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Modification of red cell membrane lipids by hypochlorous acid and haemolysis by preformed lipid chlorohydrins

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Summary Hypochlorous acid (HOCl), a strong oxidant generated by the myeloperoxidase system of neutrophils and monocytes, has been implicated in inflammatory tissue damage by these cells. Reaction of HOCl with the double bonds of unsaturated lipids produces α, β-chlorohydrin isomers. We have exposed red cell membranes to HOCl and used thin layer chromatography (TLC) of the extracted lipids and enzyme-linked immunosorbent assay (ELISA), using an antichlorohydrin monoclonal antibody, to show that fatty acyl chlorohydrins are formed. The ELISA was approximately 25 fold more sensitive than TLC, and chlorohydrins were detected when membranes from 10^6 cells were treated with ≥ 0.16 nmoles HOCl. Lipid chlorohydrins are more polar and bulky than their parent lipids and as such could affect membrane stability and function. To determine the effect of incorporation of lipid chlorohydrins into cell membranes, preformed fatty acid and cholesterol chlorohydrins were incubated with red cells. Lysis was measured as release of haemoglobin and incorporation of lipids was determined by ^{14}C scintillation counting. Addition of HOCl-treated oleic acid to red cells resulted in rapid lysis of a fraction of the cells in a concentration dependent manner. HOCl-treated cholesterol also caused a small amount of cell lysis that was predominantly due to chlorohydrin 3, one of the three major cholesterol chlorohydrin products. Chlorohydrin 3, which has a decreased planarity and polarity, was also primarily responsible for altering the critical micelle concentration of HOCl-treated cholesterol-containing liposomes.

Abbreviations CMC,critical micelle concentration; DPPC, 1,2-dipalmitoyl-phosphatidylcholine; ELISA enzyme-linked immunosorbent assay; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PBS, phosphate-buffered saline (10 mM phosphate + 140 mM NaCl, pH 7.4); TLC, thin layer chromatography

INTRODUCTION

Activated neutrophils and monocytes produce the strong oxidant hypochlorous acid (HOCl) via the myeloperoxidase-catalysed reaction of Cl^- with H_2O_2.[1] HOCl reacts readily with a multitude of biological molecules,[2,3] including unsaturated lipids.[4–10] The reaction of HOCl with the electron-rich double bonds of unsaturated fatty acids, phospholipids and cholesterol produces chlorohydrin derivatives ($-CH_2CH(OH)CH(Cl)CH_2-$) of these lipids. When cholesterol-containing liposomes are exposed to HOCl, three chlorohydrin isomers are produced; 6β-chloro-5α-cholestane-3β,5-diol (chlorohydrin **1**), its positional isomer 5α-chloro-6β-cholestane-3β,6-diol (chlorohydrin **2**) and its stereoisomer 6α-chloro-5β-cholestane-3β, 5-diol (chlorohydrin **3**).[9] Cholesterol chlorohydrins have been detected in red cells, neutrophils and MCF7 breast carcinoma cells[6] and in low density lipoprotein particles[7,8] after exposure to HOCl. The unsaturated bonds of fatty acids have a similar reactivity towards HOCl as the double bond of cholesterol.[5] Therefore, fatty acid chlorohydrins would also be expected to form under these conditions.

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Like other oxidized lipids,[11,12] lipid chlorohydrins are more polar and bulky than their parent lipids.[4,6] If formed in cell membranes they could potentially disrupt membrane structure and function, predisposing the cell to lysis. Lipid chlorohydrins are relatively stable[5,6] and so chlorohydrin cytotoxicity could occur by lipid transfer between cells and low density lipoprotein particles.[13,14] Evidence for the disruptive nature of chlorinated lipids was first proposed by Sepe and Clark[15,16] who reported that myeloperoxidase-dependent release of ^{51}Cr from cholesterol: phospholipid liposomes was due to chlorination rather than peroxidation. Red cells loaded with ^{51}Cr were used by Dallegri et al[17–19] to show that haemolysis by stimulated neutrophils was myeloperoxidase-dependent, implicating HOCl. Red cell lysis by HOCl is due to membrane changes, which cause reduced fluidity and increased permeability.[20]

Using thin layer chromatography and ELISA with a monoclonal antibody raised against oleic acid chlorohydrin[10] we have shown that fatty acyl chlorohydrins are formed in red cell membranes exposed to HOCl. To determine whether chlorohydrin formation, as distinct from other HOCl-induced modifications, could be responsible for lysis, we have examined the effects of incorporating preformed fatty acid and cholesterol chlorohydrins into red cells. Lysis was measured as release of haemoglobin and incorporation of lipids was determined by ^{14}C scintillation counting.

METHODS AND MATERIALS

Cholesterol, 1,2-dipalmitoyl-phosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), oleic acid and *o*-phenylenediamine dihydrochloride were obtained from Sigma Chemical Co. (St Louis, MO). [1-^{14}C]-Oleic acid, [4-^{14}C]-cholesterol and horseradish peroxidase-linked goat antimouse IgG were obtained from Amersham (Buckinghamshire, England). Silica gel thin layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). *N*-Phenyl-1-napthylamine and 1-chloro-2, 4-dinitrobenzene (both from Sigma) were dissolved in ethanol and further diluted in PBS before use. Hypochlorous acid (Reckitt and Coleman, Auckland, New Zealand) was diluted in phosphate buffered saline (PBS) and standardised with 5-thio-2-nitrobenzoic acid as described previously.[21]

Cholesterol chlorohydrins were synthesised by dissolving cholesterol in acetonitrile/isopropanol (1:1) and treating with a ten-fold excess of HOCl.[9] The products were extracted and purified by preparative TLC as described previously.[6] This method gives the three isomers 6β-chloro-5α-cholestane-3β, 5-diol (chlorohydrin **1**), 5α-chloro-6β-cholestane-3β,6-diol (chlorohydrin **2**) and 6α-chloro-5β-cholestane-3β, 5-diol (chlorohydrin **3**). Oleic acid chlorohydrin was synthesised by treating oleic acid epoxide (cis-9, 10-epoxystearic acid, Sigma) dissolved in acetonitrile/isopropanol (1:1) with a molar excess of HCl and extracting as for the cholesterol chlorohydrins. Half the sample was treated with anhydrous methanolic HCl to form methyl esters.[11] The chlorohydrins were pure as determined by TLC.[6]

Exposure of red cell membranes to HOCl

Red cell membranes were prepared, treated with HOCl and their total lipids extracted as described previously.[6] The phospholipids were purified from cholesterol by preparative TLC and subsequently treated with phospholipase A_2 to release free fatty acids,[4] or anhydrous methanolic HCl to produce fatty acid methyl esters.[11] These samples were analysed by TLC[6] with oleic acid chlorohydrin and its methyl ester as standards.

ELISA using an antichlorohydrin monoclonal antibody was carried out according to the method of Domigan et al.[10] Briefly, HOCl-treated cell membranes or whole cells (equivalent to 5×10^7 cells per well), were preincubated overnight at ambient temperature with an excess of monoclonal antibody, then added to ELISA wells coated with chlorohydrin-conjugated albumin. Antibody binding was detected by a horseradish peroxidase-linked secondary antibody using *o*-phenylenediamine dihydrochloride as substrate (A_{495}). POPC-chlorohydrin (0.025–2.5 nmoles/well) was used for a standard curve. Results are presented as the amount of antibody detected by ELISA, in the presence of HOCl-treated cell membranes, as a percentage of the amount of antibody detected in the presence of untreated cell membranes. In control experiments, ELISA of the total lipids, extracted from HOCl-treated cell membranes by the method of Rose and Oklander,[22] gave equivalent antibody binding to the cell membranes themselves.

Exposure of red cells to preformed lipid chlorohydrins

Oleic acid micelles, POPC liposomes and cholesterol: DPPC (1:1) liposomes were prepared by dissolving the lipids in chloroform, evaporating the solvent under nitrogen and resuspending the lipid film in PBS by vortex mixing and bath sonication. The solutions were treated with a half mole amount of HOCl relative to the unsaturated lipid and incubated for one hour at room temperature. Methionine (1 mM) was added to scavenge any residual HOCl. The 50% mixtures of lipid chlorohydrins were added at the stated concentrations (25 to 200 nmoles for fatty acid and 400 nmoles for phospholipid and cholesterol) to red cells (10^9/ml in PBS plus

5 mM glucose). The samples were incubated at 37°C with rotation. Aliquots were removed at the stated times and lysis was determined as the release of haemoglobin into the supernatant by measuring A_{416} minus A_{700} (as turbidity control). A water-lysed sample was used as 100% lysis control.

To determine the incorporation of lipid chlorohydrin into the cell membranes, ^{14}C-labelled oleic acid and cholesterol (0.5 μ Ci/ml) were incorporated into micelles and liposomes before treatment with HOCl and addition to red cells as described above. Aliquots were removed during the incubation at the stated times, diluted with PBS and centrifuged at 10 000 g for 5 min to pellet the intact cells and lysed cell membranes. The cell pellets were washed with PBS and bleached with an equal volume of H_2O_2 (15% w/v) after addition of azide (25 mM) to inhibit catalase. Scintillation counting was carried out in toluene-Triton X-100 (50% v/v) scintillant. In control experiments less than 2% of the ^{14}C-oleic acid was pelleted with the cells. Approximately 30% of the ^{14}C-cholesterol was pelleted under control conditions and this was taken as baseline.

To determine whether lipid chlorohydrins were metabolised by glutathione-*S*-transferase enzymes, red cell glutathione was selectively depleted with 1-chloro-2, 4-dinitrobenzene and the subsequent effect on lysis by chlorohydrins determined as described above. Red cells were incubated with two volumes of PBS containing 3 mM 1-chloro-2, 4-dinitrobenzene for 30 min at 37°C with gentle mixing.[23] The cells were washed twice with PBS and their glutathione content determined using monobromobimane.[24] Incubation of the cells with 1-chloro-2,4-dinitrobenzene depleted at least 95% of their glutathione.

Determination of critical micelle concentrations (CMC)

The effect of HOCl treatment on the CMC of cholesterol: DPPC liposomes was determined by a modified method of Brito and Vaz.[25] The lipids were treated with HOCl as for the red cell experiments. Purified cholesterol chlorohydrins were used in some experiments in the amounts stated (25% or 50%). The lipid solutions were diluted in a concentration range of approximately 5 mM–100 nM and *N*-phenyl-1-napthylamine (1 μM final concentration) was added. After 30 min at room temperature, fluorescence of the solutions was measured at Ex 340 nm and Em 410 nm with band slit widths of 5 nm. The CMCs of the lipids before and after treatment with HOCl were calculated by plotting the relative fluorescence against the log concentration of the lipid and determining the intersection of the two phases of the plot by solving the simultaneous equations of the slopes.

RESULTS

Detection of fatty acyl chlorohydrins in red cell membranes

Lipids were extracted from isolated red cell membranes which had been exposed to 4 and 8 nmoles HOCl per 10^6 cell membranes then hydrolyzed by either phospholipase A_2 or anhydrous methanolic HCl before separating by TLC (Fig. 1a). In each case a band that coelutes with the chlorohydrin standard, and increases in intensity with increasing HOCl exposure, is evident. The faint band in the control sample (lane 2), also coeluting with the chlorohydrin standard, is not fatty acid-related since its mobility did not change after methylation of the sample (lane 6). In the methylated control (lane 6) there appears to be a small amount of unmethylated fatty acid present. A monoclonal antibody raised against oleic acid chlorohydrin[10] showed specific recognition of the lipids purified from cell membranes exposed to HOCl, and no recognition of lipids from untreated cell membranes, as determined by ELISA. This is supportive of the products observed coeluting with the chlorohydrin standards on the TLC plate being chlorohydrins.

Red cell membranes that had been exposed to HOCl were investigated directly by ELISA, without initial extraction of the lipids (Fig. 1b). The antichlorohydrin antibody recognised treated cell membranes at approximately a 25-fold lower concentration of HOCl (8.0 nmoles per 5×10^7 cells) than seen with TLC. Treatment of the cell membranes with 80 nmoles HOCl per 5×10^7 cells gave an equivalent inhibition of antibody binding to 2 nmoles of phospholipid chlorohydrin (see inset, Fig. 1b). Chlorohydrins in cell membranes treated with up to 200 nmoles of HOCl per 5×10^7 cells have been detected in a linear fashion by ELISA using either a higher concentration of antibody or by adding less sample to the assay. Phospholipid chlorohydrins were also observed by ELISA in whole red cells exposed to HOCl, although approximately 100 times more HOCl than with isolated membranes was required to see a similar extent of modification (results not shown).

Lysis of red cells exposed to preformed lipid chlorohydrins

To determine whether uptake of polar chlorohydrins by red cells disrupts their membrane structure resulting in lysis, preformed fatty acid and cholesterol chlorohydrins were incubated with a suspension of red cells and lysis was monitored as release of haemoglobin into the supernatant. Addition of oleic acid micelles to red cells did not cause significant lysis over time as compared to the red cells alone (Fig. 2). However, addition of HOCl-modified oleic acid caused significantly more of the cells to lyse.

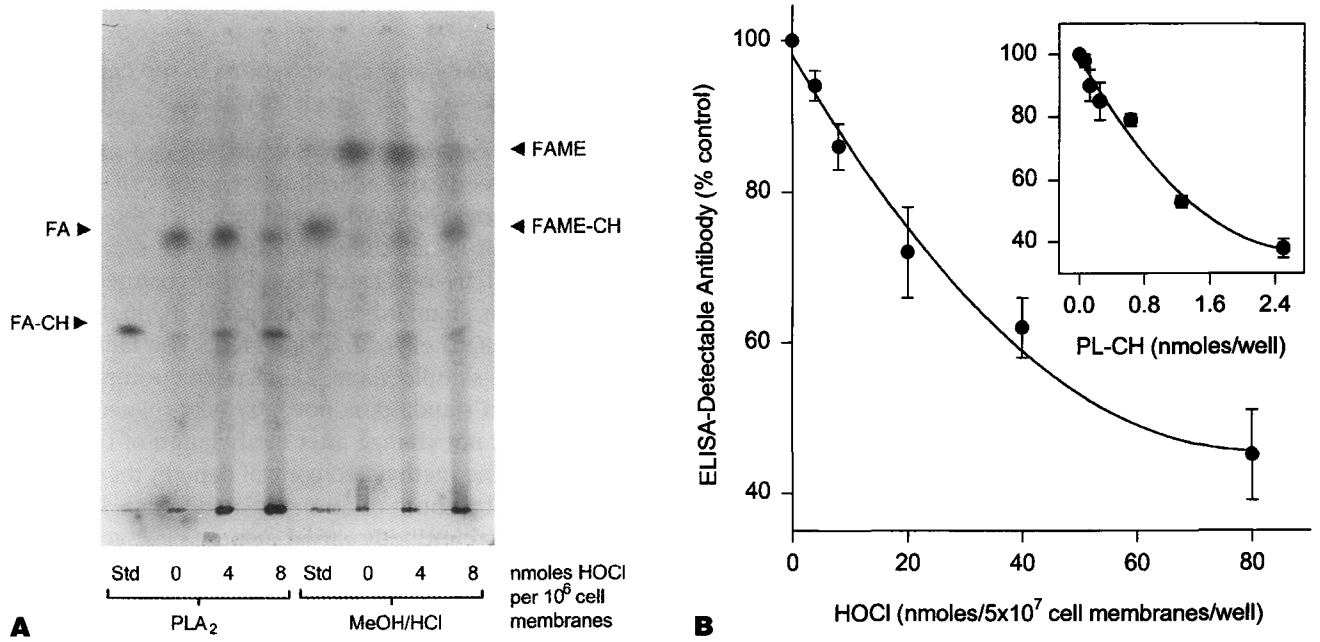

Fig. 1 (**A**) Thin layer chromatography of lipids extracted from red cell membranes (1.25×10^9/ml) which had been exposed to HOCl (0–10 nmoles). The elution solvent was diethyl ether: petroleum ether (bp 60–80°C): acetic acid (70:30:1, v/v/v) and the products were visualised by sulfuric acid (40%, v/v) charring. Lane 1; standard oleic acid chlorohydrin, lane 2; fatty acids extracted from untreated cell membranes, lanes 3–4; fatty acids extracted from cell membranes exposed to increasing amounts of HOCl, lanes 5–8; equivalent methylated samples. Std, standard; FA, fatty acid; FAME, fatty acid methyl ester; CH, chlorohydrin; PLA_2, phospholipase A_2. (**B**) ELISA of red cell membrane phospholipid chlorohydrins. Red cell membranes (equivalent to 5×10^7 cells/well), which had been exposed to HOCl, were preincubated with an excess of antichlorohydrin monoclonal antibody and applied to ELISA wells coated with albumin-conjugated oleic acid chlorohydrin. The control represents the amount of antibody binding in the presence of untreated cell membranes. Results are expressed as mean ± SEM of four experiments, each carried out in duplicate. Inset shows phospholipid chlorohydrin (PL-CH) standard curve.

Lysis occurred quickly, reaching its maximum within 10 min. Measurements taken at 1,2,4 and 6 hour time points showed no further lysis (results not shown).

In order to determine how much lipid was incorporated, ^{14}C-oleic acid was used under the same conditions as Figure 2 and the cell pellets were analysed by scintillation counting after removal of unincorporated lipid. Untreated oleic acid was incorporated rapidly into the cells with 50% being present when they were separated immediately after mixing, increasing to a maximum of 65% after 10 min. A maximum of 40% of the HOCl-treated fatty acid was incorporated within processing of the first sample ($n = 3$, results not shown).

The percentage of red cells lysed was linearly dependent on the concentration of modified oleic acid present (Fig. 3). This increase paralleled the increase in amount of fatty acid incorporated. HOCl-treated oleic acid, at all concentrations, was incorporated to about two thirds the extent of the untreated fatty acid (see inset, Fig. 3). With a decreasing cell concentration there was an increase in amount of fatty acid incorporated per cell, and a corresponding increase in lysis (Table 1).

The kinetics of lysis suggests that fatty acid chlorohydrins may be metabolized, so that their ability to

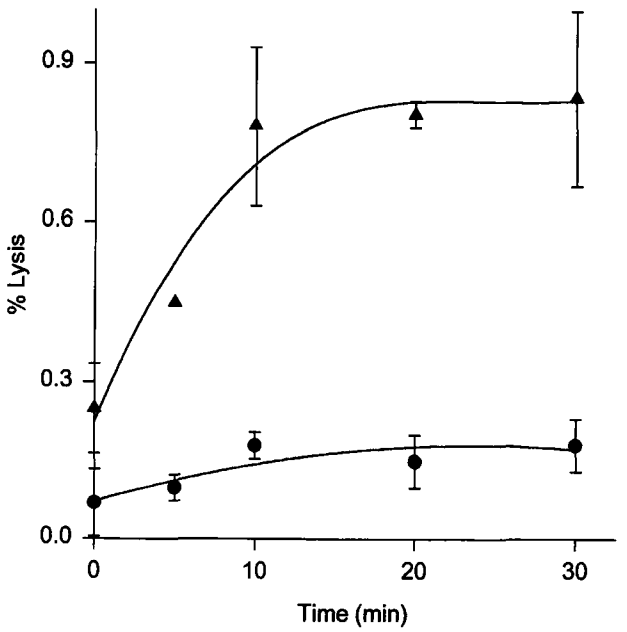

Fig. 2 Time course of red cell lysis by HOCl-treated oleic acid micelles (50 nmoles/10^9 cells in 1 ml). Results shown are the increase in lysis over red cells incubated alone (0.4% lysis) and represent mean ± SEM of four experiments, each carried out in duplicate. ● oleic acid, ▲ 50% HOCl-treated oleic acid.

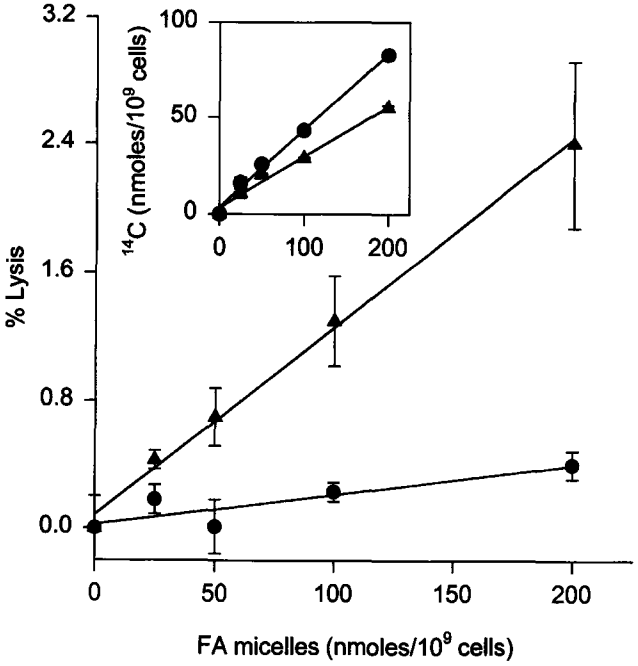

Fig. 3 Effect of the concentration of HOCl-treated oleic acid micelles on the amount of red cell lysis (0–200 nmoles micelles/10^9 cells in 1 ml). Results were determined after 30 min at 37°C and represent mean ± SEM of three experiments, each carried out in duplicate. Inset shows incorporation of ^{14}C-labelled-oleic acid (nmoles/10^9 cells) determined after dilution and sedimentation. ● oleic acid, ▲ 50% HOCl-treated oleic acid.

permeabilize the membrane is transient. Conjugation of chlorohydrins to glutathione via glutathione-*S*-transferase enzymes is one potential detoxification pathway.[26] We have investigated this possibility by selectively depleting red cell glutathione with 1-chloro-2,4-dinitrobenzene and observing the subsequent effect on lysis by oleic acid chlorohydrin. Under the conditions used in Figure 3, there was no change in lysis observed in glutathione depleted cells, as opposed to untreated cells, when they were exposed to oleic acid chlorohydrin (results not shown). Scintillation counting of red cells, before and after extraction with dichloromethane, indicated that all of the ^{14}C-labelled lipid was extracted into the solvent, with very little remaining in the aqueous phase as would be expected for the more hydrophilic glutathione conjugate (results not shown).

Cholesterol: DPPC liposomes, made up at a ratio of 1:1 to mimic the red cell membrane composition, were incubated with red cells at an equivalent concentration to red cell cholesterol (approx. 400 nmoles/10^9 cells). Figure 4a shows that untreated liposomes caused no significant lysis as compared to red cells incubated alone and in fact seemed to give slight protection. Addition of HOCl-modified liposomes caused a gradual lysis of a small proportion of cells over several hours (Fig. 4a). Approximately three times more of the HOCl-treated cholesterol was incorporated into the red cells than unmodified cholesterol, with maximum uptake corresponding to about 10% of the total cholesterol and occurring within an hour (Fig. 4b). Depletion of greater than 95% of the red cell glutathione did not affect chlorohydrin-dependent lysis as compared to unconjugated controls (results not shown).

When cholesterol: DPPC liposomes are treated with HOCl a mixture of chlorohydrins **1**, **2** and **3** are formed.[9] Chlorohydrin **3** has a decreased planarity and polarity and as such may be more disruptive to membranes than chlorohydrins **1** and **2**. When HOCl-treated cholesterol was replaced by purified chlorohydrins the increase in percent lysis over red cells incubated alone was: chlorohydrins **1** and **2** (combined); 0.16 ±0.09, chlorohydrin **3**; 0.60 ± 0.39, and an equivalent mixture of chlorohydrins **1** and **2** (combined) and chlorohydrin **3**; 0.47±0.27 after 4 h incubation at 37°C (mean ± SEM of five experiments, each carried out in duplicate). These results suggest that chlorohydrin **3** is more lytic than chlorohydrins **1** and **2**.

To confirm the hypothesis that chlorohydrin **3** is more disruptive than chlorohydrins **1** and **2**, the critical micelle concentration (CMC) of cholesterol-containing liposomes was determined. Addition of 50% chlorohydrins **1** and **2** (combined) into cholesterol: DPPC liposomes did not affect the CMC significantly compared with control liposomes (92 μM versus of 95 μM, respectively, Fig. 5). However, substitution with 50% chlorohydrin **3** caused significant reduction in CMC to 23 μM. When 25% of chlorohydrins **1** and **2** (combined) and 25% of chlorohydrin **3** were introduced together the CMC was 40 μM.

Table 1 Red cell lysis and ^{14}C-labelled fatty acid incorporation: dependence on cell number

No. cells/ml	Untreated oleic acid (% lysis)	HOCl-treated oleic acid (% lysis)	Untreated oleic acid (nmoles ^{14}C/10^9 cells)	HOCl-treated oleic acid (nmoles ^{14}C/10^9 cells)
1.0×10^9	0.17 ± 0.09	0.47 ± 0.18	25.0 ± 2.8	22.5 ± 2.8
0.5×10^9	0.33 ± 0.20	0.90 ± 0.17	41.4 ± 2.6	37.0 ± 4.4
0.2×10^9	0.63 ± 0.41	2.47 ± 0.73	74.0 ± 3.0	66.5 ± 3.5

Red cells, at the stated concentrations, were incubated for 30 min at 37°C with 50 nmoles ^{14}C-labelled oleic acid, or oleic acid which had been treated with a half mole amount of HOCl. The cell lysis and amount of radiolabel incorporated into the cells was determined as stated in Methods. Results represent mean ± SEM of three experiments, each carried out in duplicate.

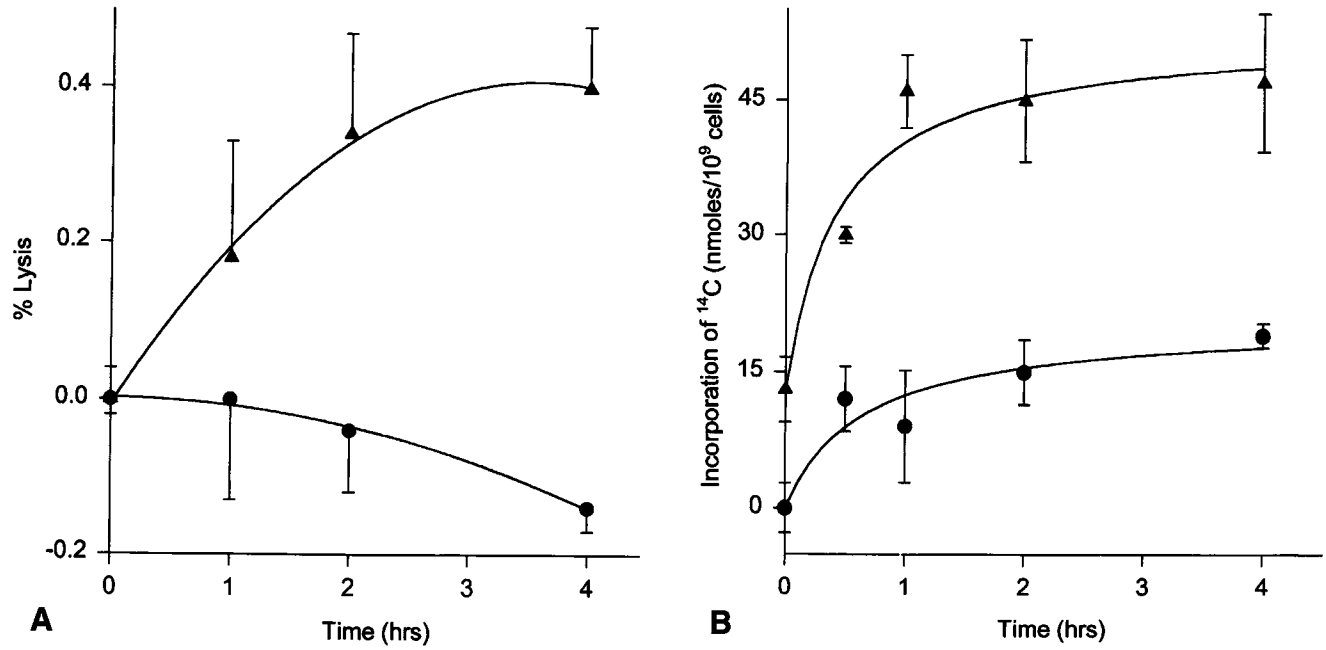

Fig. 4 Time course of (**A**) red cell lysis by HOCl-treated cholesterol:DPPC liposomes (400 nmoles/10^9 cells in 1 ml) and (**B**) incorporation of ^{14}C-labelled cholesterol into red cells. Results shown are the increase in lysis over red cells incubated alone and represent mean ± SEM of five experiments, each carried out in duplicate. ● cholesterol: DPPC, ▲ HOCl-treated cholesterol: DPPC.

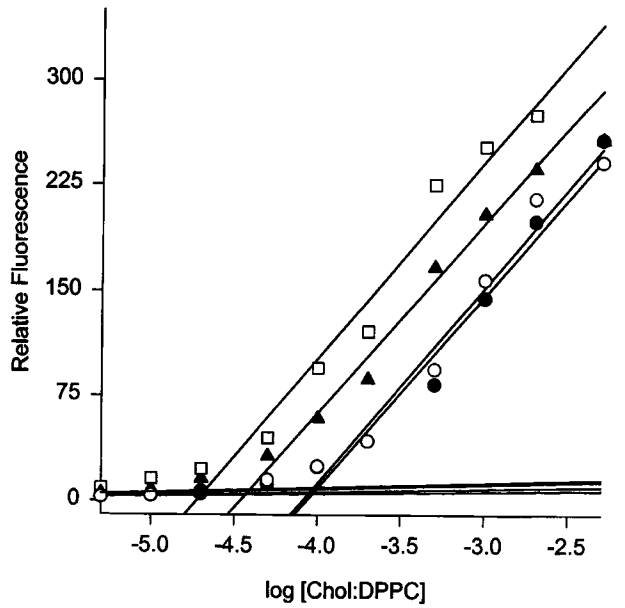

Fig. 5 Determination of critical micelle concentrations (CMC) of cholesterol-containing liposomes. Purified cholesterol chlorohydrins were incorporated into cholesterol: DPPC liposomes and their CMC's determined using the flourescent probe *N*-phenyl-1-napthylamine (Ex 340 nm and Em 410 nm). ● Cholesterol:DPPC liposomes (CMC = 95 μM), O 50% chlorohydrins **1** and **2** combined (CMC = 92 μM), □ 50% chlorohydrin **3** (CMC = 23 μM) and ▲ 25% chlorohydrins **1** and **2** combined with 25% chlorohydrin **3** (CMC = 40 μM).

This compares directly with treating cholesterol with a half mole amount of HOCl (results not shown) and indicates that the change in CMC observed after exposure to HOCl is primarily due to formation of chlorohydrin **3** rather than chlorohydrins **1** and **2**.

DISCUSSION

To assess whether the membrane effects of lipid chlorohydrins could contribute to the cytotoxicity of HOCl, we determined whether the chlorohydrins formed with isolated lipids[4,6] are also formed in more complex membrane systems. Cholesterol chlorohydrins have been observed in red cell membranes treated with HOCl.[6] We have now shown that fatty acyl chlorohydrins are formed in phospholipids at comparable amounts to cholesterol chlorohydrins (as observed with TLC). Using ELISA, formation of fatty acid chlorohydrins was detectable with approximately a 25 fold greater sensitivity than TLC. However, compared with the stoichiometric addition of HOCl to pure unsaturated fatty acids, only a few percent reacted with the membrane. This reflects a relatively slow reaction rate and competition with membrane proteins. The efficiency was even less with whole cells since about 100 times more HOCl was required to see chlorohydrins. The lower yields are presumably due to reaction of HOCl with hemoglobin and other intracellular components.

The observation that there is myeloperoxidase-dependent release of ^{51}Cr from cholesterol: egg PC liposomes [15,16] suggests that chlorohydrins may affect membrane permeability. To address this directly we exposed red cells to preformed fatty acid chlorohydrins. At concentrations at which oleic acid gave limited lysis, oleic acid chlorohydrin rapidly lysed a proportion of the cells in a concentration dependent manner. Phospholipid chlorohydrins did not cause significant lysis over 4 h (results not shown). However, this could be due to the very slow rate of phospholipid exchange between liposomes and red cell membranes.[27]

Since red cells contain approximately 400 nmoles phospholipid per 10^9 cells[27] the amount of fatty acid chlorohydrin incorporated in Figure 3 represents about 1–4% of the total fatty acyl groups present in red cell membrane phospholipids. Increasing the chlorohydrin to red cell ratio increased the number of cells lysed rather than the rate of lysis. One reason could be that incorporation does not occur homogeneously throughout the red cell population, although why this should be the case is not obvious. Alternatively, lipid chlorohydrins could be rapidly metabolised so that their effect is only transient. Glutathione *S*-transferases, which are present in red cells,[28] are responsible for detoxifying a variety of halogenated alkanes and short chain halohydrins via conjugation with glutathione.[26] They also react with endogenous lipids such as estrogen[29] and convert leukotriene A_4 to its glutathione conjugate, leukotriene C_4.[30] It is therefore possible that lipid chlorohydrins could be metabolised by this mechanism. Under the conditions employed in this study, however, we found no evidence for rapid metabolism of lipid chlorohydrins by red cell glutathione-*S*-transferases.

Cholesterol chlorohydrins significantly increased red cell lysis, but their effect was slight. One chlorohydrin isomer, chlorohydrin **3**, was presumably responsible for the increase in lysis. It, also, was the only isomer to alter the CMC of cholesterol. Chlorohydrin **3**, with its *cis* configuration between the A and B rings and its potential to hydrogen bond between the two hydroxyl groups, loses the planar structure and is less polar than cholesterol.[9] Cholesterol transfers only slowly into red cells at a rate dependent on liposome composition.[31] We saw only a small amount of native cholesterol incorporated, and as observed previously,[32] slight inhibition of lysis. Transfer of oxysterols from liposomes to red cells is dependent on their chemical structure[14] and we observed more of the chlorohydrin incorporated. For the 0.02% of lysis per hour in Figure 4, chlorohydrins equivalent to about 6% of the total cholesterol were incorporated.[27]

There have been numerous studies investigating the effects of oxidised lipids on red cells. Incorporation of oxysterols, depending on their structure, can cause cell lysis,[14,33] formation of echinocytes[34,35] and affect membrane packing and permeability.[12,36] Oxysterols have a greater surface area than cholesterol due to their perpendicular, rather than parallel, orientation at the interface[12] and as such are non-bilayer forming lipids.[35] Similarly, oxidized phospholipids have about a 50% greater molecular surface area than unmodified phospholipids due to the oxygen containing groups (-OOH and -OH) being close to the lipid-water interface.[11] The incorporation of only a few percent phospholipid hydroperoxides into red cell membranes can also cause increased permeability to small ions.[37] Our findings show that lipid chlorohydrins are able to disrupt red cell membrane structure and enhance lysis. The amount of lysis observed was small, however, especially in the case of cholesterol chlorohydrins.

Treatment with HOCl causes red cell lysis, with associated membrane changes.[20] Whether chlorohydrin formation could be responsible can be assessed from the results in Figure 1b. Quantitation by ELISA can only be approximate, because chlorohydrins would be formed not only from oleic acid but also polyunsaturated fatty acids in the red cell membrane, and the antibody has different affinities for different chlorohydrins.[10] With this proviso, our results suggest that at the highest concentration of HOCl used (80 nmoles/5×10^7 cells) in ELISA, about 10% of the total unsaturated fatty acid groups (2.0 nmoles) were modified. This is broadly consistent with the relative intensities of the TLC spots. Incorporation of oleic acid chlorohydrin corresponding to four percent of the total phospholipid into red cells caused a few percent of the cells to lyse (Fig. 3), so lysis in association with 10% chlorohydrin formation is feasible. However, about 100 fold less chlorohydrin formation was seen with whole cells as compared with isolated membranes, and 80 nmoles of HOCl per 5×10^7 red cells causes complete lysis within 2–3 h.[20] This lysis is more extensive and exhibits different kinetics than expected for the small amount of chlorohydrin formed. Chlorohydrins are, therefore, very minor products when red cells are treated with HOCl and are unlikely to be responsible for HOCl-mediated red cell lysis.

Although other modifications, such as protein crosslinking, contribute more significantly to lysis of red cells by HOCl,[38] detection of lipid chlorohydrins in cell membranes using ELISA highlights the potential of these compounds as sensitive biomarkers for oxidative damage in biological samples. Lipid chlorohydrins could have other more subtle effects in different cell types. For example, (per)oxidized lipids inhibit lipid metabolising enzymes,[39] induce apoptosis[40,41] and are mutagenic and carcinogenic.[39] The possibility of lipid chlorohydrins exhibiting these effects is currently being investigated.

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