Oxidation of neutrophil glutathione and protein thiols by myeloperoxidase-derived hypochlorous acid

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Neutrophils, when stimulated, generate reactive oxygen species including myeloperoxidase-derived HOCl. There is an associated decrease in reduced glutathione (GSH) concentration. We have shown that neutrophil GSH levels decrease on exposure to reagent HOCl, whereas the equivalent concentration of H_2O_2 had no effect. GSH loss occurred without cell lysis, was not reversible, and was accompanied by the loss of an equivalent proportion of the total protein thiols. No glutathione disulphide was formed. Studies with ^{35}S-labelled cells indicated that much of the GSH lost was accounted for by mixed disulphides with protein and a product that co-migrated on HPLC with a novel compound formed in the reaction of HOCl and pure GSH. The properties of this compound are consistent with an intramolecular sulphonamide. Neutrophils stimulated with PMA lost 30–40 % of their GSH and a similar proportion of protein thiols. Little glutathione disulphide was formed and the products were the same as seen with HOCl-treated cells. From these results and studies with inhibitors and scavengers, we conclude that HOCl was responsible for the GSH loss. Propargylglycine and buthionine sulphoximine, inhibitors of glutathione synthesis, enhanced GSH loss, but their effects were due to the production of long-lived chloramines that oxidized GSH with greater efficiency than HOCl, rather than to the inhibition of GSH synthesis. The lack of thiol selectivity by HOCl and irreversibility of oxidation means that GSH will provide limited antioxidant protection for thiol enzymes in stimulated neutrophils.

INTRODUCTION

Reduced glutathione (GSH) provides a major intracellular defence against oxidative injury. Because neutrophils generate large amounts of oxidants, they might need GSH to protect themselves against injury while performing their antimicrobial or inflammatory activities. Neutrophils defective in glutathione metabolism have impaired function [1,2]. Stimulation of neutrophils causes a decrease in their GSH concentration [3,4]. The mechanism is not fully understood but is generally attributed to removal of the H_2O_2 generated by the cells through the glutathione peroxidase pathway [3]. More recently, nitrosylation [4] and oxidation by myeloperoxidase-derived HOCl [5] have been postulated. Neutrophil stimulation has also been shown to result in formation of protein–GSH mixed disulphides, without a corresponding increase in glutathione disulphide (GSSG) [6]. Enhanced loss of GSH in the presence of buthionine sulphoximine (BSO) or propargylglycine (PAG) has been reported, and explained in terms of compensation for any loss by new synthesis [5].

Whereas the oxidation of GSH by H_2O_2 and glutathione peroxidase should be selective and reversible, HOCl is likely to be less selective and to give irreversible products. Therefore HOCl is potentially more detrimental. It undergoes a wide range of reactions with biological substrates [7–9]. The fastest of these are with thiols and thioethers, so GSH should be a favoured but not exclusive target. HOCl causes the oxidation of intracellular GSH in erythrocytes [10] and a range of cultured cells [11,12]. Furthermore the oxidation of purified GSH by HOCl produces novel products, one of which has been tentatively identified on the basis of its molecular mass (337 Da) and its lack of thiol or amine groups as an intramolecular sulphonamide [13].

In this study we have investigated whether HOCl oxidizes neutrophil GSH and protein thiols and whether it could be responsible for the thiol loss when the cells are stimulated. We have compared the effects of exogenous HOCl with H_2O_2, and investigated how various scavengers and inhibitors affect thiol loss in cells stimulated with PMA. The products of GSH oxidation have been traced by prelabelling the cells with [^{35}S]cysteine. Our results show that HOCl readily oxidizes neutrophil GSH and protein thiols and is likely to be responsible for the losses in stimulated cells.

MATERIALS AND METHODS

Materials

Sodium hypochlorite was from Reckitt and Coleman (Auckland, New Zealand). The concentration of stock solutions was determined by reaction with monochlorodimedon and measurement of the decrease in A_{290} (ϵ_{290} 19000 $M^{-1}\cdot cm^{-1}$). H_2O_2 was from BDH (Poole, Dorset, U.K.) and its concentration was determined from ϵ_{240} 43.6 $M^{-1}\cdot cm^{-1}$. Monobromobimane and *N*-monomethylarginine were from Calbiochem (La Jolla, CA, U.S.A.). GSH and GSSG (Sigma Chemical Co., St. Louis, MO, U.S.A.) were standardized with 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) and measurement of A_{412} (ϵ_{412} 14100 $M^{-1}\cdot cm^{-1}$) and with glutathione reductase and NADPH [14] respectively. Diphenyleneiodonium was a gift from Dr O. T. G. Jones (Department of Biochemistry, University of Bristol, Bristol, U.K.) and was dissolved in 10 % (v/v) dimethylformamide. Myeloperoxidase was purified from human neutrophils [15] and its concentration calculated from ϵ_{430} 91000 $M^{-1}\cdot cm^{-1}$. [^{35}S]Cysteine (1000 Ci/mmol) was obtained from NEN (Wilmington, DE, U.S.A.). Other chemicals and biochemicals were from Sigma or BDH.

Neutrophils

Neutrophils were isolated from the blood of healthy human donors by Ficoll–Hypaque centrifugation, dextran sedimentation

Abbreviations used: BSO, buthionine sulphoximine; GSH, reduced glutathione; GSSG, glutathione disulphide; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid); PAG, propargylglycine.

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of red cells and removal of contaminating red cells by hypotonic lysis [16]. They were suspended at 10^7 cells/ml in 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (0.14 M), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM) and glucose (1 mg/ml) (PBS). All reactions were performed in PBS.

Addition of oxidants to resting cells

HOCl or H_2O_2 was diluted in PBS, then added with immediate mixing to resting cells (2.5×10^6/ml) at room temperature. After 5–10 min, reagents for GSH analysis were added either to the whole reaction mixture or to pellet and supernatant fractions.

Stimulation of cells in the presence of scavengers and inhibitors

Neutrophils (2.5×10^6/ml) were preincubated at 37 °C for 20 min in the presence of the glutathione synthesis inhibitors PAG (300 μM) or BSO (200 μM). Other inhibitors and scavengers were preincubated with the cells at the stated concentrations for a minimum of 10 min at 37 °C. The neutrophils were stimulated with PMA, which was dissolved in DMSO diluted in distilled water, to give a final concentration of 100 ng/ml. The final DMSO concentration was 0.005 %. The reaction was stopped after a further 30 min by the addition of reagents for GSH analysis.

Superoxide production by stimulated neutrophils

PMA was added to neutrophils (0.5×10^6/ml) incubated at 37 °C with 150 μM cytochrome *c* in the presence of catalase (10 μg/ml). The difference between cytochrome *c* reduction with and without superoxide dismutase (20 μg/ml) was measured after 30 min from ϵ_{550} 21 100 $M^{-1} \cdot cm^{-1}$.

Chloramines

Chloramines were determined by reacting with 5-thio-2-nitrobenzoic acid and measuring A_{412} (ϵ_{412} 28 200 $M^{-1} \cdot cm^{-1}$) [17].

GSH

Analysis of intracellular GSH was performed with a modification of the monobromobimane method [18]. Briefly, neutrophil samples (2.5×10^6/ml) were brought to pH 8.0 with NaOH, an excess of monobromobimane (10 mM in acetonitrile) was added to give a final concentration of 250 μM and the samples were left at room temperature in the dark for 5–10 min. Trichloroacetic acid [final concentration 5 % (w/v)] was then added and after microcentrifugation the supernatants were subjected to HPLC analysis with authentic GSH as a standard. Basal GSH concentrations were also measured with Nbs_2 after precipitation of protein from 10^7 cells/ml with trichloroacetic acid and adjustment of the pH of the supernatant to 8.0. Results obtained with this and the monobromobimane method agreed to within 10 %.

Protein thiols

Protein thiols in the pellet obtained after monobromobimane derivatization in the GSH assay were determined by washing, redissolving in 1 % (w/v) SDS and measurement of fluorescence [18]. GSH (0–25 μM) treated with 250 μM monobromobimane and suspended in the same buffer was used as standard. Because this method has been shown with hepatocytes not to derivatize all protein thiols, analyses were also performed with 5 mM (final concentration) monobromobimane in the presence of 0.1 % Triton X-100 to maximize the exposure of thiol groups [19]. The total number of thiols detected under these conditions was three times that with the lower monobromobimane concentration, but in a control experiment it was found that the proportion of thiols lost was the same at both monobromobimane concentrations. *N*-ethylmaleimide inhibited 93 % of the fluorescence with 250 μM monobromobimane and 82 % with 5 mM monobromobimane, indicating specificity for thiols.

In some cases, protein thiols were measured as the difference between total thiols, measured by adding Nbs_2 to 10^7 cells in 1 ml of PBS containing 1 % (w/v) SDS, and the GSH content. These values were within 5 % of those measured with 5 mM monobromobimane.

Glutathione oxidation products

GSH, GSSG and glutathione sulphonic acid were measured in neutrophil samples by HPLC after derivatization with dansyl chloride [20]. Analyses were performed on the pelleted cells (10^6) as described for the original method, as well as on the supernatant to detect products released into the incubation medium. A small proportion of the GSH, up to half of the GSSG and the sulphonic acid (when present) were in the supernatant. The results are expressed as the sum of the amounts in both fractions. Authentic GSH, GSSG and glutathione sulphonic acid were used as standards.

^{35}S-labelling of cell glutathione

Neutrophils (2.5×10^6/ml) were preincubated with cycloheximide (200 μM) for 1 h at 37 °C followed by a further 1 h with [^{35}S]cysteine as described [6]. The cells were pelleted, washed and resuspended in PBS. After exposure to HOCl or stimulation with PMA, the samples were acidified with 5 % (w/v) $HClO_4$ on ice before centrifugation. The supernatants were kept for HPLC separation and scintillation counting, and the pellets were washed with ether and resuspended in 1 % (w/v) SDS for counting. Cell pellets were also resuspended in 50 mM Mops, pH 8.5, containing 20 mM dithioerythritol [21] and incubated for 1 h at 37 °C to reduce protein mixed disulphides. The samples were re-precipitated with 5 % (w/v) $HClO_4$ on ice and the proportion of radioactivity released into the supernatant was determined. Scintillation counting was performed with 0.1 ml of sample added to Bray's scintillant. Where necessary, counts were corrected for quenching by 5 % (w/v) $HClO_4$ (approx. 30 %).

HPLC separation of ^{35}S-labelled oxidation products

$HClO_4$ supernatants obtained from the S-thiolation experiments were separated by reverse-phase HPLC on a Nucleosil column with 50 mM formic acid as eluent as described [13]. Fractions were collected every 0.25 min for scintillation counting. Samples were spiked with GSH and GSSG; the retention times of ^{35}S-labelled peaks were compared with those of the products of oxidation of pure GSH with HOCl.

RESULTS

Reactions of neutrophil thiols with HOCl and H_2O_2

The addition of HOCl to resting neutrophils caused concentration-dependent losses of intracellular GSH and a proportionate number of protein thiols (Figure 1). The total number of protein thiols initially present in the neutrophils was approx. 5-fold the GSH content, so on a molar basis the loss of protein thiols was approx. 5-fold greater. Cell viability (as measured with Trypan Blue) was not affected by HOCl concentrations that gave up to 50 % GSH loss. Viability was decreased to 93 % with 50 μM and to 12 % with 100 μM HOCl. Superoxide production, measured as superoxide dismutase-

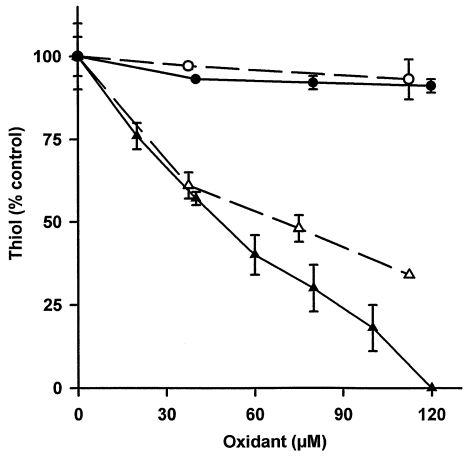

Figure 1 Loss of GSH (solid lines) and protein thiols (broken lines) in neutrophils treated with increasing concentrations of HOCl and H_2O_2

The initial GSH content was 3.22 ± 0.32 nmol per 2.5×10^6 neutrophils; the initial protein thiol content (measured with either Nbs_2 or 5 mM monobromobimane) was 17.9 ± 4.8 nmol per 2.5×10^6 neutrophils ($n = 7$). Results represent means $\pm$ S.D. for two to four samples. Symbols: ●,○, treated with H_2O_2; ▲,△, treated with HOCl.

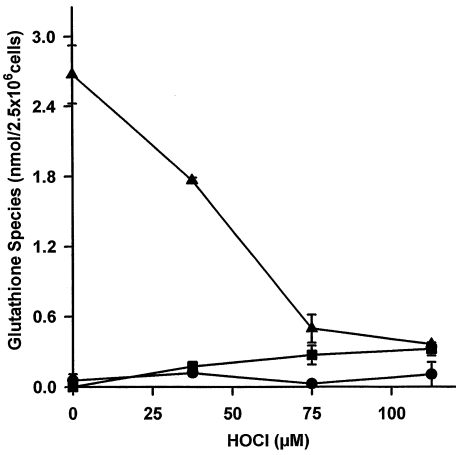

Figure 2 Glutathione species in neutrophils treated with HOCl as measured by the dansyl chloride method

Results, which represent the sum of pellet and supernatant fractions, are expressed as means and range for two representative experiments. Symbols: ▲, GSH; ●, GSSG; ■, glutathione sulphonic acid.

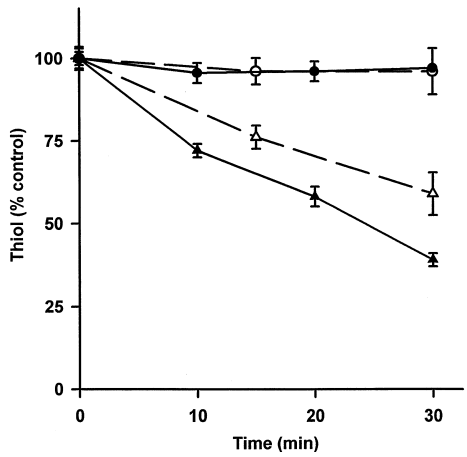

Figure 3 Loss of GSH (solid lines) and protein thiols (broken lines) from resting neutrophils and those stimulated with PMA

The initial GSH content was 3.05 ± 0.16 nmol per 2.5×10^6 neutrophils; the initial protein thiol content was 17.9 ± 4.8 nmol per 2.5×10^6 neutrophils ($n = 7$). Results represent means $\pm$ SD for two to four samples. Symbols: ●,○, resting cells; ▲,△, cells stimulated with PMA.

Table 1 Effects of inhibitors and scavengers on GSH loss in neutrophils stimulated with PMA

Results are presented as the percentage protection of GSH provided by the inhibitors and scavengers and are expressed as means $\pm$ S.D. for the number of experiments in parentheses. Control GSH levels measured with monobromobimane were 3.59 ± 0.68 nmol per 2.5×10^6 neutrophils ($n = 12$), of which 1.04 ± 0.40 nmol was lost after 30 min of stimulation. All additions gave results significantly different from PMA alone ($P < 0.05$), using paired Student's t test on individual data points.

Addition	Protection (%)
Diphenyleneiodonium (10 μM)	108 ± 33 (4)
Catalase (25 μg/ml)	36 ± 3 (4)
Azide (1 mM)	34 ± 10 (4)
Methionine (1 mM)	103 ± 15 (6)
Thiodipropionate (1 mM)	97 ± 10 (4)
Myeloperoxidase (50 nM)	−37 ± 5 (4)
Superoxide dismutase (25 μg/ml)	−37 ± 2 (4)

inhibitable cytochrome *c* reduction, was unaffected by up to 40 μM HOCl, above which it gradually declined. With lower cell concentrations, less HOCl was required for equivalent GSH loss, indicating a dependence on the amount of HOCl added per cell rather than on concentration itself. The molar ratio of GSH loss to HOCl added was approx. 1:40.

Equivalent concentrations of H_2O_2 did not decrease neutrophil GSH or protein thiol concentrations significantly (Figure 1). With azide added to inhibit catalase, 120 μM H_2O_2 still caused no detectable decrease in GSH. However, with 400 μM H_2O_2 a 15% loss was evident after 30 min at 37 °C, increasing to 25% in the presence of azide.

The GSH loss in Figure 1 was measured after 5 min at room temperature. It was the same in the presence and in the absence of glucose and was not reversed by a further 30 min of incubation at 37 °C in the presence of glucose. This was also the case if methionine, glycine and glutamic acid (1 mM each) were added as substrates for glutathione synthesis 2 min after the HOCl (results not shown).

Resting neutrophils contained low concentrations of GSSG and no detectable glutathione sulphonic acid (Figure 2). The GSH lost after treatment with HOCl was not recovered as GSSG. Although a small amount of the sulphonic acid was formed, it accounted for less than 20% of the GSH lost.

Changes in GSH in stimulated neutrophils

Stimulation of neutrophils with PMA caused time-dependent losses of GSH and protein thiols (Figure 3). The decreases over 30 min corresponded to a loss of 1.5 nmol of GSH, compared with approx. 6 nmol of protein thiols per 2.5×10^6 cells. The effects of inhibitors and scavengers of reactive oxygen species are shown in Table 1. Diphenyleneiodonium, at a concentration that

Table 2 Glutathione oxidation products in neutrophils stimulated with PMA

Glutathione derivatives were measured by HPLC after derivatization with dansyl chloride [20]. Results are expressed as nmol of GSH equivalents per 2.5×10^6 neutrophils and are means and range for two representative experiments, each performed in duplicate. The percentage recovered is the increase in GSSG plus GSO_3H as a percentage of the GSH lost.

	Concentration (nmol GSH equivalents per 2.5×10^6 neutrophils)			
Addition	GSH	GSSG	GSO_3H	Recovered (%)
None	2.70 ± 0.05	0.22 ± 0.03	0	
PMA	1.62 ± 0.14	0.22 ± 0.03	0.12 ± 0.02	11
PMA + azide	1.87 ± 0.14	0.66 ± 0.10	0.07 ± 0.02	62

inhibits the oxidative burst by at least 95% [22], prevented GSH loss, indicating that it is an oxidative process. Both catalase, which scavenges H_2O_2 and inhibits the formation of HOCl, and azide, a myeloperoxidase and catalase inhibitor, gave partial inhibition. The thioethers methionine and thiodipropionate, which are good scavengers of HOCl, were both almost completely protective. The addition of exogenous myeloperoxidase or superoxide dismutase significantly enhanced GSH loss. Azide, which prevented only one-quarter of the GSH loss (Table 1), inhibited protein thiol loss by 58 ± 10% (SD).

The GSH loss from neutrophils after stimulation with PMA was not accompanied by an increase in the disulphide (Table 2). Glutathione sulphonic acid was produced but accounted for only a small fraction of the GSH loss. When azide was present, less sulphonic acid was formed and much of the GSH loss was accounted for by an increase in GSSG.

Fate of [^{35}S]glutathione

To determine the fate of neutrophil GSH after exposure to HOCl or stimulation with PMA, the cells were preincubated with cycloheximide plus [^{35}S]cysteine to label the glutathione pool. The cells remained functional as assessed by a normal rate of superoxide generation on adding PMA. Their GSH, as measured with monobromobimane, remained within the normal range, and losses on adding HOCl or on stimulation were similar to those seen with untreated cells. After washing to remove extracellular cysteine, 40–50% of the remaining ^{35}S was co-eluted with GSH either in the monobromobimane assay (results not shown) or on reverse-phase HPLC without derivatization (Figure 4B); 10–15% was protein-associated and the remainder was eluted close to the void volume on HPLC. Gel-filtration chromatography suggested that this peak included γ-glutamylcyst(e)ine with a small amount of cyst(e)ine.

The labelled neutrophils were treated with HOCl or stimulated with PMA. Protein-bound counts were measured after precipitation and the supernatants were separated with a reverse-phase HPLC system without derivatization. As shown previously [13], the oxidation of pure GSH by HOCl gives two novel products as well as GSSG (Figure 4A). Peak B is the thiol-sulphonate; peak A has properties and molecular mass (337 Da) consistent with its being an intramolecular sulphonamide. Control neutrophils gave a broad peak at 3–5 min plus GSH, and no significant counts were eluted with GSSG (Figure 4B). Treatment with HOCl resulted in a decrease in labelled GSH, a slight shift in the initial peak to an earlier elution time, and a new peak in the position of peak A (Figure 4C). Consistent with the results with unlabelled cells, no GSSG was formed. Similar changes occurred, but to a smaller extent, when the neutrophils were stimulated (Figure 4D). Peak A was evident only as a shoulder

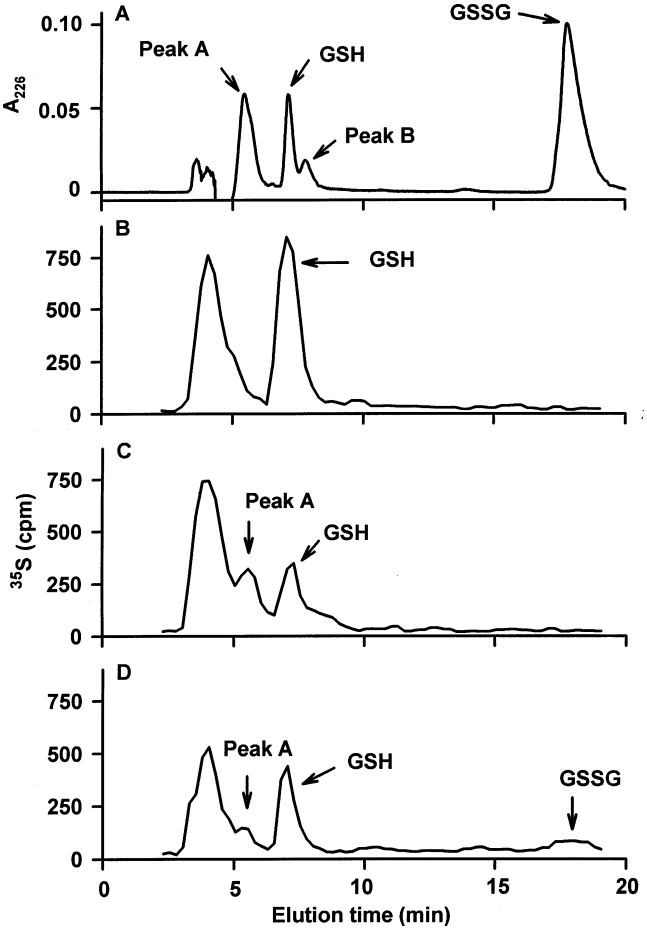

Figure 4 HPLC separation of ^{35}S-labelled glutathione

(**A**) Standard glutathione (10 mM) treated with reagent HOCl (6 mM) at pH 7.4 and separated by HPLC without derivatization. (**B**–**D**) Acid-soluble radioactive counts from neutrophils labelled with [^{35}S]cysteine in (**B**) control cells, (**C**) HOCl-treated cells and (**D**) PMA-stimulated cells. Products were separated by HPLC and fractions collected for scintillation counting as described in the Materials and methods section.

but was consistently present in all of the six cell preparations analysed. A small amount of labelled GSSG was detected after stimulation.

Fractions containing ^{35}S corresponding to each of the three peaks were pooled; the results of several experiments are

Table 3 Distribution of radiolabel in neutrophils that had been preincubated with [^{35}S]cysteine then exposed to HOCl or stimulated with PMA

Protein-bound radioactivity was determined by precipitation with $HClO_4$ and supernatants were separated as in Figure 4. Column fractions corresponding to the early peak (3–5 min), peak A (5.25–6.25 min) and GSH (6.5–8.5 min) were pooled and counted for radioactivity. Total counts represent the sum of the $HClO_4$-soluble and precipitated counts. Results are combined from three experiments and represent means ± S.D. for two to four measurements except for 15 μM HOCl, where single analyses were performed. Because of the variation in the initial percentages of counts in the different fractions between experiments, the combined results are expressed as changes in each fraction after $HClO_4$ exposure or stimulation of the cells. * $P < 0.05$ compared with no HOCl or stimulation.

Fraction	Fraction of total counts before treatment (%)	[HOCl] (mM) ...	Change in total counts after treatment (%) 15	25	50	Time of PMA stimulation ...	15	30
GSH	34–42		−8.6	−15±7	−30±3		−3.3	−12±4
Protein ppt.	8–16		+2.1	+5.0±1.9*	+9.2±3.9*		+4.3±3.0	+3.3±2.5
Peak A	1.4–4.6		+1.5	+4.4±1.9*	+9.2±3.9*		+1.6±1.4	+2.7±1.4*
Early peak	33–44		+2.0	+5.8±8.7	+17±8.3*		−1.0±4.0	+2.5±6.6

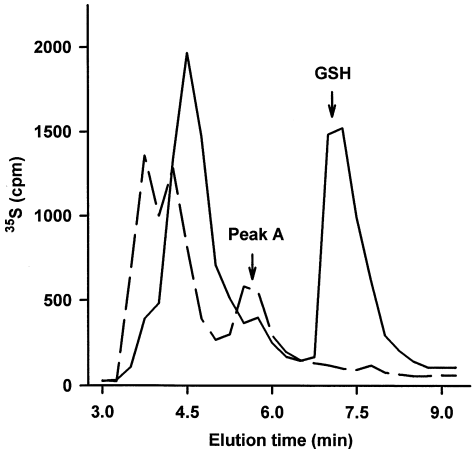

Figure 5 ^{35}S-labelled neutrophil constituents present intracellularly (solid line) and extracellularly (broken line) after treatment with HOCl

^{35}S-labelled neutrophils were exposed to HOCl (30 μM); after 3 min the cells and supernatant were separated by centrifugation. $HClO_4$ supernatants were prepared and analysed by HPLC with scintillation counting of collected fractions as in Figure 6.

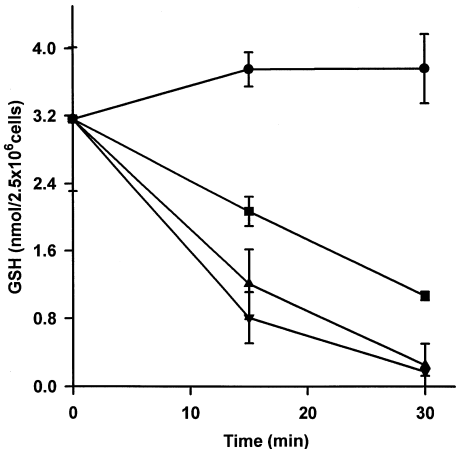

Figure 6 GSH levels in neutrophils stimulated with PMA in the presence of PAG and BSO

Results represent means and range for two representative experiments, each performed in duplicate. Symbols: ●, resting cells; ■, stimulated cells; ▲, with PAG; ▼, with BSO.

summarized in Table 3. The early peak and peak A were not fully resolved, so that for the control cells the counts in the fractions designated peak A represent a shoulder on the early peak. With both HOCl treatment and stimulation, consistent trends were apparent. Losses in GSH were accounted for by increases in protein-bound counts, peak A and early-eluted material. The increases in peak A are minimum estimates of the new product formation, as both treatments decreased the shoulder on the early peak. Most of the protein-bound counts were released by treatment with dithiothreitol and are therefore likely to be mixed disulphides as reported previously [6]. Significant increases in early-eluted material occurred only with the higher HOCl concentrations. These products were not identified, but would include the glutathione sulphonic acid that was detected by product analysis (Figure 2 and Table 2).

The soluble products of GSH oxidation were exported from the neutrophils (Figure 5). Analyses performed 3 min after treatment with HOCl showed that whereas all the remaining GSH was intracellular, almost all of peak A was in the supernatant. It also appeared that the early-eluted products of the reaction were predominantly in the supernatant, whereas the cells retained the early-eluted material that was initially present. Supernatants from untreated cells gave background counts in the position of peak A (results not shown).

Effects of inhibitors of glutathione synthesis and nitric oxide synthase on GSH loss

On the basis of studies with BSO and PAG, others have concluded that new synthesis compensates for GSH loss in stimulated neutrophils [5]. Consistent with this report, we found that preincubation with either of these inhibitors of GSH synthesis considerably enhanced GSH loss when neutrophils were stimulated with PMA (Figure 6). The GSH content of unstimulated neutrophils did not change on incubation with BSO or PAG, however, indicating no endogenous turnover of GSH. Under the conditions of Figure 6, where GSH loss in the presence of BSO and PAG was 96±2% and 89±3% respectively, methionine decreased these losses to only 17±3% and 18±3% ($n = 4$). Similar protection (12±0% GSH loss with BSO and 8±3% with PAG; $n = 4$) was observed with dithiopropionate, a thioether that can substitute for methionine as an HOCl scavenger but not as a GSH precursor.

Preincubation with BSO or PAG also enhanced GSH loss on

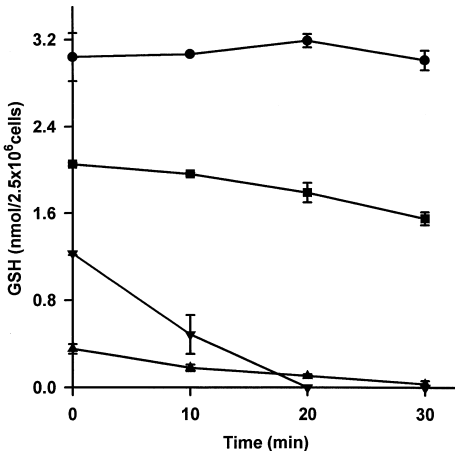

Figure 7 Glutathione levels in resting neutrophils treated with HOCl (30 μM) in the presence of PAG or BSO

Results represent means and range for two representative experiments, each performed in duplicate. Symbols: ●, without HOCl; ■, with HOCl; ▲, with HOCl and PAG; ▼, with HOCl and BSO. The time course for neutrophils treated with HOCl alone was the same whether or not glucose was present in the medium.

the addition of reagent HOCl to resting cells (Figure 7). With HOCl, the reaction was complete in the 2 min before the first sample was analysed. With PAG and BSO present, it was slower. If the effects of BSO and PAG were on GSH synthesis, synthesis would have to be complete within 2 min at room temperature and not require exogenous glucose or precursor amino acids. This is not likely.

BSO and PAG are glycine derivatives that, when mixed in 4-fold excess with HOCl, formed chloramines in stoichiometric yields. Addition of these preformed chloramines (30 μM) to resting neutrophils resulted in 80 % and 86 % losses respectively of GSH within 20 min, compared with a 30 % loss with HOCl alone. Glycine chloramine also gave more GSH loss than did HOCl. Furthermore when neutrophils were incubated with BSO or PAG and then treated with HOCl, their supernatants contained measurable chloramines that, when added to fresh cells, oxidized most of their GSH. Thus BSO and PAG form chloramines that give more efficient oxidation of intracellular GSH than that with HOCl alone.

Nitrosylation has been proposed as a mechanism for decreasing neutrophil GSH levels [4]. We found that the nitric oxide synthase inhibitor *N*-methylarginine (1 mM) protected against the GSH loss in stimulated neutrophils [29 ± 8 % protection ($n = 5$) under the conditions of Table 1], in apparent support of this possibility. However, L-arginine (31 ± 7 % protection; $n = 3$) and D-arginine (28 ± 3 % protection; $n = 3$) had similar effects. These amino compounds also scavenge HOCl to form chloramines. When arginine chloramine (100 μM) was preformed from HOCl and a 2-fold excess of arginine and then added to neutrophils, it caused a 22 % loss of GSH compared with 75 % for an equivalent concentration of HOCl.

DISCUSSION

We have shown that HOCl readily oxidizes neutrophil GSH, at concentrations where H_2O_2 has little effect. This was accompanied by a similar proportional decrease in protein thiols, which on a molar basis was severalfold greater than for GSH. Up to 50 % of the GSH was lost without lysis or impairment of superoxide generation, but there was no regeneration on incubation of the cells in the presence of nutrients. Insignificant amounts of GSSG were formed in the reaction. Of the GSH lost, 20–30 % became protein-bound. The remainder formed small amounts of sulphonic acid and other unidentified negatively charged polar compounds, and a product that co-migrated with a 337 Da putative sulphonamide shown recently to be formed from HOCl and GSH [13]. The latter accounted for at least 20 % of the GSH lost. Along with other GSH oxidation products it seems to be exported from the cells. Although more direct analyses are required to confirm the identity of this novel compound, our finding opens up the possibilities that it could have pathophysiological effects or be useful as a specific marker of HOCl generation.

We also found that neutrophils stimulated with PMA lost 30–40 % of their GSH and protein thiols. There was scarcely any GSSG formation; the product profile, including the putative sulphonamide, was similar to that seen with reagent HOCl. GSH loss [3,5] and a lack of GSSG formation [6] in stimulated neutrophils have been reported previously. Stimulation produces superoxide, H_2O_2 and HOCl, all of which could potentially be responsible for GSH oxidation in stimulated cells [7,23,24]. Reactive nitrogen species also react with thiols [25,26]. Inhibition with diphenyleneiodonium established that the process is oxidative. Diphenyleneiodonium inhibits nitric oxide synthase [27] as well as the NADPH oxidase but we found no evidence that NO-dependent oxidation or nitrosothiol formation [4] contributed to the GSH loss. The slight inhibition by *N*-methylarginine was also seen with arginine and can be explained by scavenging of HOCl to form chloramines that are less efficient than HOCl at penetrating neutrophils and oxidizing their GSH.

Our results strongly favour myeloperoxidase-derived HOCl as being primarily responsible for the GSH loss in stimulated neutrophils. The GSH oxidation products and the large amount of protein thiol oxidation were similar for HOCl-treated and stimulated cells, and the HOCl scavengers methionine and thiodipropionate were fully protective. Catalase, and azide, which inhibits myeloperoxidase and allows H_2O_2 to accumulate [28], both partly inhibited GSH oxidation. In the presence of azide there was more GSSG formed and less protein thiol loss, as expected for a switch from oxidation by HOCl to H_2O_2 acting with glutathione peroxidase. The slight enhancement by superoxide dismutase can be explained by an increase in myeloperoxidase-dependent HOCl formation [29]. The results with amino compounds, added with the original intention of inhibiting other cell enzymes, also support the involvement of HOCl. These formed chloramines with reagent HOCl. The arginine chloramines gave less oxidation of neutrophil GSH than HOCl, whereas the chloramines of the glycine analogues gave more. This can be explained by differences in membrane permeability, and the chloramines' being longer-lived and therefore more efficient than HOCl at oxidizing GSH relative to competing extracellular reactions, provided that they can get into the cells. Different chloramines have been shown to vary in their abilities to penetrate cells, and this is a factor in their relative toxicities [30].

The increase in protein-bound ^{35}S seen both with HOCl-treated and stimulated cells most probably represents the formation of mixed disulphides with GSH, as reported by Chai et al. [6]. The extent of protein thiol loss was severalfold greater than the GSH loss, indicating that internal disulphides or other oxidation products must also be formed. The comparable proportions of GSH and protein thiol loss is an expected consequence of the reaction being non-enzymic and HOCl having a low selectivity for different thiols.

The lack of GSSG formation in neutrophils contrasts with the response of red blood cells to HOCl, where GSH is converted mostly into GSSG and regenerated on incubation with glucose [10]. It also contrasts with the reaction of HOCl with pure GSH, for which GSSG is the major product [13]. It might be that the 5-fold excess of protein thiols over GSH in neutrophils favours mixed disulphide formation.

The enhanced loss of GSH in the presence of BSO and PAG has been interpreted previously as indicating that stimulation induces neutrophils to synthesize GSH to compensate for the loss [5]. Our findings do not support this interpretation. BSO and PAG both enhanced the immediate GSH loss in resting cells treated with HOCl, and their effects were prevented by the HOCl-scavenging thioethers methionine and thiopropionate. They formed chloramines capable of oxidizing neutrophil GSH, and supernatants of cells treated with BSO or PAG and HOCl oxidized GSH in fresh cells. This evidence all supports the conclusion that BSO and PAG act by trapping HOCl as chloramines that can enter the neutrophils and cause more GSH oxidation. Because the effect of methionine was mimicked by thiodipropionate, the previous interpretation of its acting as a substrate for GSH synthesis [5] is incorrect.

Our findings with both the inhibitors of glutathione synthesis and the inhibitors of nitric oxide synthase raise a note of caution that amino compounds used to probe neutrophil function can act through an unexpected mechanism involving HOCl scavenging and chloramine production. This is true not only for inhibitors but also buffers such as Hepes that can form chloramines. Discrepancies between different studies can often be explicable on this basis.

We have shown that HOCl readily oxidizes neutrophil GSH, producing no GSSG but a variety of other products. These include protein adducts and one with the same HPLC mobility as a putative intramolecular sulphonamide recently characterized as a product of GSH oxidation. Similar products are seen when neutrophils are stimulated and we conclude that HOCl is primarily responsible for the GSH loss in these cells. This oxidation has been traditionally attributed to H_2O_2 but it would seem that most of the H_2O_2 generated by neutrophils is intercepted by myeloperoxidase or catalase rather than reacting with glutathione peroxidase. The result is that the major oxidant formed is the less selective HOCl, which causes substantial oxidation of protein thiols as well as GSH. GSH becomes a sacrificial rather than a recyclable antioxidant and will provide limited antioxidant protection for thiol enzymes. Extracellular amines can enhance GSH oxidation if they form chloramines that can penetrate the cells, so oxidation of GSH and other intracellular thiols could account for a significant proportion of the HOCl that neutrophils produce. This could occur not only in neutrophils but also other target cells.

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