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Effect of N-chloroamino acids on the erythrocyte

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Abstract
Amino acids present in blood plasma may be targets for oxidation and chlorination by $HOCl/OCl^-$. N-Chloroamino acids have been reported to be less reactive, but more selective than $HOCl/OCl^-$ in their reactions; therefore, they may act as secondary mediators of $HOCl/OCl^-$-induced injury. This study compared the effects of five N-chloroamino acids (AlaCl, LysCl, SerCl, AspCl and PheCl) on erythrocytes with the action of $HOCl/OCl^-$. The N-chloroamino acids differed in stability and reactivity. They had a weaker haemolytic action than $HOCl/OCl^-$; $HOCl/OCl^-$, AlaCl and PheCl increased osmotic fragility of erythrocytes at a concentration of 1 mM. Oxidation of glutathione, formation of protein-glutathione mixed disulphides and efflux of GSSG from erythrocytes were observed for erythrocytes treated with all the employed chloroderivatives, while increased oxidation of 2′,7′-dichlorofluorescin was detected only after treatment of the cells with 1 mM $HOCl/OCl^-$, AlaCl and PheCl. Generally, the reactivity of at least some N-chloroamino acids may be not much lower with respect to $HOCl/OCl^-$.

Keywords: *Hypochlorous acid, chloramines, amino acids, erythrocyte, glutathione, oxidative stress*

Abbreviations: *OPA,* o*-phtalaldehyde; PBS, phosphate-buffered saline; RBC, red blood cell; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; TNB, 5-thio-2-nitrobenzoic acid; RQB, red-ox quenching buffer; GSSG, glutathione disulphide*

Introduction

Hypochlorous acid, produced by myeloperoxidase in activated phagocytes [1,2], is an important means of chemical defense against pathogens and a major bactericidal factor [3–5]. However, hypochlorous acid can also injure host cells, especially under pathological conditions when it can reach even millimolar concentrations at the site of inflammation [6,7].

Hydrochloric acid, partly dissociated to hypochlorite ($pK_a = 7.6$) at physiological pH, acts both as a strong, highly reactive oxidant and a chlorinating factor [8], reacting with protein thiol groups, thioethers and glutathione and converting them to protein or glutathione disulphides, sulphonamides, mixed protein-glutathione disulphides and dehydroglutathione [9–11]. The best known chlorination reactions are those with free amine groups of protein or amino acids [8], double bonds of unsaturated fatty acids and cholesterol [9,12], tyrosyl residues [13,14] and purine and pyrimidine bases [15–18]. The chlorination products so formed have been reported to be less reactive, but more specific in their reactions than hypochlorous acid [19].

Amino acids present in human plasma and interstitial fluid are potential targets of $HOCl/OCl^-$ oxidation and chlorination. The major reaction is chlorination of amine groups with formation N-chloro- or N,N-dichloroamino acids [20]:

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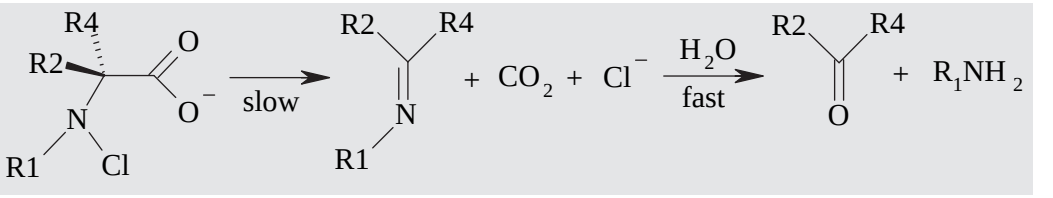
$$R_1NH_2 + HOCl \; R_1NHCl + H_2O \rightarrow$$

$$R_1NHCl + HOCl \; R_1NCl_2 + H_2O \rightarrow$$

N-Monochlororamino acids are formed when the ratio of amino acid concentration to $HOCl/OCl^-$ concentration is 1:1 or higher. When hypochlorous acid is present in excess, N,N′-dichloroamino acids are generated. The stability of N-chloramino acids depends on their structure and environmental conditions. In the absence of other reagents, in near-neutral medium, they undergo fragmentation yielding aldehydes or ketones, carbon dioxide, ammonia or primary amines and chloride ion.



N-Chloroamino acids have been reported to be selective for different cellular thiols. Their reactivity correlates inversely with the pK_a value of thiols which is dependent in turn on the presence of charged residues in the close environment [21,22]. The transchlorination reactions were observed between N-chlorohistamine (HisCl), N-chloroglycine (GlyCl) and N-chlorotaurine and were postulated to be an important facet of the cytotoxicity of these compounds [23]. There is also evidence that N-chloroamino acids react with NADH yielding NADH-chlorohydrins as products [24]. Because of lower reactivity, they may be dangerous mediators of $HOCl/OCl^-$-induced lesions and damage of intracellular macromolecules by oxidation and chlorination [23].

While the cellular effects of $HOCl/OCl^-$ have been extensively studied, the effects of N-chloroamino acids on cells are relatively less documented. This study was aimed at definition of some general properties of N-chloroamino acids and a comparison of their action and that of $HOCl/OCl^-$ on a simple model cell, the human erythrocyte.

Materials and methods

Reagents

2′,7′-dichlorofluorescin diacetate were obtained from Molecular Probes (Leiden, Netherlands). *o*-Phtalaldehyde was purchased from POCh (Gliwice, Poland). All other materials were obtained from Sigma (Deisenhofen, Germany).

Synthesis of N-chloroamino acids

The synthesis of N-chloroamino acids was carried out immediately before use. $HOCl/OCl^-$ at a concentration of 40 mM in phosphate-buffered saline (145 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4; PBS), obtained by dilution of a concentrated stock solution of sodium hypochlorite, was added to amino acid solutions in PBS (40 mM) at a volume (and molar) ratio of 1:5, mixed and incubated for 2 min. Only monochloramines of amino acids are formed under these conditions [25].

Erythrocytes

Blood of healthy donors anti-coagulated with 3.2% sodium citrate was obtained from Central Blood Bank in Lodz. The erythrocytes were isolated and purified by three repeated cycles of centrifugation ($2000 \times g$, 4°C, 5 min) and resuspension in 20 volumes of PBS. The buffy coat was taken off carefully and the residual leukocytes were removed by passing the cell suspension through a column of HBS cellulose.

Determination of concentration and stability of N-chloroamino acids

N-Chloroamino acids were quantified by the reaction with 5-thio-2-nitrobenzoic acid (TNB), prepared from 5,5′-dithio-2-nitrobenzoic acid (1 mM) by alkaline hydrolysis with an equal volume of NaOH (50 mM) for 5 min and dilution with 100 mM phosphate buffer (pH 7.4). TNB solution was added with freshly prepared N-chloroamino acids at a ratio of 500:1 and incubated at room temperature for 15 min. The decrease in absorbance at 412 nm was read and N-chloroamino acid concentration was calculated using the molar absorption coefficient of 13 600 M^{-1} cm^{-1}. In order to ascertain the lack of $HOCl/OCl^-$ in the reaction mixture, absorption spectra of the solutions were taken in a range of 200–500 nm 2 min after addition of $HOCl/OCl^-$.

Oxidation of 2′,7′-dichlorofluorescin

The ability of N-chloroamino acids to oxidize molecules used for detection of reactive oxygen species was studied using 2′,7′-dichlorofluorescin. The fluorogenic probe (final concentration of 5 μM) was mixed with N-chloroamino acids or $HOCl/OCl^-$ (final concentrations of 100 μM) and the rate of fluorescence increase was monitored at room temperature ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 538$ nm) for 10 min.

Concentration of the oxidized probe was read from calibration curve obtained with 2′,7′-dichlorofluorescein as a standard [26,27].

2′,7′-dichlorofluorescin was obtained after 1-h alkaline hydrolysis of 5 mM 2′,7′-dichlorofluorescin diacetate with 2 M NaOH in the presence of methanol, neutralization with 2 M HCl and dilution with 0.1 M phosphate buffer, pH 7.0.

Haemolysis and osmotic fragility

For studies of haemolysis induced by $HOCl/OCl^-$ and N-chloroamino acids, erythrocyte suspensions (hematocrit of 16%) were incubated in the presence and absence of the chlorinating agents at room temperature for 1, 5 and 10 h. Then the samples were centrifuged. The absorbance of the supernatants and of haemolysed red cell pellets was read after centrifugation ($5000 \times g$, 23°C, 10 min) at 540 nm. Percentage haemolysis was calculated on the basis of haemoglobin release

$$H\,(\%) = [A_{540\ \text{supernatant}}/A_{540\ \text{supernatant}} + A_{540\ \text{haemolysed pellet}}] \times 100\%$$

Osmotic fragility was determined after 30-min incubation of erythrocytes (hematocrit of 16% in PBS) with $HOCl/OCl^-$ or N-chloroamino acids. The erythrocytes were washed three times with PBS, then 25 µl of RBC suspension was added to 975 µl of NaCl solutions (40–100 mM in 10 mM sodium phosphate buffer pH 7.4, final hematocrit of 0.4%), mixed, incubated at 23°C for 5 min and centrifuged ($500 \times g$, 23°C, 5 min). Percentage haemolysis was estimated as above. For each sample the c_{50} value (NaCl concentration causing 50% haemolysis) was estimated.

Content of glutathione, glutathione disulphides and glutathione-protein mixed disulphides

Suspension of erythrocytes (RBCs) in PBS (hematocrit of 16%) treated with different concentration of $HOCl/OCl^-$ and N-chloroaminoacids at room temperature was centrifuged ($500 \times g$, 5 min, 23°C). The supernatant was transferred to new test-tubes and the pellet was washed three times with PBS. Proteins of the supernatant and of the erythrocyte pellet precipitated with RQB-TCA buffer (20 mM HCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM ascorbic acid, 5% trichloroacetic acid) were then centrifuged ($5000 \times g$, 23°C, 10 min) and the supernatant was taken for GSH and GSSG assay [28].

For GSH determination, 2 µl of deproteinized supernatant diluted to 25 µl with RQB-TCA were put on two wells (denoted '+' and '−') of a 96-well black plate. The sample '−' was added with 4 µl of 7.5 mM N-ethylmaleimide in RQB-TCA, both samples added with 40 µl of 1 M potassium phosphate buffer, pH 7.0, mixed for 1 min and incubated at room temperature for 5 min. Then 160 µl of 0.1 M potassium phosphate buffer was added, followed by 25 µl of 0.5% *o*-phtalaldehyde in methanol and the plate was shaken (1 min). After 30-min incubation (room temperature) the fluorescence was read at 355 nm/460 nm. The value obtained for the '−' sample was subtracted from that obtained for the '+' value and GSH concentration was read from a calibration curve obtained with glutathione as a standard.

For determining GSSG concentration, two paired samples, '+' and '−', each containing 25 µl of deproteinized supernatant, were added with 4 µl of 7.5 mM N-ethylmaleimide in RQB-TCA and 40 µl of 1 M potassium phosphate buffer. Then, 5 µl of 100 mM sodium dithionite in RQB-TCA was introduced into the sample '+'. The mixture was incubated at room temperature for 60 min. The remaining part of the procedure was the same as for GSH estimation. The calibration curve was prepared with GSSG.

For determination of glutathione-protein mixed disulphides, proteins were precipitated with RQB-TCA buffer, centrifuged ($500 \times g$, 23°C, 10 min), washed twice with RQB-TCA and once with distilled water. The pellet was dissolved in 8 M urea in 0.2 M phosphate buffer, pH 7.4, mixed with 0.1 M potassium borohydride in 0.2 M phosphate buffer, pH 7.4, at a volume ratio of 1:3 and incubated at 40°C for 30 min. The excess of borohydride was decomposed by lowering the pH to ~2.6 with 6 M HCl and 1-h incubation. After this time, 5 M NaOH was added to bring the pH to ~8 and the procedure for GSH estimation was carried out as above.

Glutathione and glutathione disulphide content was also estimated in supernatants obtained by centrifugation of RBC suspension after 1-h incubation with the chloroderivatives.

Oxidation of 2′,7′-dichlorofluorescin in erythrocytes treated with chloroderivatives

For estimation of reactive oxidants inside erythrocytes the 2′,7′-dichlorofluorescin diacetate was used as a probe. After 1-h incubation with chloroderivatives, RBC suspension (hematocrit of 16%) was washed three times with PBS (centrifugation: $500 \times g$, 25°C, 5 min), diluted to a hematocrit of 5% with PBS, pipetted onto a 96-well black plate and added with 2′,7′-dichlorofluorescin diacetate (5 µM final). After 30-min incubation at 36°C the fluorescence was read at 485 nm/538 nm and the concentration of the oxidized probe was read from a calibration curve prepared with 2′,7′-dichlorofluorescein as a standard.

Haemoglobin concentration

Haemoglobin concentration was estimated by absorbance measurements at 540 nm [29].

Results

Formation and stability of N-chloroamino acids

To exclude the possibility of incomplete consumption of $HOCl/OCl^-$ in the reaction of N-chloroamino acid production, its presence in the post-reaction mixture was verified spectrophotometrically. For each amino acid, molar absorption at ~250 nm increased after HOCl addition, indicating formation of N-chloroderivative. For no amino acid, the presence of residual $HOCl/OCl^-$ was found after 2-min reaction time as judged from the disappearance of $HOCl/OCl^-$ spectrum and appearance of spectrum typical for N-chloroamino acid (not shown). The yield of formation of N-chloroamino acids, estimated on the basis of TNB decolourization, was different for various amino acids. The lowest yield was observed for alanine (0.57 mol/mol $HOCl/OCl^-$) and the highest for lysine and serine (0.81 and 0.70 mol/mol HOCl, respectively; Figure 1).

N-chloroamino acids differed in stability. Their concentrations decreased rapidly in PBS (pH 7.4) at 23°C and 30°C. N-chloroalanine and N-chloroaspartic acid were the most and the least stable, respectively. The half-life time was ~37 min and 9 min for N-chloroalanine and N-chloroaspartic acid at 30°C, respectively (Figure 2).

Oxidation of 2′,7′-dichlorofluorescin by N-chloroamino acids

$HOCl/OCl^-$ caused immediate 2′,7′-dichlorofluorescin oxidation (within 1 min). The concentration of the oxidized probe after 1 min incubation with $HOCl/OCl^-$ at room temperature was 308.2 ± 47.8 μmol/mol $HOCl/OCl^-$ and only a slight increase was observed upon further incubation (405.8 ± 65.4 and 450.8 ± 44.2 after 5 min and 10 min, respectively). N-chloroamino acids also oxidized the fluorogenic probe but at lower rates. The increase of fluorescence of the product of this reaction, 2′,7′-dichlorofluorescein, was a linear function of time. The highest rate of oxidation of 2′,7′-dichlorofluorescin was found for N-chlorolysine (Figure 3).

Haemolysis

N-chloroamino acids showed a weak haemolytic activity. Treatment of erythrocytes with N-chloroamino acids followed by 10-h incubation at room temperature did not lead to any significant increase in haemolysis except for the highest concentrations of N-chloroalanine and N-chlorophenylalanine tested. $HOCl/OCl^-$ was much more potent, causing concentration- and time-dependent haemolysis starting from the time of 1 h (Table I). N-chlorolysine, N-chloroserine and N-chloroaspartic acid did not cause haemolysis at any concentration tested (data not shown).

Osmotic fragility

Hypochlorous acid increased osmotic fragility of erythrocytes. Its effect was concentration-dependent and became statistically significant at the concentration of 1 mM. The effects of various N-chloroamino acids on the osmotic fragility differed. No tendency to increase the osmotic fragility was found for N-chlorolysine, N-chloroaspartic acid and N-chloroserine (data not shown) but N-chloroalanine and N-chlorophenylalanine had an effect similar to that of $HOCl/OCl^-$, bringing about a significant increase of osmotic fragility at the concentration of 1 mM (Table II).

Glutathione content

Exposure of erythrocytes to N-chloroamino acids resulted generally in a concentration-dependent decrease in the glutathione content. The diminution of cellular glutathione content was most prominent for N-chlorophenylalanine and for N-chloroalanine

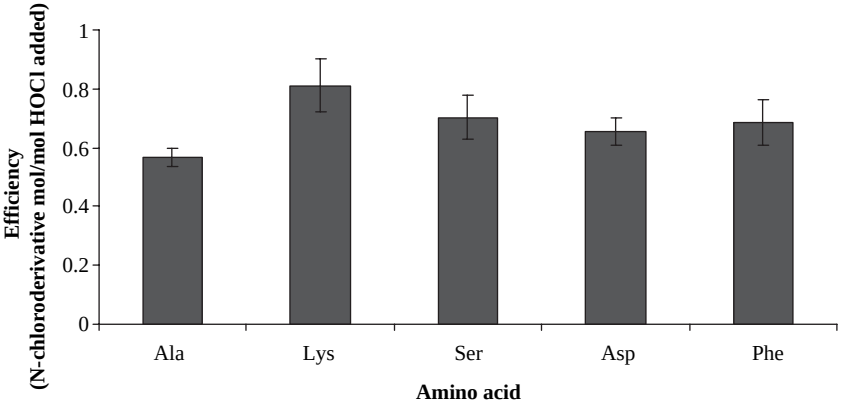


Figure 1. Yield of N-chloroamino acids after treatment of various amino acids with $HOCl/OCl^-$. 40 mM $HOCl/OCl^-$ was added to amino acid solutions in PBS (40 mM) to obtain a final molar ratio of 1:5 and incubated for 2 min. The concentration of N-chloroamino acids was determined with TNB. Mean ± SD; $n \geq 3$.

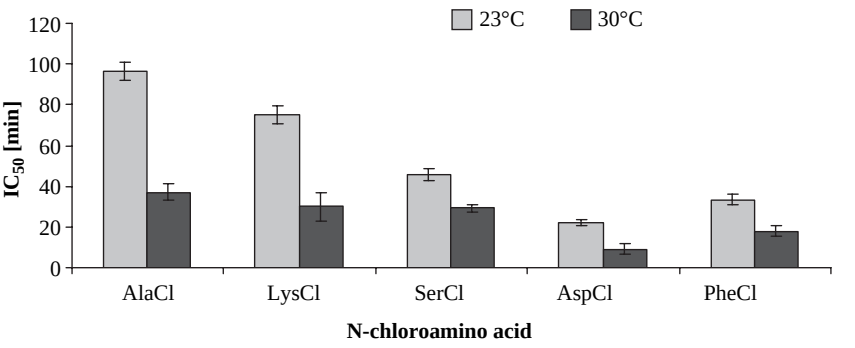


Figure 2. Stability of N-chloroamino acids. The value of IC_{50} for each was estimated on the basis of reduction of the N-chloroamino acids concentration as a function of time, determined with TNB decolourization assay for each chlorocompound.

and exceeded that induced by $HOCl/OCl^-$, N-chlorolysine, N-chloroserine and N-chloroaspartic acid (Table III).

Glutathione disulphide content

N-chloroamino acids and $HOCl/OCl^-$ have the ability to oxidize intracellular glutathione and bring about glutathione disulphide (GSSG) formation. The highest GSSG concentration was found for 1 mM N-chlorophenylalanine, N-chloroalanine and N-chlorolysine (Table III).

Glutathione and glutathione disulphde efflux from erythrocytes

The loss of cellular content of glutathione induced by N-chloroamino acids and $HOCl/OCl^-$ was also due to its efflux outside the RBCs. The GSSG made up the large majority of the total pool of exported glutathione. N-chloroserine, N-chloroaspartic acid and N-chlorophenylalanine induced the highest extrusion of glutathione disulphide ($\sim$1 μmol/g Hb) (Table IV).

Content of glutathione-protein mixed disulphides

Apart from glutathione disulphide formation, an increase in the level of mixed protein-glutathione disulphides was noticed. N-Chloroalanine (1 mM) was the most effective, followed by N-chlorolysine, inducing mixed disulphides at the levels of 1.43 ± 0.16 and 1.22 ± 0.12 μmol/g Hb, respectively. The glutathione-protein mixed disulphides were formed already at the lowest concentrations of all the chloroderivatives used (Table III).

Oxidation of 2,7-dichlorofluorescin in erythrocytes treated with the chloroderivatives

The increased level of reactive oxidants inside erythrocytes incubated for 1 h with N-chloroamino acids or $HOCl/OCl^-$, estimated on the basis of 2′,7′-dichlorofluorescein formed, was observed for 1 mM concentrations of $HOCl/OCl^-$, N-chlorophenylalanine and N-chloroalanine. The remaining chlorocompounds did not bring about significant changes in the oxidation of 2′,7′-dichlorofluorescin in erythrocytes (Figure 4).

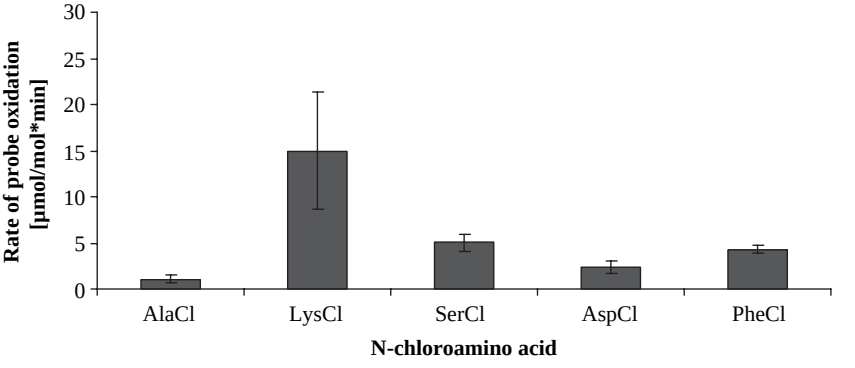


Figure 3. 2′,7′-dichlorofluorescin oxidation by N-chloroamino acids. Oxidation rate of 2′,7′-dichlorofluorescin was followed at room temperature at 485 nm/538 nm for 10 min and expressed as the amount of the probe oxidized by 1 mol of analysed compounds per 1 min. Autoxidation of 2′,7′-dichlorofluorescin was subtracted. 2′,7′-dichlorofluorescein concentration was determined from a standard curve.

Table I. Induction of haemolysis by N-chloroamino acids and $HOCl/OCl^-$.

N-chlorocompound concentration (μM) / Time (h)	Haemolysis (%)			
	125	250	500	1000
N-chloroalanine				
1 h	0.11±0.05	0.12±0.04	0.13±0.04	0. 15±0.05
5 h	0.18±0.08	0.18±0.07	0.19±0.04	0.16±0.06
10 h	0.21±0.03	0.25±0.06	0.33±0.07	0.58*±0.10
N-chlorophenylalanine				
1 h	0.13±0.03	0.14±0.05	0.15±0.05	0.16±0.04
5 h	0.15±0.06	0.17±0.08	0.18±0.09	0.20±0.10
10 h	0.17*±0.03	0.27±0.01	0.29±0.06	0.41*±0.05
$HOCl/OCl^-$				
1 h	0.86*±0.03	1.94*±0.59	3.87*±0.96	7.62*±0.35
5 h	1.26*±0.10	1.93*±0.36	5.19*±1.30	8.92*±1.71
10 h	1.41*±0.08	2.67*±0.37	6.21*±0.31	9.91*±2.03

Haemolysis was estimated on the basis of haemoglobin release after various times of incubation in the presence or absence of N-chloroamino acids and $HOCl/OCl^-$ at room temperature. For the control, haemolysis was 0.14±0.04%, 0.24±0.02% and 0.29±0.03% after 1, 5 and 10 h, respectively. Mean±SD; $n \geq 3$. *$p < 0.05$.

Discussion

The oxidative damage of cells and their constituents by $HOCl/OCl^-$ is well described and understood. It has been suggested and demonstrated for many systems that N-chloroamino acids, formed upon reaction with $HOCl/OCl^-$, decrease the concentration of this chlorinating agent but exert similar effects themselves. Taurine has been reported to prevent the production of nitrite and TNF-α by lung cells in the presence of myeloperoxidase-H_2O_2-halide system via N-chlorotaurine formation [30], but on the other hand N-chlorotaurine has also been implicated in intracellular glutathione loss [31] and apoptosis induction by triggering the p-53 dependent pathway [32].

Amine group of proteins, apart from thiols, are the major targets of $HOCl/OCl^-$ acid attack in proteins [8]. In free amino acids which (except for cysteine) lack thiol groups, mostly amine groups are attacked by HOCl. When amino acids react with HOCl at a ratio of 5:1, only N-monochloramines are formed [25]. In these experiments, N-chloroamino acid formation was confirmed by the appearance of a characteristic absorption peak with a maximum at ~250 nm in the absorption spectrum of amino acids after hypochlorite addition [33] (not shown). This absorption peak is often employed for the determination of chloramines concentration [34]; however, this approach cannot be used with respect to aromatic amino acids. Therefore, TNB decolourization assay [25] was employed for determination of concentration of N-chloroamino acids in this study.

As it has already been reported for histamine and glycine, N-chloroamino acids show relatively low stability [23]. The general mechanism proposed for their decomposition postulates formation aldehydes or ketones, carbon dioxide, ammonia or primary amines and chloride ion [20]. However, other authors demonstrated also other ways of their decomposition leading to formation of nitrogen-centred radicals as a result of cleavage of the N-Cl bond [35]. Results of this study demonstrate that the rate of decomposition varies significantly between the chloroderivatives of various amino acids and seems to be dependent on their structure or chemical properties. The experiments conducted at 23°C and 30°C indicate that N-chloroalanine is the most stable of all the analysed N-chloroamino acids and N-chloroaspartic acid is the least stable, which is in good agreement with previously published results [33].

2′,7′-dichlorofluorescin was employed as a model chemical target for the oxidative action of the analysed chlorocompounds. We found that this fluorogenic probe is oxidized by all N-chloroamino acids. Interestingly, the reactivity of N-chloroamino acids did not correlate with their stability in the absence of other reactants. Positively charged N-chlorolysine was found to be the most reactive

Table II. Effect of N-chloroamino acids and $HOCl/OCl^-$ on the osmotic fragility of erythrocytes.

Chloroderivative	c_{50} (mM)
AlaCl (250 μM)	58.4±0.5
AlaCl (500 μM)	58.4±1.0
AlaCl (1000 μM)	60.7±0.6*
PheCl (250 μM)	58.1±0.9
PheCl (500 μM)	59.6±2.4
PheCl (1000 μM)	61.1±2.1*
$HOCl/OCl^-$ (250 μM)	57.4±0.2
$HOCl/OCl^-$ (500 μM)	58.2±0.2
$HOCl/OCl^-$ (1000 μM)	62.5±2.5*

Osmotic fragility of erythrocytes, treated with N-chloroamino acids or $HOCl/OCl^-$ for 30 min was estimated by measurement of percentage haemolysis in hypotonic NaCl solutions (40–100 mM). For each sample the c_{50} value (NaCl concentration which causing 50% haemolysis) was determined. The value for control was 57.2±0.5 mM. Each result represents mean±SD; $n \geq 3$. *$p < 0.05$.

Table III. Effect of N-chloroamino acids and HOCl/OCl^- on the erythrocyte content of reduced glutathione, glutathione disulphides and mixed protein-glutathione disulphides.

Chlorocompound (μM)	AlaCl	LysCl	SerCl	AspCl	PheCl	HOCl/OCl^-
Glutathione (μmol/g Hb)						
125	4.71±0.40*	5.20±0.32	4.73±0.36*	4.80±0.37*	3.97±0.40*	4.21±0.50*
250	2.77±0.37*	4.09±0.56*	3.99±0.49*	3.86±0.35*	2.28±0.33*	3.63±0.57*
500	1.38±0.17*	3.60±0.57*	3.33±0.26*	2.68±0.32*	0.43±0.15*	3.36±0.31*
1000	0.57±0.19*	2.22±0.19*	2.38±0.33*	2.13±0.16*	0.17±0.09*	2.26±0.26*
Glutathione disulphide (nmol/g Hb)						
125	499.8±71.0*	265.3±57.8*	56.5±27.4	57.4±29.0	307.0±25.5*	257.9±36.3*
250	794.1±86.7*	362.5±77.6*	99.8±30.7	164.1±25.7*	715.7±97.8*	248.1±87.1*
500	1045.1±103.2*	481.1±71.0*	98.3±21.0	206.6±53.6*	1113.6±178.9*	337.2±56.5*
1000	1159.8±104.8*	1138.2±103.8*	123.6±34.2*	309.4±54.1*	1564.9±121.7*	496.4±68.5*
Glutathione-protein mixed disulphides (nmol/g Hb)						
125	404.5±78.0*	252.3±69.1*	269.1±71.4*	233.2±19.5*	220.4±64.6*	306.0±76.1*
250	526.0±99.8*	327.5±78.8*	354.7±82.0*	243.2±13.1*	390.4±68.0*	458.5±76.5*
500	844.8±95.1*	615.0±107.9*	593.3±96.6*	398.1±86.8*	680.0±113.5*	518.2±76.7*
1000	1427.9±164.5*	1220.5±117.4*	772.1±120.9*	705.1±115.6*	979.5±136.2*	839.6±26.1*

Glutathione, glutathione disulphide and protein-glutathione mixed disulphides were determined after 1-h incubation of erythrocytes in PBS with various concentrations of chloroderivatives analysed at room temperature. Control values were: glutathione, 5.97±0.31 μmol/g Hb; glutathione disulphide, 36.8±15.3 nmol/g Hb; protein-glutathione mixed disulphides, 44.9±10.2 nmol/g Hb. Each result represents mean±SD; $n \geq 3$. *$p < 0.05$ with respect to untreated control.

towards anionic 2′,7′-dichlorofluorescin, while N-chloroalanine and N-chloroaspartic acid showed low reactivity (Figures 2 and 3).

HOCl has been reported to cause red blood cell lysis and induce numerous alterations in erythrocyte membranes including inhibition of ATPase activities (especially Na^+/K^+-ATPase), changes of membrane surface area, oxidation of thiol groups and tryptophan, pre-haemolytic K^+-leak and inhibition of aminophospholipid translocase [36]. In this study, HOCl/OCl^- appeared to be the most efficient haemolytic factor, leading to time- and concentration-dependent RBC lysis, already after 1 h incubation of RBCs with HOCl/OCl^- at a concentration of 125 μM (i. e. at a ratio of $\sim 4*10^7$ molecules/cell). The haemolytic action of HOCl/OCl^- has been ascribed to its ability to induce membrane pore formation. The percentage of haemolysed erythrocytes (hematocrit of 10%) was found to reach ~11% when measured 1 h after 10 min incubation with 1 mM HOCl/OCl^- [37]. Our experiments indicate that 60-min incubation of RBCs with 1 mM HOCl/OCl^- causes haemolysis reaching ~7.5% for hematocrit of 16%. N-chloroamino acids showed a weaker haemolytic action than that of HOCl/OCl^- (Table I). N-chlorolysine, N-chloroserine and N-chloroaspartic acid did not increase erythrocyte susceptibility to haemolysis and for N-chloroalanine and N-chlorophenylalanine a slight effect was observed only at the highest concentration used and the longest incubation time. In another study, the haemolytic action of monochloramine (NH_2Cl) has been documented [38]. NH_2Cl was 10 times less effective than HOCl as a haemolytic agent. Monochlorated taurine has also been reported to cause oxidative events inside erythrocyte, but only little haemolysis [39]. HOCl/OCl^-, N-chloroalanine and N-chlorophenylalanine were also found to increase erythrocyte osmotic

Table IV. Glutathione and glutathione disulphide in the extracellular medium of erythrocytes treated with chlorocompounds.

Chlorocompound (μM)	AlaCl	LysCl	SerCl	AspCl	PheCl	HOCl
Glutathione in the extracellular medium (nmol/g Hb)						
125	1.14±0.23	3.79±0.42	4.17±4.04	5.68±2.05	0.76±0.19	6.06±0.61
250	1.52±0.72	2.27±0.66	3.03±0.38	6.44±0.55	3.03±0.38	2.65±0.54
500	5.30±2.05	7.58±3.30	12.10±3.76	12.50±7.07	4.55±0.64	7.95±2.24
1000	1.52±0. 71	9.09±6.04	5.30±0.50	6.06±0.54	1.52±0.97	3.03±0.35
Glutathione disulphide in the extracellular medium (nmol/g Hb)						
125	21.2±10.0	17.0±1.1	246.9±1.8*	230.7±20.1*	190.6±42.5*	26.5±15.8
250	157.3±38.6*	103.7±74.8	447.0±65.1*	358.9±58.3*	570.4±60.6*	84.7±84.3
500	393.5±61.0*	198.5±43.4*	618.0±76.5*	732.7±83.3*	508.4±69.4*	94.3±29.9*
1000	597.9±86.2*	219.4±50.2*	1055.4±122.2*	979.6±73.9*	929.4±93.8*	234.1±49.2*

The concentration of glutathione and glutathione disulphide was estimated in supernatants of centrifuged erythrocyte suspensions after 1-h incubation with chlorocompounds and calculated per intracellular haemoglobin. Control values: glutathione, 4.9±3.7 nmol/g Hb; glutathione disulphide, 24.9±8.7 nmol/g Hb. Each result represents mean±SD; $n \geq 3$. *$p < 0.05$ with respect to untreated control.

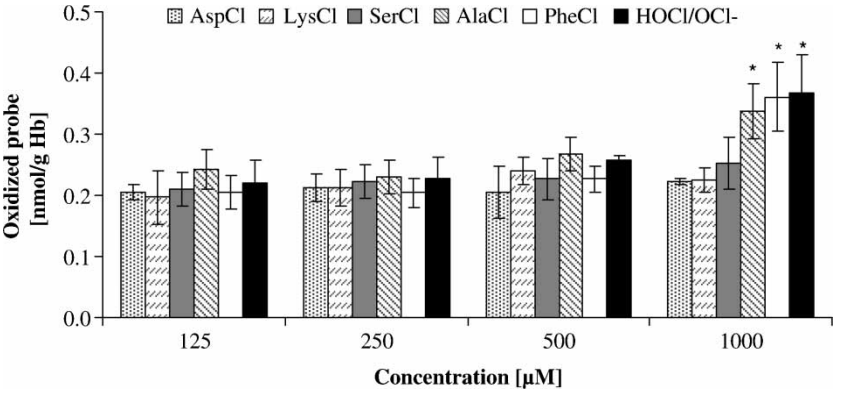


Figure 4. 2′,7′-dichlorofluorescin oxidation in erythrocytes treated with chloroderivatives. Oxidation of 2′,7′-dichlorofluorescin in erythrocytes was measured 1 h after cell treatment with chloroderivatives. The value for control samples was 0.184 ± 0.011 nmol/g Hb. Each result represents mean $\pm$ SD; $n \geq 3$. $^{*}p < 0.05$.

fragility (Table II), but only after 30-min incubation at the highest concentration employed (1 mM). The greatest shift in the c_{50} value was observed for HOCl/OCl^-. It should be mentioned, however, that blood plasma concentrations of HOCl/OCl^- and chloramines can reach even millimolar values at the sites of inflammation [7].

The covalent bond between chlorine and the amine group nitrogen alters the charge of amino acid molecules, preventing zwitterion formation [39]. It gives the possibility for N-chloroamino acids to be transported across the erythrocyte membrane by the anion transport system which was observed for taurine chloramine and demonstrated to lead to depletion of intracellular glutathione and ATP and to haemoglobin oxidation [39]. N-chloroamino acids can probably be substrates for amino acid transport systems present in the erythrocyte membrane or transchlorinate erythrocyte components. Results of this study suggest an efficient transport of N-chloroamino acids into the erythrocyte since they oxidized intracellular glutathione. Interestingly, N-chloroalanine and N-chlorophenylalanine were even more efficient in oxidation of erythrocyte glutathione than HOCl/OCl^-, in spite of their weak haemolytic action (Table III).

It has been demonstrated previously that exposure of human erythrocytes to HOCl/OCl^- resulted in loss of intracellular glutathione and its conversion into glutathione disulphide [40]. The present experiments document and quantify also the formation of protein-glutathione mixed disulphides. Harwood et al. [11] postulated also two other possible products of myeloperoxidase-derived oxidant reaction with glutathione under acellular conditions: sulphonamide and dehydroglutathione. One of them, sulphonamide, was detected also in endothelial cells treated with HOCl/OCl^- and determined to be a major product of glutathione oxidation in these cells. We calculated that the ratio of HOCl/OCl^- employed to oxidized glutathione is 4:1, in excellent agreement with previous reports [41]. The decrease of glutathione concentration after HOCl/OCl^- treatment was similar to that observed for N-chlorolysine, N-chloroserine and N-chloroaspartic acid at the highest concentration used. Our results indicate that for HOCl/OCl^- the process of mixed disulphide formation seems to play the major role in the decrease of glutathione concentration, while formation of glutathione disulphide and its export are of secondary importance (Tables III and IV).

The protein-glutathione mixed disulphides were reported to be the major products of glutathione oxidation in human fibroblasts treated with N-chlorohistamine [42]. Our study shows that the concentration and type of glutathione oxidation products depend on the N-chloroamino acid used, apparently due to differences in their stability, reactivity, membrane permeability and selectivity towards substrates. N-chloroamino acids in contrast to HOCl/OCl^- demonstrate higher selectivity in reactions with thiol-containing molecules [21] and this may be the main reason of relatively small decrease of glutathione in RBCs treated with HOCl/OCl^-, a stronger but less selective oxidant. Membrane permeability seems to be also important, since the easily permeable monochloramine caused a higher glutathione loss than the less membrane-permeable N-chlorotaurine. We did not observe any correlation between the ability of the chloroderivatives to oxidize the fluorogenic probe 2′,7′-dichlorofluorescin, their stability and the content of intracellular glutathione, so other factors seem to play a more important role in these reactions.

Mostly GSSG rather than GSH was found in the extracellular medium of erythrocytes treated with the highest concentrations of all chloroderivatives. The highest concentrations of GSSG outside the cells were found for N-chloroserine, N-chloroaspartic acid and N-chlorophenylalanine and, in the case of the

first two N-chloroamino acids, GSSG excretion seems to be the main process responsible for the low intracellular GSSG content. $HOCl/OCl^-$ did not induce such a high increase of GSSG level in the extracellular medium as most of the employed N-chloroamino acids (except for N-chlorolysine). The GSSG efflux from RBCs treated with $HOCl/OCl^-$ can be explained as a result of its haemolytic effect, while in the case of N-chloroamino acids, which are much weaker haemolytic agents, the active export of oxidized glutathione can be a predominant mechanism [43,44]. $HOCl/OCl^-$ has been reported to inhibit the multi-drug resistance-associated protein activity and active export of glutathione conjugates and glutathione disulphide from the erythrocytes [41] which explains the lower level of GSSG in the extracellular medium of $HOCl/OCl^-$ treated cells.

In erythrocytes treated with the highest concentrations used of N-chloroalanine, N-chlorophenylalanine and $HOCl/OCl^-$, increase of intracellular reactive oxidants could be demonstrated by augmented 2′,7′-dichlorofluorescin oxidation (Figure 4). This effect could contribute to the decrease in glutathione content, especially for AlaCl and PheCl which caused the strongest glutathione depletion (Table III) and a significant augmentation of 2′,7′-dichlorofluorescin oxidation (Figure 4). Other N-chloroamino acids studied did not increase oxidation of the fluorogenic probe significantly. 2′,7′-Dichlorofluorescin can be oxidized by the chlorocompounds themselves (especially by $HOCl/OCl^-$) and by radical products of their decomposition. Formation of secondary reactive oxygen species in cells treated with the chloroderivatives cannot also be excluded, so the nature of the oxidant responsible remains elusive but increased oxidation of the probe in RBCs treated with N-chloroamino acids and $HOCl/OCl^-$ is evidence of oxidative stress persisting 1 h after the treatment.

N-Chloroderivatives of non-polar amino acids (AlaCl and PheCl) were most effective in glutathione depletion and intracellular 2′,7′-dichlorofluorescin oxidation (Figure 4, Table III). A previous study has demonstrated that only N-chloroderivatives of low lipid solubility can protect cells against myeloperoxidase-H_2O_2-Cl^- system, while membrane-permeable N-chloroderivatives were cytotoxic towards leukocytes [45]. Thus, amino acid hydrophobicity seems to be an important factor determining whether N-chloroamino acid formed is a mediator of $HOCl/OCl^-$ damage or plays a protective action against hypochlorous acid/hypochlorite.

In summary, our data demonstrate that N-chloroamino acids differ in stability and reactivity with respect to erythrocyte components but generally their reactivity may be not much lower with respect to $HOCl/OCl^-$. The relative reactivity of individual N-chloroamino acids for erythrocytes depends on the parameter studied. While N-chloroalanine and N-chlorophenylalanine were most effective in decreasing the glutathione content and increasing osmotic fragility of erythrocytes, N-chloroalanine and N-chlorolysine increased the content of protein-glutathione mixed disulphides to the highest levels while N-chloroserine, N-chloroaspartic acid and N-chlorophenylalanine were most efficient in stimulating GSSG efflux. Therefore, they may be important players in the undesired effects of chlorination reactions at the site of inflammation. The susceptibility of erythrocytes to the action of $HOCl/OCl^-$ and N-chloroamino acids may contribute to the whole-body effects of chlorination reactions proceeding locally at inflammation sites.

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