different modifications, due to the relative

Correspondence: Gustavo González, Instituto de Química, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Chile. Fax: 56-32-2273422. Email: ggonzale@ucv.cl

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reactivity they have towards the erythrocytes components, which could increase or decrease the lytic activity of the St II toxin.

In erythrocytes, hypochlorous acid oxidizes glutathione (GSH) [11], decreases both membrane proteins-SH groups [12] and cell deformability [13], and increases osmotic fragility [14] as well as lipid oxidation [12,13,15] without modifying band 3 protein transport capacity [15] or haemoglobin oxidation status [11]. On the other hand, peroxynitrite oxidizes haemoglobin [16], aggregates membrane proteins [17,18] and decreases GSH and membrane protein-SH groups [19] with a low level of lipid oxidation [20]. Furthermore, oxidation by peroxynitrite induces both an activation of K^+ passive transport [21] and a decrease of the band 3 protein transport capacity [18].

Considering the differences in the oxidative effects on RBC of hypochlorous acid and peroxynitrite, which may give rise to a differential response of these cells to the toxin haemolytic action, we have evaluated the action of St II on pre-oxidized cells with these two oxidant agents. The results obtained show that the variety of cellular factors that condition the toxin activity leads to a marked specificity of the RBC response to the damage elicited by the different endogenous oxidative agents.

Materials and methods

Materials

4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and 5,5'-dithiobis(2 nitrobenzoic acid) (Ellman's reagent) were obtained from Sigma (St. Louis, USA). Hypochlorous acid, from Merck (Darnstadt, Germany), was employed as received from manufacturer. 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-y)amino] ethanosulphonate (NBD-taurine) was synthesized according to Eidelman and Cabantchik [22]. St II toxin (Swiss Data Protein Bank P07845) was purified according to Lanio et al. [23]. Other chemicals were from standard commercial sources.

Peroxynitrite synthesis

Peroxynitrite was synthesized in a quenched-flow reactor as described by Beckman et al. [24], with modifications according to Saha et al. [25], and stored at –80°C. Peroxynitrite anion concentration was determined spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}) in 1 mole/L NaOH. Its concentration in the top layer of a partially frozen solution was 180–216 mmole/L. A small aliquot of this solution was added to a RBC suspension in buffer A (NaCl 80 mmole/L, phosphate buffer, pH 7.4). Experiments with decomposed peroxynitrite were performed by adding the peroxynitrite to buffer A and incubating the solution at 25°C for 5 min prior to the RBC addition.

Hypochlorous acid solutions

Hypochlorous acid concentration was evaluated by measuring the absorbance at 292 nm at pH 12 [ε_{292} = 350 M^{-1} cm^{-1}] [26]. Stock solutions of hypochlorous acid were diluted with 145 mmole/L NaCl, 10 mmole/L phosphate buffer pH 7.4. These solutions were prepared immediately prior to its addition to the RBC suspension.

Blood samples

Heparinized fresh blood was collected from healthy volunteers following informed consent. After centrifugation at 12 000 g for 30 s, plasma and buffy coat were removed and erythrocytes were washed three times by centrifugation in 145 mmole/L NaCl, 5 mmole/L phosphate buffer, pH 7.4 (PBS) [27] or in buffer A prior to peroxynitrite treatment.

Erythrocytes oxidation

Prior to the toxin haemolytic activity evaluation, RBC were oxidized employing peroxynitrite or hypoclorous acid. Oxidations were carried out at 25°C with a fixed number of oxidant molecules per RBC (1 nmole of oxidant per 10^6 cells).

Erythrocytes oxidation by peroxynitrite

Resuspended erythrocytes in buffer A (1% haematocrit) were exposed for 3 min at 25°C to a bolus of peroxynitrite (final concentration: 100 μmole/L). Immediately after, erythrocytes suspensions were cooled at 0°C, kept at this temperature for 5 min, washed three times by centrifugation with cold buffer A and then suspended in PBS buffer to the desired haematocrit according to the study to be performed.

Experiments were also carried out employing decomposed peroxynitrite. The results were similar to those obtained in control assays employing non-oxidized erythrocytes.

Erythrocytes oxidation by hypochlorous acid

Erythrocyte suspensions in PBS buffer were treated with hypochlorous acid at 25°C for 10 min (final haematocrit was 10% and final hypochlorous acid concentration was 1 mmole/L). Mixing was performed by slowly pouring 2 mL of 2 mmole/L hypochlorous acid on 2 mL of the 20% haematocrit RBC suspension under gentle stirring conditions. Cells were then washed

three times with excess cold PBS and suspended in PBS buffer to the desired haematocrit according to the study to be performed.

Toxin haemolytic activity assay

RBC suspensions were diluted to an absorbance of 1.0 at 620 nm with addition of PBS buffer. Lytic activity was evaluated turbidimetrically at 620 nm at room temperature (25 ± 1°C) in a Labsystems microplate reader (Finland). Assay was started by adding the corresponding volume of St II solution to the RBC suspensions (285 µl final volume) and the decrease in absorbance was recorded as a function of time with intermittent shaking. Loss of turbidity was quantitatively related to lytic activity [23]. Complete haemolysis was achieved by adding a large excess of toxin. Toxin concentration was adjusted to that rendering t_{50} lysis values in the 300–500 s range (t_{50} is defined as the time required to get 50% lysis of the RBC suspension).

K^+ loss measurement

K^+ release from RBC was measured with an ion selective electrode (Cole Palmer 27502-39, Illinois, USA) coupled to an Orion 420A millivoltimeter. Lineal relationships were obtained between voltage and log K^+ concentration employing KCl in PBS solution. Plot slope was that expected from a Nernst type relationship. Increase of external K^+ concentration after exposing a RBC suspension to St II was followed under constant stirring. Total K^+ release was estimated by addition of a large toxin excess. All K^+ loss measurements were performed simultaneously with an assessment of the toxin lytic activity.

Osmotic fragility assay

Five microlitres of pre-oxidized or control RBC suspensions in PBS (20% haematocrit) were added to 500 µL of solutions containing increasing NaCl concentrations (from 0–0.9 g/dL). After 60 min incubation at room temperature, suspensions were centrifuged and haemolysis (percentage) was determined spectrophotometrically from supernatant readings at 405 nm in a Labsystems microplate reader [28].

NBD-taurine transport assay

Band 3 protein anion transport capacity can be estimated from the rate of NBD-taurine anion transport. NBD-taurine efflux was assessed by the continuous monitoring of NBD-taurine transport by a fluorescence method, as previously described [29] under symmetric conditions ($Cl^{-1}{}_{in}/Cl^{-1}{}_{out}$). Efflux of NBD-taurine increases its fluorescence which was quenched by the intracellular haemoglobin. The increase in fluorescence can then be quantitatively related to the NBD-taurine anion transport. The probe was incorporated to erythrocytes by incubating a cell suspension in PBS at 8% haematocrit with NBD-taurine (0.8 mmole/L) at 37°C during 60 min in the dark with gentle shaking. No cell haemolysis was observed during the incubation. Extracellular NBD-taurine was removed by three washes with cold PBS by centrifugation at 4°C. Sediments were resuspended in the same buffer to 4% haematocrit. A suspension aliquot of 40 µl was added to 3 ml PBS in a cuvette and the efflux of NBD-taurine to the extracellular medium leads to a time-dependent increase in fluorescence intensity due to intracellular NBD-taurine fluorescence quenching by haemoglobin. Fluorescence intensity was continuously recorded at 37°C in a Spex Fluorolog spectrofluorometer at 540 nm, with an excitation wavelength of 478 nm. NBD-taurine efflux was ended when cells were lysed with 0.01% Triton X-100, which released the remaining intracellular probe. Exit rates were obtained from the fluorescence/time traces and analysed in terms of the NBD-taurine first order efflux (k_{efflux}), defined by the equation

$$K_{efflux}(t) = \frac{1}{F_\infty - F(t)} \times \frac{dF(t)}{dt}$$

in which F_∞ is the maximum fluorescence intensity and $F(t)$ is the fluorescence measured at the corresponding time [30].

Efflux rates were also evaluated in the presence of DIDS (1 mmole/L) to inhibit band 3 protein transport and to assess anion transport through other pathways present in erythrocyte membrane. Control k_{efflux} of NBD-taurine efflux was $2.1 \pm 0.1 \times 10^{-3}$ s^{-1} ($n = 7$). In the presence of DIDS, k_{efflux} decreases to $4.0 \pm 0.7 \times 10^{-5}$ s^{-1} ($n = 6$).

Haemoglobin oxidation

Haemoglobin oxidation was assessed spectrophotometrically in erythrocyte lysates of treated and untreated red blood cells, as described by Winterbourn [31].

Glutathione oxidation

Glutathione level was determined by the method of Ellman [32]. Briefly, 0.2 ml of 25% trichloroacetic acid was added to 2 ml of the RBC suspension (10% haematocrit) and centrifuged. To 1 ml of the supernatant, 1 ml of 1 mole/L phosphate buffer (pH 7.8) and 0.1 ml Ellman's reagent (10^{-3} mole/L) were added for GSH determination. The concentration of GSH was monitored spectrophotometrically at 412 nm using an extinction coefficient of 13.6 mM^{-1} cm^{-1}.

A correction was introduced to take into account a possible contribution of haemoglobin's Soret band to the absorption measured at 412 nm. To this effect, GSH contribution was equated to the difference in absorbances measured prior and after addition of Ellman's reagent. This correction amounts to ~ 10% of the measured absorbance.

Statistical analysis

Results are expressed as mean ± SEM over the different blood samples. On each sample, duplicate or triplicate measurements were performed. Statistical significance of results was assessed by Mann-Whitney test.

Results

Peroxynitrite or hypochlorous acid effect on St II haemolytic activity

Pre-exposure of the RBC ensemble to peroxynitrite or hypochlorous acid (1 nmole of oxidant per 10^6 cells) modify the resistance of the cell to the lytic action of St II. Results obtained employing 0.3 nM St II indicate that pre-exposure to peroxynitrite decreases the susceptibility of the RBC ensemble to the lytic action of the toxin, whereas pre-exposure to hypochlorous acid renders the cell more prone to the toxin-induced lysis (data not shown). This qualitative difference takes place independently of the toxin concentration considered. Typical examples carried out at toxin concentrations that generate measurable rates of haemolysis, are shown in Figures 1A and B, respectively, and emphasizes the opposite effects of these two oxidant agents. These effects can be quantified in terms of t_{50} values (Table I). Similar tendencies are observed at higher oxidant agent concentrations (data not shown).

Haemolysis observed in Figure 1 can be ascribed to the toxin haemolytic activity since negligible haemolysis was observed in this time scale in control experiments. In fact, after 30 min incubation of untreated RBC in the absence of toxin, haemolysis was undetectable. Similarly, percentage of haemolysis of pre-oxidized RBC was also very low (undetectable in hypochlorite pre-treated RBC and less than 2% in peroxynitrite pre-treated RBC).

Peroxynitrite or hypochlorous acid effect on K^+ loss induced by St II

The effect of RBC pre-oxidation on K^+ loss induced by St II is shown in Figure 2. It can be seen that pre-incubation with both oxidants increase the rate of K^+ efflux, although the process is more affected when peroxynitrite is employed. This effect can be quantified in terms of t_{50} values, defined at the time required to release 50% of the intracellular K^+ (Table I). It must be noticed that RBC oxidation promotes K^+ efflux even in the absence of St II.

It has been reported that different oxidants increase K^+ efflux [7,21]. In particular, lysis promoted by hypochlorous acid is preceded by an increase in K^+ efflux [13]. However, this K^+ efflux occurs at a longer time scale, as stressed by the fact that no significant changes are observed in the intracellular K^+ concentration of pre-oxidized RBC at the moment of the toxin addition (Table I, K content). Furthermore, data given in Figure 2 show that the rate of K^+ efflux in control experiments is considerably smaller than that promoted by the toxin.

In order to analyse the possible factors determining the different behaviour of St II activity on oxidized

Table I. Effect of RBC oxidation by peroxynitrite or hypochlorous acid.[#]

	Peroxynitrite	Hypochlorous acid
Haemolysis and K loss promoted by St II toxin		
Lysis ($t_{50}/t_{50control}$)	1.31 ± 0.10 (9)**	0.55 ± 0.05 (9)**
K loss ($t_{50}/t_{50control}$)	0.43 ± 0.14 (6)*	0.71 ± 0.05 (5)*
Osmotic fragility ($C_{50}/C_{50control}$)	1.02 ± 0.02 (6)^{ns}	1.07 ± 0.04^{ns} (5)
Anion transport $(k_{efflux})/(k_{efflux})_{control}$		
In the absence of DIDS	0.67 ± 0.03 (4)	0.96 ± 0.06 (3)
In the presence of DIDS	0.83 ± 0.14 (3)	2.43 ± 0.32 (3)
Methemoglobin formation (%)	25.3 ± 1.3 (9)**	0.0 (8)
Glutathione decrement (%)	63 ± 5 (4)	43 ± 4 (9)**
K content (K/Hb)/$(K/Hb)_{control}$	0.9 ± 0.04 (3)	0.93 ± 0.05 (5)^{ns}

$^{*}p < 0.01$, $^{**}p < 0.0001$, ns = non-significant, with respect to non-oxidized RBC. Significance applies to differences regarding control values (non-oxidized RBC).

$c_{50}/t_{50control}$ corresponds to the t_{50} value normalized with respect to the control value.

$c_{50}/c_{50control}$ corresponds to the t_{50} value normalized with respect to the control value.

$(k_{efflux})/(k_{efflux})_{control}$ corresponds to the k_{efflux} value normalized with respect to the control value.

(K/Hb) corresponds to the normalized K cell content with respect to cell haemoglobin. $(K/Hb)/(K/Hb)_{control}$ corresponds to the (K/Hb) normalized value with respect to the control value.

[#]Reported values are relative to controls measured in the same blood sample.

Values between parentheses correspond to the number of independent assays from different donors.

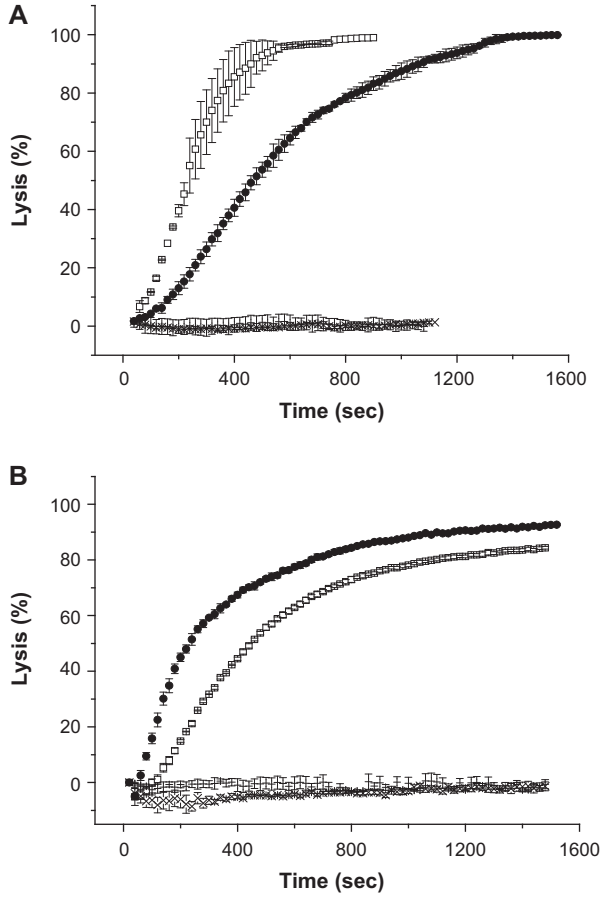

Figure 1. Time course of haemolysis induced by St II in RBC pre-treated with peroxynitrite (A) or with hypochlorous acid (B). Untreated erythrocytes (□), peroxynitrite or hypochlorous acid treated erythrocytes (●). Profiles shown in (A) or (B) represent triplicates obtained employing treated and untreated cells from a given donor at the corresponding toxin concentration. Similar lytic time-profiles were observed in nine independent assays from different donors; 2.3×10^6 cells/mL in PBS buffer, pre-oxidized with peroxynitrite or hypochlorous acid were used. St II was 0.5 nM (final concentration) for peroxynitrite pre-oxidized RBC and 0.3 nM (final concentration) for hypochlorous acid pre-oxidized RBC. Profiles obtained in toxin absence are: (+) for untreated cells and (×) for pre-oxidized cells.

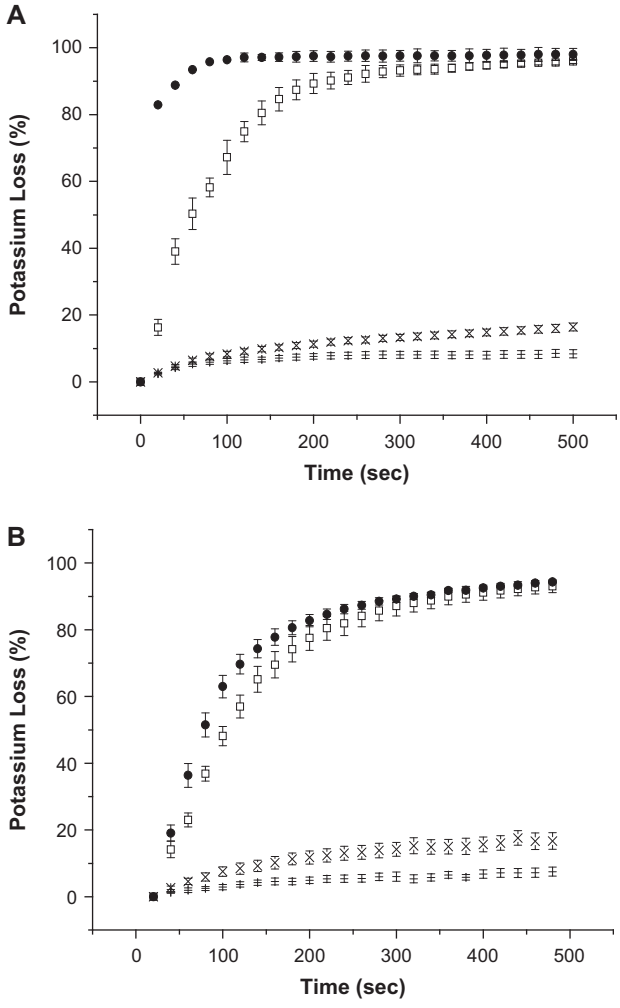

Figure 2. Time course of K^+ loss induced by St II in RBC pre-treated with peroxynitrite (A) or with hypochlorous acid (B). Untreated erythrocytes (□), peroxynitrite or hypochlorous acid treated erythrocytes (●). Profiles shown in (A) or (B) represent duplicates obtained employing treated and untreated cells from a given donor at the corresponding toxin concentration. Similar time profiles were observed in at least five independent assays from different donors; 2.3×10^6 cells/ml in PBS buffer, pre-oxidized with peroxynitrite and hypochlorous acid were used. St II was 0.5 nM (final concentration) for peroxynitrite pre-oxidized RBC and 0.3 nM (final concentration) for hypochlorous acid pre-oxidized RBC. Profiles obtained in toxin absence are: (+) for untreated cells and (×) for pre-oxidized cells.

RBC, we evaluated the peroxynitrite or hypochlorous acid effect on erythrocytes osmotic fragility and anion transport capacity.

Peroxynitrite or hypochlorous acid effect on RBC osmotic fragility

In order to evaluate erythrocytes susceptibility to osmotic shock, lysis extent was measured in relation to an applied osmotic gradient and the results obtained for erythrocytes exposed to peroxynitrite or hypochlorous acid are shown in Figures 3A and B, respectively. Figure 3A shows equivalent profiles irrespective of RBC pre-oxidation, which implies that pre-incubation with peroxynitrite under described experimental conditions is not altering cells response to the volume changes. This is appreciated in the C_{50} relative values shown in Table I. C_{50} is defined as the NaCl concentration at which 50% of the cell population is lysed. On the other hand, osmotic fragility profiles of pre-oxidized erythrocytes with hypochlorous acid show significant differences with the control at NaCl concentrations higher than 0.4 g/dL (Figure 3B). Although C_{50} value does not significantly vary with the treatment (Table I), profiles show a partial lysis, even in iso-osmotic conditions, of ~ 13% of cells population. This would imply that, after the treatment, at least two cell populations would exist, a predominant population with properties

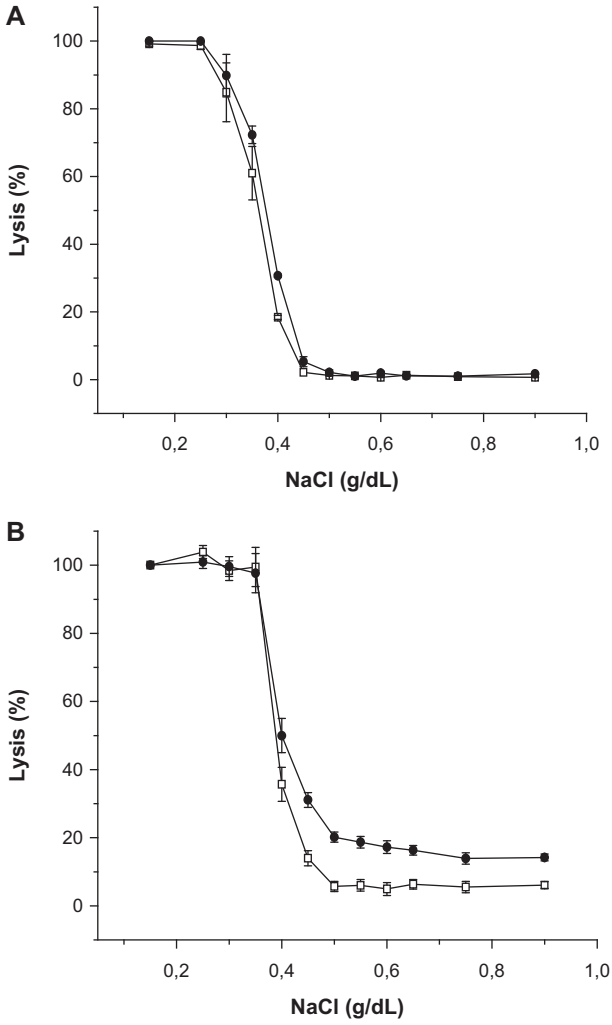

Figure 3. Osmotic fragility of a suspension of RBC pre-oxidized with peroxynitrite (A) or with hypochlorous acid (B). Untreated erythrocytes (□), peroxynitrite or hypochlorous acid treated erythrocytes (●). Profiles shown in (A) or (B) correspond to duplicate assays carried out on the same blood sample. Similar time profiles were observed in at least five independent assays from different donors; 2×10^7 cells/ml were used in the assays.

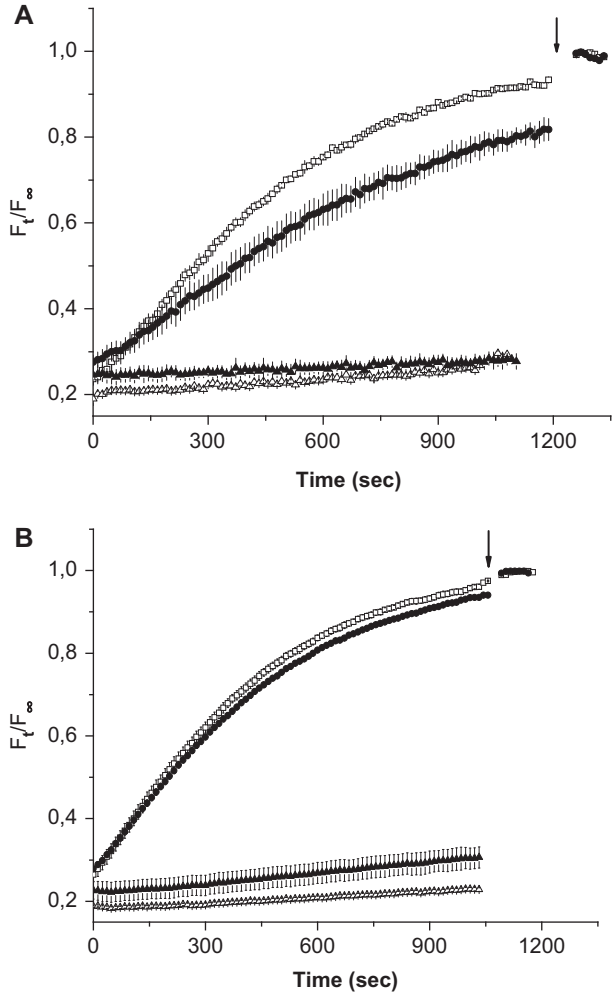

Figure 4. Time-dependent NBD-taurine efflux from RBC pre-oxidized with peroxynitrite (A) or with hypochlorous acid (B). Untreated erythrocytes (□), peroxynitrite or hypochlorous acid treated erythrocytes (●), untreated erythrocytes in the presence of DIDS (△), peroxynitrite or hypochlorous acid treated erythrocytes in the presence of DIDS (▲). Profiles shown in (A) or (B) correspond to duplicate assays carried out on the same blood sample. Similar time profiles were observed in at least three independent assays from different donors. Arrow indicates Triton-X100 addition.

similar to the control cells and other more sensitive that lyses even during incubation under iso-osmotic conditions. Other authors have also reported that erythrocytes exposure to hypochlorous acid results in an increase in cell osmotic fragility [14].

Peroxynitrite or hypochlorous acid effect on NBD-taurine transport

Previous works have shown that one of the factors conditioning the haemolytic response to St II is the cell capacity for anion transport, in particular through band 3 anion transport protein [5]. Results obtained measuring NBD-taurine transport after RBC pre-oxidation with peroxynitrite or hypochlorous acid are shown in Figures 4A and B, respectively. Figure 4A shows NBD-taurine efflux in control erythrocytes and in peroxynitrite pre-oxidized erythrocytes, in the presence and absence of DIDS, a specific band 3 anion transport inhibitor [33]. Anion transport capacity through band 3 is diminished by 33% in pre-oxidized erythrocytes and the unspecific anion transport is slightly altered (17% decrease) (Table I). This result is concordant with our previous report in which a decrease in sulphate anion transport was observed in peroxynitrite-oxidized erythrocytes [18]. Results obtained with erythrocytes pre-oxidized with hypochlorous acid under the described experimental conditions are markedly different since band 3 protein anion transport capacity is not altered. This finding is concordant with results reported by Vissers et al. [15], in which RBC sulphate anion transport was not affected by hypochlorous acid. However, the unspecific anion transport increases 2.4-fold, a result suggesting that

hypochlorous acid produces defects in the membrane which facilitate anion passage.

Peroxynitrite or hypochlorous acid effect on glutathione and haemoglobin oxidation

In order to evaluate the access of oxidants and/or its reactive metabolites to the cell under the described experimental conditions, the decrease of GSH and the increase of methemoglobin were measured (Table I). The strong decrease of intracellular GSH produced by both oxidant agents would imply that they have access to the cell interior. These results agree with previous reports which show that RBC oxidation by peroxynitrite induces oxidation of GSH and haemoglobin [19], whereas oxidation by hypochlorous acid depletes intracellular reduced GSH and does not produce haemoglobin oxidation [11,14]. The fact that hypochlorous acid does not originate measurable methemoglobin concentrations suggests that it is reacting predominantly with the globin component of haemoglobin. This is compatible with the high reactivity of hypochlorous acid towards different amino acids [34,35].

Discussion

In order to allow a meaningful comparison of the effect of RBC pre-exposure to peroxynitrite or hypochlorous acid, similar doses (defined in terms of oxidant added per cell) were employed. The data obtained, presented in Table I and Figure 2, show that both oxidants increase the rate of intracellular K^+ efflux promoted by St II addition to the RBC ensemble. This K^+ efflux, that takes place prior to haemolysis, can be employed as a marker of pore formation [4]. The presence of these pores allows the exit of K^+ directly through them and/or as a consequence of transporters activation triggered by the cell volume increase associated to the osmotically driven water influx [5]. This early K^+ efflux can act as a protective response of RBC to delay the osmotically driven haemolysis.

Formation of a competent pore in model membranes requires the adsorption of the toxin to the bilayer and its subsequent insertion and organization [36]. If the same is assumed for RBC, t_{50} values are more a reflection of the rate of St II organization than a measure of the number of pores formed in the cells.

Data obtained in this work show that, in spite that pre-oxidation with peroxynitrite and hypochlorous acid promotes pore organization, as sensed by the rate of K^+ efflux [4], the effect upon the toxin lytic activity strongly depends on the oxidant considered (Table I and Figure 1). In fact, while pre-oxidation with hypochlorous acid promotes the lysis (t_{50} value is considerable smaller than that of the control), the opposite happens when peroxynitrite is employed. In fact, pre-oxidized RBC with peroxynitrite increases in ~ 30% the time required to disrupt 50% of the cells. This different behaviour can be interpreted in terms of the variety of factors that determine the rate of the lytic process. Among these factors we can mention the cell membrane osmotic resistance, the rate of water influx, the rate of K^+ efflux (to decrease the osmotic imbalance) and the rate of Na^+ influx (to maintain the osmotic imbalance) [37]. Associated to this, anion exchange promoted by band 3 protein can contribute to maintain the osmotically driven water influx that finally leads to the cell disruption [5].

Peroxynitrite promoted pre-oxidation of the RBC ensemble

Results obtained after pre-exposure of RBC ensemble to peroxynitrite, shown in Table I and Figures 3A and 4A, allow one to conclude that:

i) Peroxynitrite (and/or their oxidant metabolites) enters RBC (see glutathione and haemoglobin modification);
ii) Membrane characteristics are barely modified (see osmotic fragility and unspecific anion transport in presence of DIDS); and
iii) Anion transport through band 3 protein is inhibited.

The increase rate of K^+ efflux and the reduction in anion transport rate can explain the slower rate of cell disruption by St II observed in the RBC ensemble pre-exposed to peroxynitrite.

Hypochlorous acid promoted pre-oxidation of the RBC ensemble

The response of the RBC ensemble pre-oxidized with hypochlorous acid to the St II lytic action is opposite to that discussed in the previous section (Table I and Figures 3B and 4B). It is relevant to point out that the changes in haemolytic activity produced by hypochlorous acid are not a result of secondary processes mediated by chloramines. In fact, t_{50} values obtained after RBC ensemble exposure to hypochlorus acid were independent ($n = 6$) of the time elapsed (3 or 10 min) between oxidation and toxin evaluation of its haemolytic activity: $(t_{50})_{10\ min}/(t_{50})_{3\ min} = 1.03 \pm 0.03$).

The main characteristics of the RBC ensemble after its hypochlorous acid promoted oxidation are:

i) Hypochlorous acid enters the red cell (see GSH loss in Table I) without modifying haemoglobin status;
ii) A disruption of the membrane organization and/or its resistance to osmotic shock (see the osmotic fragility profile and the noticeable

increase in the unspecific anion transport in presence of DIDS); and

iii) There is no modification of the anion transport through the band 3 protein complex.

The increase in membrane permeability (for example to water) with band 3 protein activity could provide a naïve explanation for the increased lytic activity of St II in cells pre-exposed to hypochlorous acid.

We can conclude that the effect of pre-oxidation of the RBC ensemble upon the haemolytic activity of St II is determined by the employed oxidant. This is further sustained if the present data are compared to those obtained after pre-oxidation of the RBC with AAPH, a non-penetrating oxidant [38]. An increase in St II haemolytic activity has been reported after AAPH treatment, an effect attributed to the oxidant-promoted loss of internal K^+. This effect is not relevant under the conditions employed in the present work (Table I). The present data, obtained employing endogenous oxidants, emphasizes the fact that the response of the RBC to their oxidation is extremely dependent of the oxidant considered. This conclusion, obtained employing a widely studied model haemolytic toxin, can be related to the variety of factors that conditions the sensitivity of the cell to the toxin activity. Studies employing other biological relevant oxidants, such as singlet oxygen, superoxide and hydroxyl radicals, will contribute to our understanding of the relationship between oxidative status and sensitivity of the RBC ensemble to the action of haemolytic toxins.

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Declaration of interest

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