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Hypochlorous acid-induced membrane pore formation in red blood cells

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Abstract

The hyperproduction of hypochlorous acid (HOCl), an extremely toxic biological oxidant generated by neutrophils and monocytes, is involved in the pathogenesis of many diseases. In these studies, we attempted to determine the membrane and cellular events associated with HOCl-induced erythrocyte impairment and haemolysis. In vitro human erythrocyte exposure to HOCl (0.1–1.0 mM) resulted in rapid oxidation of reduced glutathione, an increase in cell osmotic fragility and the formation of transient membrane pores. The process of glutathione oxidation depended on the [oxidant]/[cell number] ratio. The HOCl-induced haemolysis observed was apparently mediated by pore formation and altered membrane electrolyte permeability. The estimated pore radius was approximately 0.7 nm and the average number per cell was 0.01. The rate constant of HOCl-produced haemolysis depended on pH. There were significant differences in haemolysis of HOCl-treated erythrocytes which had maximal stability at pH 7.2–7.3.

Keywords: Hypochlorous acid; Haemolysis; Glutathione; Membrane pore; Red blood cell

1. Introduction

The respiratory burst of stimulated neutrophils and monocytes, cells present under inflammatory conditions, produces substantial amounts of hypochlorous acid (HOCl). The mechanism is via a myeloperoxidase-catalysed reaction of H_2O_2 with Cl^- [1,2]. HOCl, a highly reactive biological oxidant, is thought to play an important role in both microbial killing (bactericidal action of phagocytic cells) and inflammatory tissue injury by neutrophils [1,2]. Under pathological conditions, HOCl/OCl^- is found at concentrations up to 200 μM [3], however in some tissues it can be as high as 3–8 mM [4]. HOCl reacts with a wide range of biological target molecules, including lipids, proteins and DNA, to form long-lived N-chloramines (RNHCl) in a reaction with amine groups [5–7].

Toxic effects of HOCl have been studied on different cell types [5,7–13]. HOCl induces general dramatic cell impairment [1,2]. It also interferes specifically with intracellular signal transduction pathways [11].

Red blood cells (RBCs) have been used extensively as a model system for investigating common mechanisms of neutrophil-mediated cell injury [5,7,14,15]. Vissers and Winterbourn [7] have shown that exposure of RBCs to low concentrations of HOCl resulted in the loss of intracellular reduced glutathione (GSH) that preceded oxidation of membrane thiols and the formation of chloramines. The well-known end result of erythrocyte exposure to HOCl is cell haemolysis [14,15]. HOCl treatment causes an immediate change in the RBC membrane structure that affects membrane deformability and permeability. These changes are followed by gradual cell swelling and K^+—leakage and then lysis [14]. Electron microscopy showed extensive disruption of the erythrocyte membrane [14]. But the exact mechanism of RBC damages and haemolysis is poorly understood.

Perhaps hypochlorous acid-produced impairment of erythrocyte membranes resulting from a direct interaction between neutrophils and RBCs in circulation may play a role in the pathogenesis of diseases that are associated with

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RBC membrane defects, such as haemolytic anemia and vascular complications. Free-radicals and oxidants produce the membrane defects and subsequent haemolysis that occur in stored blood, as well as in the blood of haemodialysed patients and in blood of hypochlorite-treated septic patients. The aim of the present study was to assess the size and the number of membrane defects (pores), formed by HOCl treatment of RBCs in vitro and their relationship to subsequent erythrocyte lysis.

2. Materials and methods

2.1. Chemicals

Sodium hypochlorite (NaOCl), 5,5′-dithio-bis (2-nitrobenzoic acid) (Ellman's reagent) and valinomycin were from Sigma-Aldrich, Germany; the fluorescence probe 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) was from Molecular Probes, Eugene, OR, USA. All other reagents were from POCh (Gliwice, Poland) and were of analytical grade.

2.2. Blood samples

Blood from healthy donors was purchased from the Central Blood Bank in Lodz. Blood was taken into 3.2% sodium citrate. After removing plasma and the leukocyte layer, erythrocytes were washed three times with cold (4 °C) phosphate buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). Erythrocytes were used immediately after isolation.

2.3. Susceptibility of erythrocytes to hypochlorous acid-induced damage

Suspensions of RBCs in PBS (haematocrit 20%, 10% or 5%) were treated with different concentrations of hypochlorous acid at 22 ± 1 °C for 5 or 10 min. Then the cells were washed three times with excess of cold PBS and resuspended in PBS (haematocrit 10%). At pH 7.4, this solution contained approximately 1:1 ratio of HOCl and OCl^- and was subsequently referred to as HOCl [15]. The concentration of OCl^- was determined spectrophotometrically using an absorbance coefficient of 350 $M^{-1}cm^{-1}$ (292 nm) at pH 9.0 [16].

The susceptibility of erythrocytes to HOCl-induced oxidative damage was measured in terms of the apparent rate constant of cell haemolysis, changes of cell osmotic fragility and intracellular GSH level.

The process of haemolysis was monitored by haemoglobin release: after various time periods of incubation, 50 μl of pretreated RBS suspension (haematocrit 10%) was added to 1 ml of PBS and centrifuged (1.000 × *g*, 5 min). The haemoglobin content in the supernatants was measured spectrophotometrically by absorbance at 414 nm. The haemoglobin content after haemolysis of the same volume of the RBC suspension by addition of 1 ml of water was assumed to be equal to 100%.

To measure the pH-dependence of haemolysis rate constant, HOCl-treated RBCs were resuspended in different buffered solutions (PBS, pH 5.8–8.4).

2.4. Assay for intracellular GSH concentration

The GSH level was determined by the method of Ellman et al. [17]. Briefly, 0.2 ml of 25% trichloroacetic acid was added to 2 ml of final RBC suspension (10% haematocrit) and centrifuged. To 1 ml of the supernatant, 1 ml of 1 M phosphate buffer (pH 7.8) and 0.1 ml Ellman's reagent (10^{-3} M) were added for GSH determination.

The concentration of GSH was monitored spectrophotometrically at 412 nm using the extinction coefficient of 13.6 mM^{-1} cm^{-1}.

2.5. Osmotic fragility assay

To determine osmotic fragility, HOCl-treated erythrocytes were washed and diluted to the 0.25% haematocrit with NaCl solutions of various concentrations (50–150 mM) buffered with 5 mM sodium phosphate (pH 7.4). After 20 min of incubation, the cell suspensions were centrifuged and the percentage of osmotically lysed cells was estimated.

2.6. The membrane potential measurement

The membrane potential was measured using the optical probe $DiSC_3(5)$ which responds to the membrane potential of the cell according to a widely used method [18] and calculated using the Nernst equation. Briefly, erythrocytes before or after HOCl treatment and washing were suspended at a haematocrit of 0.2% in buffered saline, containing 10 mM Tris–HCl, pH 7.4 and 150 mM (KCl + NaCl) with K^+ concentrations ranging from 50 to 140 mM. To all suspensions of cells the fluorescent dye was added to give a final concentration of 2 μM. After the time required for stabilizing the fluorescence intensity of the dye, valinomycin at a concentration of 1 μM was added to the sample. The value of 150 mM was used as the internal K^+ concentration in RBCs. Fluorescence was excited at 625 nm and registered at 660 nm with a Perkin-Elmer LS-5B spectrofluorimeter.

All the results were expressed as the means of three to five replicates ± S.E.M. and the statistical analysis was conducted using analysis of variance (ANOVA).

3. Results

Treatment of RBCs with HOCl and subsequent washing to remove unreacted oxidant and its products, resulted in a time-dependent process of cell haemolysis. Fig. 1

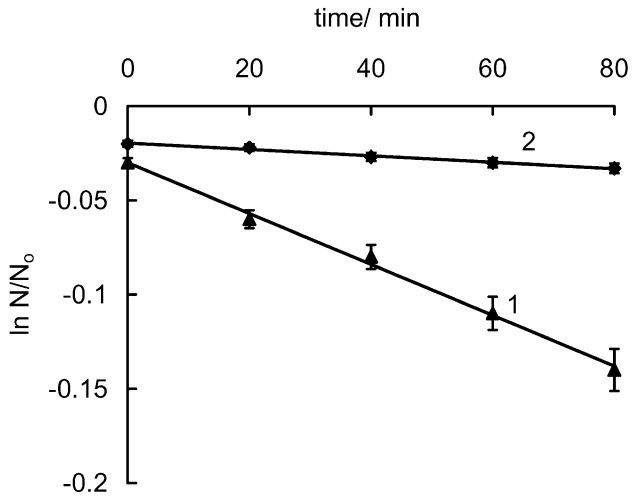

Fig. 1. Time courses of RBC destruction after erythrocyte exposure to HOCl (1.0 mM) and following resuspension in PBS, pH 7.4, (1) and in 300 mM mannitol buffered with 5 mM sodium phosphate, pH 7.4, (2); time of cell exposure to oxidant—10 min; PBS, pH 7.4; 22 ± 1 °C, haematocrit—10%. N/N_0—the fraction of cells lysed.

represents the kinetic curves of erythrocyte lysis after treatment and resuspension in different media. According to our measurements, the apparent rate constant of haemolysis of HOCl-treated (1.0 mM) RBCs in buffered 150 mM NaCl, pH 7.4 (PBS), was $(21.3 \pm 3.4)\cdot 10^{-6}$ s^{-1} and that in buffered 300 mM mannitol, pH 7.4 was $(2.5 \pm 0.1)\cdot 10^{-6}$ s^{-1}. Substitution of mannitol for PBS resulted in a significant diminution in the rate of haemolysis (Fig. 1). This effect can be rationalized assuming that like many other haemolytic agents, i.e. γ-radiation [19] or ethanol [20], oxidation by HOCl produces a limited number of pores in a cell membrane through which molecules of various diameters permeate at different rates. We calculated the size and the average number of pores formed as a result of RBC treatment with HOCl. This was accomplished using our data on the inhibition of haemolysis when HOCl-treated cells were suspended in mannitol containing solution instead of NaCl and the equation of Lieber et al. [21].

The apparent hole area would be:

$$A = d[(V^*)^2 - (V_0)^2]/2DV_0t_{50},$$

where d is the membrane thickness ($d = 6$ nm) [21]; V^* is the average erythrocyte aqueous volume of the maximally swollen cell (critical haemolytic volume); V_0 is the average RBC aqueous volume before the entry of the permeable osmotic solute; D is the diffusion coefficient of the solute in the medium; and t_{50} is the time required for 50% haemolysis (s) [19–21].

It can be assumed for human erythrocytes that $V_0 = 9.8 \times 10^{10}$ nm^3 and the osmotically active volume of the cell is 0.7 of its total volume [22].

V^* can be determined from the osmotic fragility curve (Fig. 2) as a sum of the osmotically inactive volume (V_{ina}) and the osmotically active volume (V_a) corresponding to 50% osmotic haemolysis, assuming that the osmotically active volume of the RBC behaves as a perfect osmometer and the osmotically inactive volume is not changed after HOCl-treatment [22].

The RBCs exposure to HOCl resulted in cell membrane damage and increased RBC osmotic fragility. Fig. 2 shows the osmotic fragility curves of erythrocytes treated with various doses of HOCl. The concentration of NaCl corresponding to 50% osmotic haemolysis of native erythrocytes was 63.5 ± 1.7 mM, whereas that corresponding to 50% haemolysis of cells treated with 1.0 mM HOCl was 67.5 ± 1.7 mM. Taking into account the osmolarity of the buffer, these values correspond to the osmolarity of 139.5 and 147.5 mosM, respectively. The critical haemolytic volume would be:

$$V^* = V_{ina} + V_{a\ iso}\left(\frac{C_{iso}}{C_{50\%}}\right),$$

where C_{iso} is the osmolarity of isoosmotic medium (300 mosM), $C_{50\%}$ is the osmolarity of the medium of 50% osmotic haemolysis. V^* was equal to $17.7\cdot 10^{10}$ nm^3 for nontreated cells and to $16.9\cdot 10^{10}$ nm^3 for damaged cells at 1.0 mM HOCl, respectively. Cell oxidation decreased the average aqueous haemolytic volume due to decreasing the membrane stability and deformability.

For the pair of solutes (mannitol and NaCl) of Stokes radii and diffusion coefficients of $a = 0.13$ nm and $D_a = 12.6 \times 10^{-10}$ m^2/s (NaCl) and of $b = 0.42$ nm and $D_b = 6.13 \times 10^{-10}$ m^2/s (mannitol) [19–21], t_{50} was

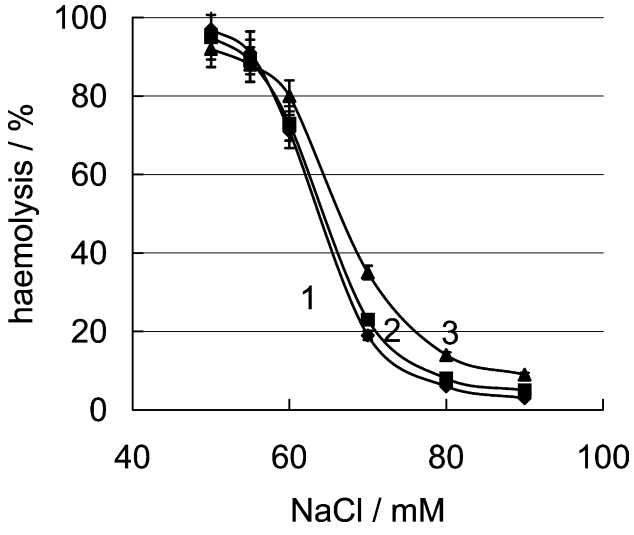

Fig. 2. Osmotic fragility curves of red blood cells treated with various doses of HOCl: control erythrocytes (1); 0.1 mM HOCl (2); 1.0 mM HOCl (3); exposure conditions: time of cell exposure to oxidant—10 min; PBS, pH 7.4; 22 ± 1 °C, haematocrit—10%.

measured to be 32343 s for NaCl solution and 276000 s for mannitol solution (Fig. 1). Two different A values $A_a = 1.42 \times 10^{-2}$ nm^2 (NaCl) and $A_b = 0.34 \times 10^{-2}$ nm^2 (mannitol) were obtained, respectively.

The true mean radius (r) and the number (n) of the pores can be calculated from:

$$r = (b\sqrt{A_a} - a\sqrt{A_b})/(\sqrt{A_a} - \sqrt{A_b}),$$

$$n = A_a/[\pi(r-a)^2] = A_b/[\pi(r-b)^2].$$

According to our calculations HOCl (1.0 mM) treatment of RBCs induces membrane pores with the apparent radius $r = 0.7$ nm, and the average pore number per cell is $n = 0.01$.

There were significant differences in the rate of haemolysis of RBCs when they were haemolysed at different pH. All cells were exposed to oxidant treatment in PBS at pH 7.4. Aliquots of these oxidant treated cells were evaluated for their rates of haemolysis at different pH's and significant differences were observed. The maximal stability of cells was observed at pH 7.2–7.3 (Fig. 3).

Shown in Fig. 4 is the loss of intracellular reduced glutathione after RBC exposure to HOCl (35–800 μM). The process of GSH oxidation is rapid, occuring in 1–2 min (data not shown). In our experiments GSH loss is linearly dependent on the ratio of HOCl/cell number. No significant changes in this dependence were observed at different cell concentrations (Fig. 4).

Using $DiSC_3(5)$, a fluorescence dye to measure membrane potential, we evaluated human RBC transmembrane electrical potential. It was equal to 12.6 ± 1.5 mV and

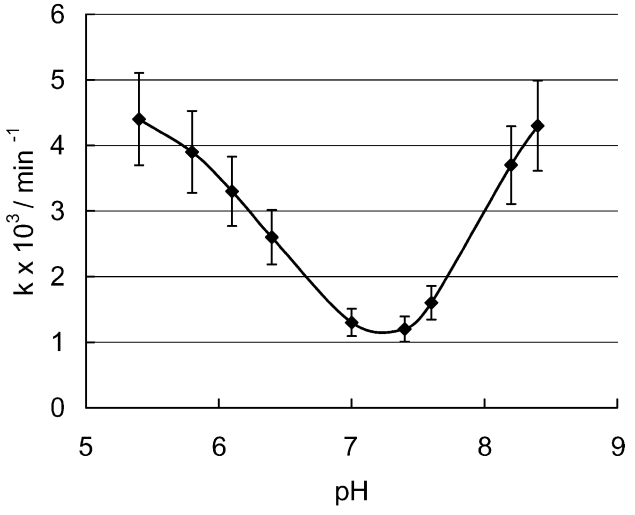

Fig. 3. Effect of incubation medium pH (PBS) on the apparent rate constant of HOCl-induced haemolysis; exposure conditions: 1.0 mM HOCl, time of cell exposure to oxidant—10 min; PBS, pH 7.4; 22 ± 1 °C, haematocrit—10%.

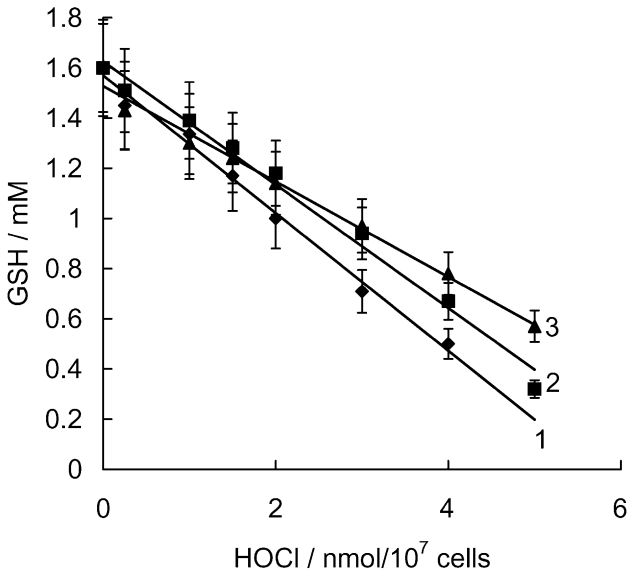

Fig. 4. Oxidation of red blood cell reduced glutathione caused by HOCl at different haematocrit—20% (1), 10% (2) and 5% (3); exposure conditions: time of cell exposure to oxidant—5 min; PBS, pH 7.4; 22 ± 1 °C.

treatment with hypochlorous acid did not produce any significant changes in it.

4. Discussion

Vissers and Winterbourn [7] showed that HOCl can easily penetrate the red blood cell membrane and cause GSH oxidation. The amount of the oxidant per cell rather than its concentration in cell suspension was important for cell injury (Fig. 4). There was almost a complete loss of intracellular GSH at 6–8 nmol of HOCl per 10^7 red blood cells (Fig. 4).

Vissers et al. [14] reported that the reduced glutathione and membrane thiol oxidation was not associated with cell lysis. This is in contrast to studies by others [12] who suggested that membrane thiol oxidation by HOCl resulted in lysis of P388D1 cells. In earlier studies, we demonstrated that HOCl treatment resulted in oxidation of RBC membrane thiols and tryptophan residues, membrane chloramine formation, inhibition of Na^+, K^+- and Mg^{2+}-ATPases, morphological transformation, including cell swelling as a result of water influx, and haemolysis [23].

The increase in osmotic fragility (Fig. 2) reflected membrane impairment and cell destabilization produced by HOCl. However, no significant changes of RBC membrane electrical potential were observed following HOCl-treatment. From that, we believe that the pore formed is not ion-selective and that K^+ efflux through the damaged membrane is probably compensated by Na^+ influx.

The pH-dependence of the apparent rate constant of haemolysis (Fig. 3) might be the result of pH-induced structural transition of the RBC membrane. Earlier we observed a similar pH-dependence for the rate of ethanol-

induced haemolysis of human erythrocytes [24]. Likewise maximal RBC stability was between 7.2 and 7.3.

We conclude that the pore formation and disturbance in passive ion permeability is the reason of the HOCl-induced haemolysis (earlier we showed that the disturbance in active membrane ion flows is also due to HOCl induced membrane impairment [23]). This agrees with earlier data indicating that HOCl-induced haemolysis occurred by a colloid–osmotic mechanism that involved disruption of the membrane [14,15]. The disruption causes K^+-leakage and gradual cell swelling [14,15]. Modification of RBC membrane protein and cross-link formation might result in clustering of band 3 and other membrane and cytoskeletal proteins to form haemolytic pores [15].

HOCl-treatment of RBCs induces damage of membrane proteins and lipids, which eventually bring about formation of transient dynamic non-selective short-lived pores. The average number of pores per cell is less than 1.00. Similar numbers of pores are formed following exposure of RBCs to γ-irradiation [20] or photosensitization [25]. For example, in recent studies we have shown that photosensitized lysis of RBCs occurs due to the formation of the short-lived pores. In those studies, the average pore number per cell was 0.02 and the apparent pore radius was estimated to be 1.1 nm [25]. The relatively low number of pores may be the result of the small fraction of time during which at least one haemolytic pore is present in the HOCl-treated cell membrane. In summary, HOCl-treatment seems to create transient pores. Such pores remain open for a limited fraction of time and allow electrolyte movement through the membrane. The process of pore opening is pH-dependent, which agrees with earlier conclusions that conductivity and selectivity of the plasma membrane pores are dependent on ionisation state of neighbouring groups in the membrane [26].

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