Oxidation of intracellular glutathione after exposure of human red blood cells to hypochlorous acid

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Exposure of human red blood cells to low doses of hypochlorous acid (HOCl) resulted in the loss of intracellular GSH. Oxidation occurred less than 2 min after the addition of HOCl, and required approx. 2.5 mol of HOCl per mol of GSH lost. Loss of GSH preceded oxidation of membrane thiols, the formation of chloramines and haemoglobin oxidation. The susceptibility of intracellular GSH to oxidation by HOCl was two-thirds that of GSH in cell lysates. These results indicate that HOCl can penetrate the red cell membrane, which provides little barrier protection for cytoplasmic components, and that GSH oxidation by HOCl may be a highly selective process. Virtually all of the GSH lost was converted into GSSG. If glucose was added to the medium, most of the GSH oxidized by low doses of HOCl was rapidly regenerated. At higher doses, recovery was less efficient. However, when HOCl was added as a slow infusion rather than in a single bolus, there was increased recovery at higher doses. This indicates that in metabolically active cells regeneration is rapid and GSH may protect cell components from damage by HOCl. HOCl-induced lysis was only slightly delayed by adding glucose to the medium, indicating that lytic injury is not ameliorated by GSH.

INTRODUCTION

Hypochlorous acid (HOCl) is a potent oxidant with broad-ranging microbicidal properties which are exploited both domestically and commercially [1,2]. It is also generated in high concentrations by polymorphonuclear leucocytes (neutrophils) in response to a phagocytic challenge. These cells undergo a respiratory burst and produce H_2O_2 which is converted into HOCl by the granule enzyme myeloperoxidase, according to the following reaction [3]:

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

HOCl is thought to be the major strong oxidant produced by neutrophils. As well as being a potent microbicidal agent it is also implicated as a cause of neutrophil-mediated tissue injury [2,4]. Numerous studies have demonstrated its ability to damage cells [5–7] and extracellular matrix proteins [2,8,9], and to inactivate enzymes [10].

HOCl reacts readily with a range of biological molecules, particularly thiols and thioethers [11], with FeS centres in proteins [12,13], amino groups [14], haem proteins [15] and alkene groups in phospholipids and cholesterol [16]. This last reaction results in saturation of fatty acids and introduces chlorohydrins into lipid bilayers. Thiol groups are approximately 100-fold more reactive than amines or haem proteins [11]. Reaction with amino groups results in the formation of chloramines [12]:

$$R\text{-}NH_2 + HOCl \rightarrow R\text{-}NHCl + H_2O$$

Chloramines are long-lived species which retain the oxidizing equivalents of HOCl and can react with thiols and other groups [14,17], thereby extending the toxicity of HOCl.

Several studies have been carried out to determine the sites of action of HOCl on tumour cells [18], bacteria [19,20] and erythrocytes [13,21,22]. One major consequence of exposure to HOCl is lysis. Because of the reactivity of HOCl, the cell membrane is considered to be the primary site for reaction and is thought to act as a protective barrier for cytoplasmic components [12,18,23]. In support of this, several studies have focused on susceptible membrane targets [12,18]. In bacteria, respiratory enzymes and membrane transporters were inactivated by HOCl [12,19,24,25]. In cultured tumour cells, HOCl caused disturbance of various plasma-membrane functions and this was attributed to membrane protein thiol oxidation [18].

If the plasma membrane did not constitute a protective barrier to HOCl, GSH, because of its high reactivity, would be expected to be one of the first constituents lost on exposure to HOCl. We have investigated whether this is the case by exposing human red blood cells to HOCl and relating the dose–response for GSH loss to modification of other intracellular and membrane targets. We show that the membrane does not prevent GSH from being a preferential target.

MATERIALS AND METHODS

Materials

HOCl was obtained from Reckitt and Coleman, Avondale, Auckland, New Zealand, and its concentration determined by reaction with thionitrobenzoic acid (TNB) [26]. Stock solutions of HOCl were diluted with 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM $MgCl_2$ (PBS), and the pH was adjusted to 7 immediately before use. TNB was generated from 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by briefly raising the pH to 10 with dilute NaOH, and then returning it to 7 [27]. The concentration was determined from the absorbance at 412 nm, using an absorption coefficient of $1.36 \times 10^4\ M^{-1} \cdot cm^{-1}$ [26]. Other reagents were from Sigma, St. Louis, MO, U.S.A.

Preparation of red cells

Normal human red blood cells were obtained from consenting donors, spun to remove the plasma and buffy coat layers, and then washed three times with PBS. The cells were suspended in PBS at a known haematocrit which was determined by measuring the haemoglobin content of the cell suspension with Drabkins reagent [28] and assuming that packed red cells contain 30%

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, thionitrobenzoic acid.

haemoglobin. A 5% haematocrit suspension of red cells therefore contained 5 ml of packed cells/100 ml of PBS. Lysed red cells were prepared from a suspension of known haematocrit by centrifuging the cells and replacing the PBS with an equal volume of 5 mM sodium phosphate buffer, pH 7.4.

Reaction of red cells with HOCl

In most of the experiments, HOCl was added to the red cell suspension as a single bolus. Thorough mixing of the cell suspension and HOCl in PBS was required to ensure uniform reaction and consistent results. Equal volumes of HOCl and red cells were mixed while vortexing, and were incubated at 37 °C for periods of between 2 and 30 min before further analysis. In some experiments, red cell suspensions were incubated with 50 μM diisothiocyanostilbenedisulphonate (DIDS) for 5 min before the addition of HOCl.

To determine whether the effect of HOCl on red cell GSH depended on whether it was added as a bolus or over a period of time, 7.5 ml of HOCl solution in PBS was added to 7.5 ml of red cells at 2% haematocrit, either as a single addition or as a slow pump infusion over a period of 15 min. Experiments were carried out at 37 °C, with final concentrations of up to 300 nmol of HOCl/ml of cell suspension. GSH was measured with monobromobimane immediately after addition of HOCl and after a further 30 min incubation (bolus addition experiments). For the infusion experiments, GSH was analysed at the end of 30 min, which included 15 min infusion time and 15 min incubation. Rates of subsequent lysis were measured by taking samples at intervals up to 9 h and measuring A_{700} as an index of turbidity [26].

GSH measurement

Red cell GSH was determined by reaction with either DTNB, using the method of Beutler [28], or with monobromobimane. When monobromobimane was used, 20 μl (10 mM in acetonitrile) was added to 2 ml of red cells at 1% haematocrit. After 10 min in the dark at room temperature, the protein was precipitated with trichloroacetic acid (2.5%, w/v, final concentration) and the fluorescence of the supernatant was read at 394 nm (excitation wavelength) and 480 nm (emission wavelength). The concentration of GSH per ml of cell suspension was calculated from a standard curve for GSH which was run with each experiment [29].

Membrane thiols

Haemoglobin-free ghosts were prepared [30], suspended at 10% (v/v) in PBS and solubilized with 0.5% (w/v) SDS. Thiol concentration was measured by reaction with DTNB [28] and related to the protein concentration of the sample, determined by the method of Lowry et al. [31].

Determination of GSSG

Formation of GSSG was determined after exposure of red cells at 12.5% haematocrit to HOCl. After approx. 15 min, the cells were spun down, incubated at 0 °C for 10 min with 40 mM *N*-ethylmaleimide and assayed for GSSG by the glutathione reductase method described by Beutler [28].

Chloramine formation

To determine whether long-lived chloramines were formed by reaction with free amino groups, red cells at 12.5% haematocrit were exposed to between 1 and 5 mM HOCl and incubated for 1 h. The cells were lysed with 0.07% SDS and 1 ml of 0.5 mM TNB was added per ml of lysate. To separate the haemoglobin from the TNB fraction, the sample was passed through a Sephadex G-25 column (20 ml). The TNB fraction was collected, adjusted to 10 ml and the absorbance read at 412 nm. The difference in absorbance between the control sample (no HOCl) and the HOCl-treated red cells was taken as an indication of the level of chloramines in the sample.

Reaction of HOCl with haemoglobin

After exposure to HOCl, red cells were lysed with 5 mM phosphate buffer, pH 7.4, and this lysate was combined with the supernatant from the cells if these were already partially lysed. The proportions of oxy- and met-haemoglobin were quantified spectrophotometrically from the absorbances at 700, 630, 577 and 560 nm [26]. The formation of mixed disulphides between haemoglobin and GSH was investigated by electrophoresis on cellulose acetate [32], and the formation of haemoglobin cross-links was estimated by electrophoresis of globin.

RESULTS

Addition of low doses of HOCl to intact red blood cells resulted in loss of GSH (Figure 1). The stoichiometry of the initial loss was 1 mol of GSH lost per 2.5 mol of HOCl added. We have previously shown that HOCl-mediated injury to red cells is dependent on the absolute amount of HOCl rather than its concentration [33]. This was also the case in the present study: the mole ratio of 2.5 was obtained whether GSH was measured with DTNB, at a haemotcrit of 12.5%, or with monobromobimane, which required only a 1% haematocrit of red cells. We have previously quantified the effect of HOCl on red blood cells as nmol of HOCl/10^7 cells [33]. With red cells at 12.5% haematocrit, there was almost complete loss of GSH with 1 mM HOCl, corresponding to 8 nmol of HOCl/10^7 cells.

Using monobromobimane, GSH loss was shown to occur in less than 2 min after the addition of HOCl (Figure 2). Taken together, the results indicate that the reaction is both sensitive and rapid. At the doses used to achieve complete loss of GSH, other effects on the red cells were less pronounced. With 1 mM HOCl and red cells at 12.5% haemotocrit, haemolysis and

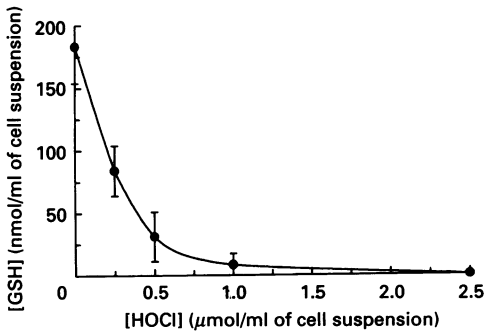

Figure 1 Loss of red cell GSH caused by increasing concentrations of HOCl

Red cells at 12.5% haematocrit were exposed to HOCl for 15 min at the concentrations indicated, and the GSH content of the cell suspension was measured by reaction with DTNB, as described in the Materials and methods section. It should be noted that 1 μmol of HOCl/ml is equivalent to 8 nmol of HOCl/10^7 cells. The results are the means ± S.D. of seven experiments.

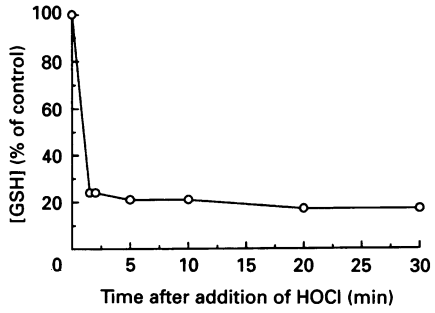

Figure 2 Time course of GSH oxidation on exposure of red cells to HOCl

The GSH content of red cells at 1% haematocrit was measured by reaction with monobromobimane at various times after the addition of 50 nmol of HOCl/ml of red cells. For comparison with Figure 1, this is equivalent to an HOCl dose of 5 nmol/10^7 cells. The results are from one of two similar experiments.

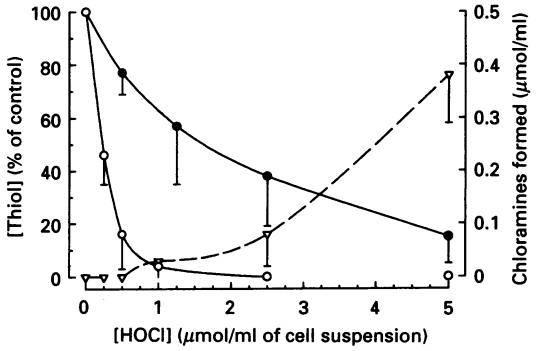

Figure 3 Thiol loss and chloramine formation on exposure to HOCl

Red cells at 12.5% haematocrit were allowed to react with various concentrations of HOCl, and the levels of GSH (○), membrane thiols (●) and chloramines (▽) were determined as described in the Materials and methods section. The results represent means ± S.D. of seven (thiols and GSH) and two (chloramines) experiments.

significant K^+ leakage occurred only after 4–8 h, and membrane cross-linking was relatively minor [33].

GSH was more vulnerable than membrane thiols, such that when 80% of the GSH was oxidized, approx. 20% of the membrane thiols were lost (Figure 3). Similar results were obtained whether DTNB or monobromobimane was used for the assay, and, as monobromobimane reacts with intracellular GSH while the cells are intact, it is unlikely that GSH is oxidized by regeneration of membrane thiols by GSH during processing for analysis. Chloramines were detected only after all the GSH was lost (Figure 3). It is possible that chloramines were formed at lower concentrations but, as they readily undergo secondary reactions with available thiols, they would not be detected until all thiol groups are oxidized. However, even at higher levels of HOCl exposure, the amount of chloramine detected represents less than one-tenth of amount of HOCl used. Conversion of oxyhaemoglobin into methaemoglobin was detectable only at relatively high concentrations of HOCl, when all the GSH was oxidized. With red cells at 12.5% haematocrit, methaemoglobin levels remained at less than 1% until the concentration of HOCl exceeded 5 mM. This is in accord with our previous results [33].

To determine whether the cell membrane constituted a protective barrier for GSH oxidation, a comparison was made

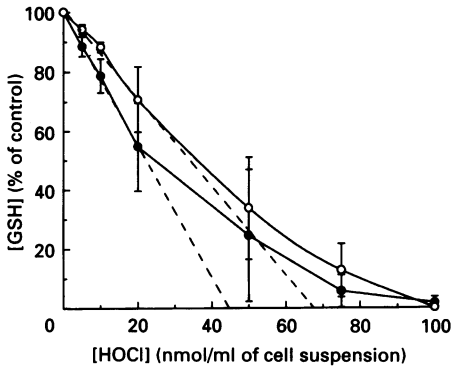

Figure 4 HOCl-mediated oxidation of GSH in intact (○) and lysed (●) red blood cells

Suspensions of approx. 1% haematocrit, or an equivalent volume of cell lysate, were exposed to increasing concentrations of HOCl, and the GSH content was measured by reaction with monobromobimane. The concentration of GSH measured in the control suspensions was 30 ± 6 μM. The results are the means ± S.D. of five experiments. For all experiments, the slopes of the lines were measured. A paired t test showed that the difference between intact and lysed cells was statistically significant ($P < 0.05$). Similar results were obtained at 12.5% haematocrit when DTNB was used as the detection agent.

between lysed and intact cells (Figure 4). From the slopes of the initial linear portions of the curves, it can be calculated that for the equivalent GSH loss, 1.5 times more HOCl was required for intact cells than when cell lysate was used. This represents an HOCl/GSH stoichiometry of approx. 1.7 for the lysate, and suggests that the membrane provides only a partial barrier to HOCl.

To determine the extent of this barrier function in the presence of other extracellular targets, we compared the ability of GSH, present in cell lysates and in intact red cells, to inhibit ascorbate oxidation by HOCl. Red cells at 5% haematocrit, containing the equivalent of 125 nmol of GSH/ml of cell suspension, inhibited oxidation of 22 μM ascorbate by 23% (GSH/ascorbate molar ratio 5:1). By contrast, equivalent inhibition by lysate was seen with a GSH/ascorbate ratio of 1:12. This indicates that the membrane does constitute a barrier to GSH-scavenging HOCl if other reactive species are present extracellularly.

At pH 7.4, HOCl and OCl^- exist in approximately equimolar ratio. In order to determine which species was able to penetrate the cell membrane, we measured the rate of HOCl-induced lysis in red cells incubated with the anion channel-blocking agent, DIDS. At three concentrations of HOCl (0.25, 0.5 and 1 mM), there was no change in the rate of lysis measured over a 6 h period. We also determined the reactivity of GSH in intact and lysed cells at pH 6.6 and pH 8.0. This represents a 25-fold difference in the HOCl/OCl^- ratio. At both pH values, results were similar to those at pH 7.4: there was 1.2–1.5 times greater GSH loss in lysates than in intact cells.

Over a range of HOCl concentrations that resulted in up to 90% GSH loss, virtually all the GSH lost was accounted for as GSSG (Table 1). We also investigated the possible formation of mixed disulphides of GSH with haemoglobin, which has two exposed cysteine residues per tetramer, and the formation of haemoglobin cross-links. Cellulose acetate electrophoresis revealed no mixed disulphides, which would run as a more anionic band than HbA, and electrophoresis of globin chains (results not shown).

The ability of the cells to regenerate GSH from GSSG was investigated. When HOCl-treated red cells were incubated in

Table 1 Formation of GSSG after treatment of red cells with HOCl, and regeneration of GSH in the presence of glucose

Red cells at 12.5% haematocrit were allowed to react with HOCl and subsequently analysed for GSH with DTNB and for GSSG using glutathione reductase, as described in the Materials and methods section. For ease of comparison, GSSG was calculated as GSH equivalents. n.d., not determined. To measure regeneration of GSH, 5 mM glucose was added to the cells 5 min after exposure to HOCl, and incubation continued at 37 °C for 30 min. The results shown are means ± S.D. of three experiments (GSH/GSSG ratio) and six experiments (regeneration with glucose).

HOCl concn. (mM)	GSH lost/GSSG gained	GSH regenerated with glucose (%)
0.25	0.9 ± 0.1	90 ± 20
0.5	1.1 ± 0.1	76 ± 12
1.0	1.2 ± 0.3	38 ± 10
2.5	n.d.	0.6 ± 0.0

Table 2 Rates of lysis of red cells treated with HOCl administered in a single bolus or by infusion

Red cells at 1% haematocrit were treated with HOCl administered in a single bolus or by pump infusion over 15 min as detailed in the Materials and methods section. The rates of lysis were measured over a period of 8 h by monitoring the turbidity of the sample at 700 nm as previously described [26]. The results shown are means ± S.D. from three experiments.

HOCl concn. (nmol/ml)	Relative lysis rates in the presence and absence of glucose	
	Bolus addition	15 min infusion
60	0.60 ± 0.16	0.46 ± 0.13
100	0.79 ± 0.08	0.80 ± 0.21
200	0.96 ± 0.05	0.92 ± 0.11
300	1.00 ± 0.0	0.88 ± 0.03

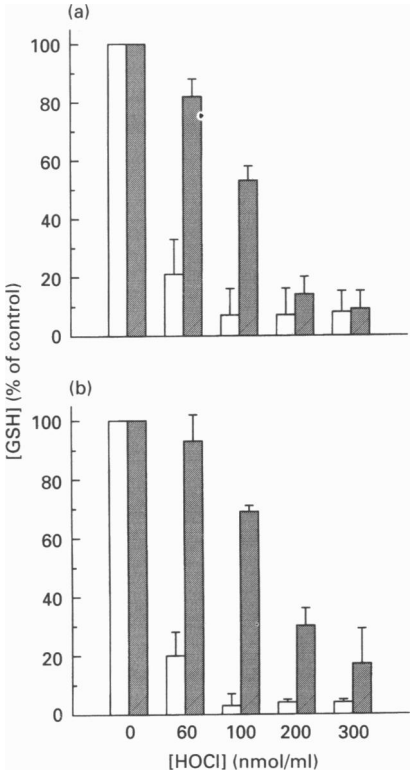

Figure 5 Regeneration of GSH in red cells exposed to HOCl (a) as a bolus addition or (b) by slow infusion

Red cells at 1% haematocrit were exposed to various concentrations of HOCl in a single addition or over a 15 min infusion period. GSH was measured with monobromobimane after 30 min incubation in the absence (□) or presence (▨) of glucose. The results shown are the means and ranges of two experiments. A paired *t* test of the GSH levels in the presence of glucose after bolus addition or by infusion of HOCl indicated that the difference between them was significant ($P < 0.01$).

PBS supplemented with 1 mM glucose, most of the GSH was recovered (Table 1). At higher levels of HOCl exposure, however, there was only partial recovery of GSH. As at these doses of HOCl most of the oxidized GSH was present as GSSG (Table 1), failure of the cells to regenerate the GSH was probably due to HOCl-induced inactivation of the repair enzymes.

In our experiments, we added HOCl to the red cell suspension as a single bolus. *In vivo*, however, it is more likely that target cells are exposed to a lower concentration of oxidant over a longer period of time. To determine whether HOCl affects red cells differently under these conditions, we administered HOCl as a bolus or as a slow infusion over 15 min. Whereas the extent of GSH oxidation was identical under the two conditions (Figure 5), the ability of the cells to regenerate GSH differed markedly. There was no regeneration of GSH in the absence of glucose, but when glucose was present, red cells that had been exposed to HOCl administered by slow infusion retained their capacity to regenerate GSH (Figure 5). This was particularly evident at higher doses of HOCl. Regeneration was also rapid, and the GSH levels measured immediately after the 15 min infusion increased only by around 20% on further incubation. Although glucose improved the ability of the cells to regenerate GSH, it did not affect the rate of lysis at HOCl concentrations above 100 μM (Table 2). At lower HOCl concentrations lysis was slowed in the presence of glucose, but there was no difference whether HOCl was administered as a bolus or by infusion.

DISCUSSION

In this study we have shown that HOCl, when added to red blood cells, readily oxidizes intracellular GSH. GSH loss occurred concomitantly with loss of membrane thiols and was complete at HOCl concentrations at which membrane thiols were still detectable. HOCl has been shown to cause red cell lysis, associated with K^+ leakage and swelling, and membrane cross-linking and lipid modification [33]. However, these changes only became apparent at concentrations equivalent to 0.5–1 mM HOCl with red cells at 12.5% haematocrit, i.e. when all the GSH was oxidized. We have also shown that GSH was only 1.5 times more susceptible to oxidation in red cell lysates than in intact cells.

Taken together, our results indicate that HOCl penetrates into the cells without the membrane acting as a major barrier and that the membrane does not prevent HOCl reacting with intracellular GSH. This suggests that the most important determinant of targets for HOCl is their relative reactivity, rather than intracellular versus extracellular location. As GSH is one of the most reactive biological molecules with HOCl, it would be expected to be preferentially oxidized by this compound. The membrane does constitute a partial barrier, however, and the reactivity of HOCl with intracellular GSH was almost two orders of magnitude less than with GSH free in solution. Competition experiments with extracellular ascorbate also demonstrated that

red cell GSH will not compete effectively in the presence of a highly reactive external substrate.

These results could be explained by the uncharged free acid, HOCl, passing through the hydrophobic lipid bilayer while undergoing minimum reaction with membrane constituents. Although HOCl should be freely permeable, this is somewhat surprising. It has been thought that, because of the reactivity of HOCl, the cell membrane would be the prime target and would provide a protective barrier for cytoplasmic components. This has been suggested previously. Grisham et al. [15] described one experiment in which GSH was more readily lost from red cell lysates than from intact cells. Schraufstatter et al. [18] reported that the membrane thiols in cultured tumour cells were oxidized at the same rate as GSH, and that membrane protein residues were oxidized before those from the cytoplasm or other cellular compartments. They also found that glyceraldehyde-3-phosphate dehydrogenase was much more susceptible to inactivation by HOCl in lysed than in intact cells [18]. In a recent study [34], epithelial cells were protected from HOCl-induced toxicity by taurine. Although it was not suggested in this latter study, the implication was that epithelial cells are permeable to HOCl.

If, as our results suggest, HOCl is able to permeate the lipid bilayer, such a route should be common to all membranes. Yet the results of Schraufstatter et al. [18] with tumour cells and our own observations with neutrophils (C. C. Winterbourn and A. Carr, unpublished work) suggest that some cells may allow much more restricted access of HOCl to intracellular components. Alternatively, OCl^- could gain access through the anion channel, which is particularly active in red cells, and this could explain their relatively greater permeability. Two lines of evidence argue against this. First, the anion channel blocker, DIDS, had no effect on HOCl-mediated red cell lysis, and secondly, there was no difference in the results obtained from experiments carried out at pH 6.5 or 8, conditions under which the relative concentrations of HOCl and OCl^- vary greatly. Another possibility is that GSH oxidation occurs indirectly by transmembrane disulphide exchange. Red cells can use intracellular GSH to regenerate membrane thiols oxidized by treatment with external DTNB [35]. However, in this case, regeneration occurred over a 30 min period, compared with the 2 min or less required for HOCl-mediated GSH loss in our study. Moreover, the concentration of membrane thiols is only a small percentage of the GSH concentration, and many exchange cycles would be necessary to consume all the GSH. Therefore this appears to be an unlikely explanation of our results.

Almost all of the GSH lost on treatment of the red cells with HOCl was accounted for as GSSG. The GSH/HOCl stoichiometry for this reaction is 0.5, yet we measured 2.5 with the intact cells. This implies other targets for HOCl. The 1.5-fold greater reactivity of GSH in cell lysates indicates that almost half of the extra HOCl reacted with membrane constituents. The haem groups and the two accessible β-chain cysteine residues of haemoglobin are also potential targets. However, at concentrations of HOCl that caused complete GSH oxidation, haem oxidation was insignificant, electrophoresis showed no formation of mixed disulphides between haemoglobin and GSH, and β-chain dimers were virtually undetectable. We detected chloramines, but only at concentrations of HOCl above those required to oxidize all available thiols. This does not preclude the formation of chloramines as initial products, as these would react with any available GSH to regenerate the original amine groups.

GSH could be regenerated from GSSG when red cells exposed to lower concentrations of HOCl were incubated with glucose. This did not occur at higher levels of exposure, even though GSSG was the major product, suggesting that there was damage to the components of the GSH reductase system. Regeneration of GSH was more effective when HOCl was administered by slow infusion rather than as a bolus addition. This may be because regeneration is rapid, and a sufficient concentration of GSH is maintained to protect the components of the regeneration pathway from damage.

Addition of glucose to the medium also delayed lysis slightly, but only at the lower levels of exposure to HOCl or when higher amounts of HOCl were administered by infusion, i.e. conditions under which the cells maintained their capacity to regenerate GSH. Hence it seems that GSH is unable to prevent the membrane changes responsible for lysis, and that lytic injury and GSH oxidation occur concurrently on exposure to HOCl. However, metabolically active cells such as those incubated in the presence of glucose may be slightly better at counteracting injury, e.g. by Na^+ pump activity, and this may explain their slightly increased resistance to lysis.

Our results have implications for the mechanism of HOCl-mediated toxicity. On exposure of red cells to HOCl, susceptible targets will be determined by their reactivity to HOCl as well as location. Reaction with some membrane components is likely, but intracellular constituents that are highly reactive with HOCl, such as thiols and methionine groups [11], should also be preferentially oxidized. We have previously shown that, when thiol groups are blocked, red cells are more susceptible to HOCl [33]. It may therefore be possible that intracellular GSH could serve as a defence mechanism against HOCl, particularly when cells are supplied with a source of energy.

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