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QUARTERLY

Review

The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids★✪

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Myeloperoxidase (MPO), an abundant enzyme in phagocytes, has been implicated in the pathogenesis of various inflammatory diseases including atherosclerosis. The major oxidant produced by MPO, hypochlorous acid (HOCl), is able to modify a great variety of biomolecules by chlorination and/or oxidation. In this paper the reactions of lipids (preferentially unsaturated fatty acids and cholesterol) with either reagent HOCl or HOCl generated by the MPO-hydrogen peroxide-chloride system are reviewed.

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Abbreviations: ESMS, electrospray mass spectrometry; HNE, 4-hydroxynonenal; HOCl, hypochlorous acid; LC-MS, liquid chromatography-mass spectrometry; LDL, low density lipoprotein; MPO, myeloperoxidase; MS, mass spectrometry; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; TBArS, thiobarbituric acid reactive substances.

One of the major issues has been whether the reaction of HOCl with lipids of low density lipoprotein (LDL) yields predominantly chlorohydrins or lipid hydroperoxides. Electrospray mass spectrometry provided direct evidence that chlorohydrins rather than peroxides are the major products of HOCl- or MPO-treated LDL phosphatidylcholines. Nevertheless lipid peroxidation is a possible alternative reaction of HOCl with polyunsaturated fatty acids if an additional radical source such as pre-formed lipid hydroperoxides is available. In phospholipids carrying a primary amino group such as phosphatidylethanolamine chloramines are the preferred products compared to chlorohydrins. Cholesterol can be converted by HOCl to great variety of oxysterols besides three isomers of chlorohydrins.

For the situation *in vivo* it appears that the type of reaction occurring between HOCl and lipids would very much depend on the circumstances, e.g. the pH and the presence of radical initiators.

The biological effects of lipid chlorohydrins are not yet well understood. It has been shown that chlorohydrins of both unsaturated fatty acids as well as of cholesterol may cause lysis of target cells, possibly by disruption of membrane structures.

Early studies have shown that stimulated phagocytes (neutrophils and monocytes) cause lipid peroxidation [1–4]. Both Cathcart *et al.* [5] and Wieland *et al.* [6] found that both neutrophils and monocytes oxidise low density lipoprotein (LDL) making it cytotoxic. In a rat model of acute inflammation activated neutrophils form the chemotactic lipid peroxidation product 4-hydroxynonenal (HNE) [7].

The strong oxidising and chlorinating species hypochlorous acid (HOCl), which is formed from H_2O_2 and chloride, has been implicated in lipid peroxidation by phagocytes. The formation of HOCl is catalysed by the enzyme myeloperoxidase (MPO), which appears to be of major importance in inflammatory processes such as atherosclerosis. Catalytically active MPO is a component of human atherosclerotic tissue [8], and specific products of MPO damage such as 3-chlorotyrosine have been detected during all stages of the development of atherosclerosis [9]. The *in vivo* relevance of MPO damage is demonstrated by the observation that HOCl modified epitopes of LDL have been detected in human atherosclerotic lesions using a specific monoclonal antibody [10], although the precise modification recognised by the antibody has not yet been identified.

HOCl, which exists in equilibrium with its anion and molecular chlorine [11], is produced by MPO in micro-molar concentrations. In incubations with LDL *in vitro*, HOCl was found to be consumed rapidly in a concentration-dependent manner by reaction with LDL [12]. At low concentrations of HOCl the oxidation of thiol and methionyl residues as well as the formation of chloramines dominate in LDL modification [13, 14]. A wealth of evidence indicates that LDL must be oxidised to promote vascular disease [15, 16] and a physiologically relevant mechanism could include efficient modification of both the lipid and the protein moiety of LDL by the $MPO/H_2O_2/Cl^-$ system.

As far as the analysis of oxidative damage by MPO activity is concerned, van den Berg *et al.* [17] described the measurement of reaction products from HOCl and unsaturated lipids and a collection of methods for identifying and quantifying oxidative modification of cholesterol, proteins and LDL has been published recently by Hazen *et al.* [18].

LIPIDS ARE PEROXIDISED BY HOCl

Several studies have been performed to clarify the effect of HOCl on the lipid moiety of LDL. In lipids the major sites of attack by HOCl are the double bonds of unsaturated fatty acids and cholesterol, leading to either chlorohydrin formation or peroxidation.

Carlin & Djursaeter [3] observed peroxidation of phospholipids by the MPO/H_2O_2/io-

dide system in terms of the production of thiobarbituric acid reactive substances (TBArS) and Stelmaszynska *et al.* [19] reported that liposomes containing egg yolk phosphatidylcholine are oxidised either by the $MPO/H_2O_2/Cl^-$ system or stimulated rat neutrophils as measured by TBArS formation. Since virtually no oxidation occurred in the absence of chloride and lipid hydroperoxide formation could be inhibited by the HOCl scavenger taurine, it was concluded that HOCl was the oxidising species. When the influence of the H_2O_2 concentration on TBArS formation was studied, a bi-phasic dependence was found with the $MPO/H_2O_2/Cl^-$ system indicating self-destruction of the enzyme at higher substrate concentrations. The occurrence of lipid peroxidation was supported by the finding that HNE, another secondary product, was formed. Most significantly, HNE formation was associated with a marked lag phase of about one hour (Fig. 1).

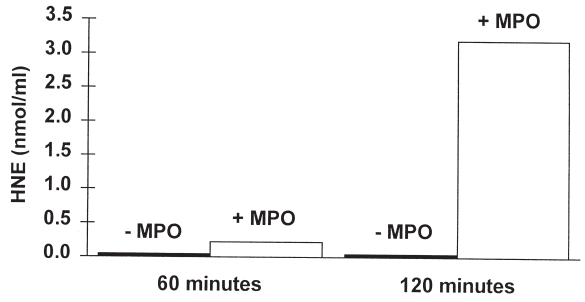

Figure 1. Formation of 4-hydroxynonenal in phosphatidylcholine liposomes by the $MPO/H_2O_2/Cl^-$ system.

2 μmol/l liposomes and 10.6 μg MPO in 0.5 ml of 0.06 mol/l phosphate buffer with 0.16 mol/l NaCl were incubated at 37°C inside a dialysis bag (0.8 cm diameter) in 5 ml of an external fluid containing 2.5 μmol H_2O_2. In the control samples (without MPO) only traces of HNE were detectable; an upper value of 0.05 nmol/ml HNE is shown [19].

Panasenko *et al.* [20–22] also reported that reagent HOCl or HOCl generated by the $MPO/H_2O_2/Cl^-$ system can initiate lipid peroxidation both in lipoproteins and liposomes. In the reagent HOCl concentration range 0.1–1.0 mmol/l a linear increase of TBArS formation was observed, while at concentrations above 1 mmol/l the yield of TBArS decreased (Fig. 2). Several other pero-

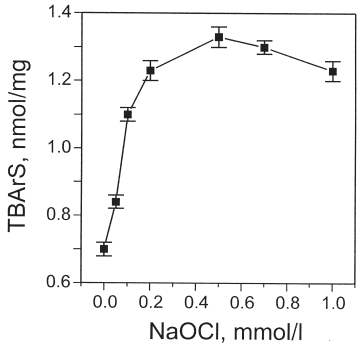

Figure 2. Dependence of TBArS formation in phosphatidylcholine liposomes on the hypochlorite concentration.

Phosphatidylcholine liposomes (2 mg/ml) were prepared in 0.14 mol/l NaCl, 10 mmol/l phosphate (pH 7.4) and incubated for 40 min with NaOCl at 37°C. TBArS were determined and expressed as malondialdehyde equivalents (mean and S.D., n = 4) [23].

xidation products were detected including primary products (conjugated dienes and oxygen containing species), aldehydes [21–24] and Schiff bases [21]. A concomitant decrease of double bonds was noted and the formation of TBArS could be inhibited by the free radical scavengers α-tocopherol and butylated hydroxytoluene, indicating a free radical process [23].

HOCl-induced lipid peroxidation was found to be pH-dependent. Using phospholipid liposomes Panasenko *et al.* [25] found that an increase in pH values led to an increase in various products of lipid peroxidation.

ALTERNATIVELY LIPID CHLOROHYDRINS ARE FORMED BY HOCl

Recently it has been reported that treatment of LDL with HOCl concentrations as low as 5 μmol/l results in overall aggregation of LDL as detected by dynamic light scattering [26].

According to the findings of Hazell *et al.* [27, 28], this aggregation after exposure of LDL to reagent or enzymatically generated HOCl is mediated by modification of lysine residues rather than by lipid oxidation. Lysine residues quantitatively represented the major target and were oxidised to approximately the same extent with reagent or enzymatically generated HOCl. Chemical modification of lysine residues before oxidation with HOCl prevented aggregation. LDL lipid oxidation was less favoured than protein oxidation, as judged by the amounts of lipid hydroperoxides, chlorohydrins, cholesterol or fatty acid oxidation products formed [27]. In a kinetic study protein modification was found to be rapid and followed by an extended period of lipid peroxidation during which further protein oxidation did not occur [28]. Similarly, Jerlich *et al.* [12] observed a low rate of TBArS formation in LDL by the $MPO/H_2O_2/Cl^-$ system, but a rapid degradation of apo B-100 tryptophan residues under the same conditions.

Since unsaturated fatty acids are consumed to a much greater extent than TBArS were formed [12] it was assumed that chlorohydrin formation accounts for the difference. HOCl reacts with olefinic double bonds of lipids to yield chlorohydrins [29–31].

Winterbourn *et al.* [29] studied the reactions of HOCl with oleic, linoleic, and arachidonic acids both as free fatty acids or bound in phosphatidylcholine. Oleic acid was converted to the two 9,10-chlorohydrin isomers in near stoichiometric yield. Linoleic acid, at low HOCl:fatty acid ratios, yielded predominantly a mixture of the four possible mono-chlorohydrin isomers. Bis-chlorohydrins were also formed, in increasing amounts at higher HOCl concentrations. Arachidonic acid gave a complex mixture of mono- and bis-chlorohydrins, the relative proportions depending on the amount of HOCl added. Reactions of oleic and linoleic acids with MPO, hydrogen peroxide, and chloride gave chlorohydrin products identical to those with HOCl. According to van den Berg *et al.* [32], cholesterol and several unsaturated fatty acids exhibited comparable sensitivities towards HOCl.

Formation of cholesterol chlorohydrins has been studied by van den Berg *et al.* [32], Heinecke *et al.* [30] and Carr *et al.* [33, 34]. Three different positional and steric isomers are formed by addition of HOCl to the C=C double bond in cholesterol. Heinecke *et al.* [30] identified an α- and a β-chlorohydrin (6-β-chlorocholestane-3β,5α diol and 5α-chlorocholestane-3 β,6 β-diol). The structure of chlorohydrin 3 (6 α-chloro-5 β-cholestane-3 β,5-diol) was elucidated by Carr *et al.* [34] using various spectroscopic techniques.

The mechanism of chlorohydrin formation is a two step reaction involving an electrophilic addition reaction of HOCl (Fig. 3). One molecule of HOCl is added to the C=C double bond. The Cl^+ ion of HOCl serves as an electrophilic species; it polarises the double bond and is added to one of the carbon atoms. The other carbon atom then bears a positive charge (carbenium ion) and the unsaturated nature of the bond is lost. Then the remaining OH^- ion is added in a second step. However, in some studies other reaction products of the $MPO/H_2O_2/Cl^-$ system have been reported. Instead of OH^-, other anions like Cl^- can be added to the carbenium ion, resulting for example in the appearance of di-chlorinated substances. Hazen *et al.* [35] found a di-chlorinated reaction product at acidic pH while studying the chlorination of cholesterol in LDL by the myeloperoxidase system. This sug-

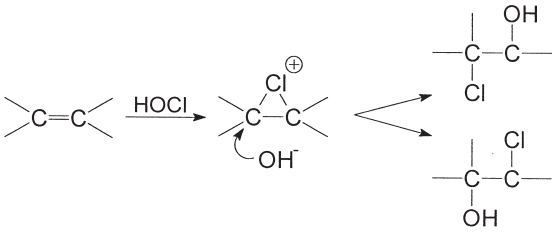

Figure 3. Electrophilic addition of HOCl to olefins (unsaturated fatty acids and cholesterol).

For details see text.

gested that molecular chlorine could also be formed and act as the reactive species.

As known from organic chemistry, chlorohydrins can be further converted into epoxides and *trans*-dihydroxy compounds under alkaline conditions. Formation of lipid epoxides by HOCl has been observed by Heinecke *et al.* [30] and Carr *et al.* [33]. Heinecke *et al.* [30] described the formation of α- and β-epoxides in cholesterol-phosphatidylcholine multi-lamellar vesicles as reaction products of cholesterol and the MPO/ H_2O_2/Cl^- system besides chlorohydrins. Carr *et al.* [33] found that chlorohydrins of cholesterol are readily converted into their respective epoxides during analysis by MS.

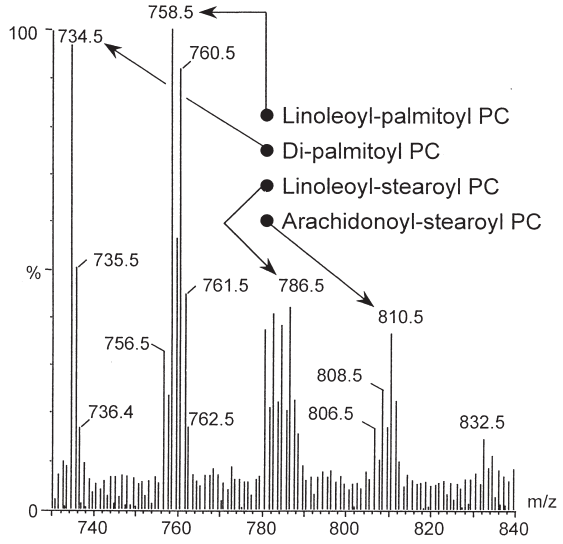

Figure 4. HPLC/ESMS of native LDL from human plasma.

The lipid profile of native LDL shows the presence of a number of phosphatidylcholine species with varying fatty acid chain lengths and degrees of unsaturation. Linoleoyl-palmitoyl phosphatidylcholine appears at 758 *m/z* and linoleoyl-stearoyl phosphatidylcholine appears at 786 *m/z* [36].

A COMPARATIVE LC-MS INVESTIGATION

Since both peroxidation and chlorohydrin formation have been described for lipids the question arises as to whether HOCl preferentially either chlorinates or peroxidises polyunsaturated fatty acids (PUFAs) in LDL, rendering this lipoprotein atherogenic.

High performance liquid chromatography in connection with electrospray mass spectrometry (HPLC/ESMS) in the positive ion mode was used by Jerlich *et al.* [36] to detect chlorohydrin and peroxide formation with comparable sensitivity in phosphatidylcholines (PC) derived in human LDL modified by reagent HOCl. This study provided the first direct chemical evidence that treatment of LDL with micromolar quantities of reagent HOCl or the $MPO/H_2O_2/Cl^-$ system is able to generate phospholipid chlorohydrins under conditions where no lipid hydroperoxides were detectable.

In native LDL PC-species containing saturated (palmitoyl, stearoyl) and unsaturated (linoleoyl, arachidonoyl) fatty acids were observed (Fig. 4). Incubation of LDL with HOCl resulted in a concentration-dependent depletion of the native lipid peaks and the concomitant appearance of new species at +52 *m/z* corresponding to the addition of HOCl to the molecule as shown for linoleoyl-palmitoyl PC in Fig. 5. This is consistent with the formation of chlorohydrins.

Furthermore, spectra of the region of the new peak appearing at a retention time of 6.4 min clearly showed a compound with a mass of 810 *m/z* corresponding to the ^{35}Cl isotope peak and its ^{37}Cl isotope peak at 812 *m/z*. Bis-chlorohydrinated products were also observed, but did not represent a significant proportion of the modified lipid [36].

INFLUENCE OF FREE RADICAL GENERATORS

Nevertheless lipid peroxidation is a possible alternative reaction of HOCl with polyunsaturated fatty acids if an additional radical source is available. Panasenko & Arnhold [37]

have shown that the reaction of HOCl with a pre-formed lipid hydroperoxide of linoleic acid yields free radicals able to cause further oxidation of lipid molecules. The yield of TBArS continuously increased with higher amounts of hydroperoxide after the initiation of lipid peroxidation by HOCl producing systems (Fig. 6). The accumulation of TBArS was inhibited by scavengers of free radicals such as butylated hydroxytoluene and by the HOCl scavengers taurine and methionine.

In a number of experiments it has been shown that the increased rate of lipid peroxidation induced by HOCl is caused neither by catalytically active traces of metal ions nor by singlet oxygen that can result from the reaction of hypochlorous acid with hydrogen peroxide [23, 24, 38].

The mechanisms of the reaction of lipid hydroperoxides with hypochlorous acid is still unknown. A possible mechanism for the free radical formation in this reaction has been proposed by Arnhold *et al.* [38] (Fig. 7) which is based on the finding that di-alkyl-peroxides have been identified as side-products. It is assumed that hydroperoxides are chlorinated by HOCl according to reaction (3) and the resulting chlorinated intermediates decompose yielding alkoxy radicals. The reaction (2), analogous to the well known reaction (1) of H_2O_2 with HOCl, was excluded due to the fact that no singlet oxygen formation was detect-

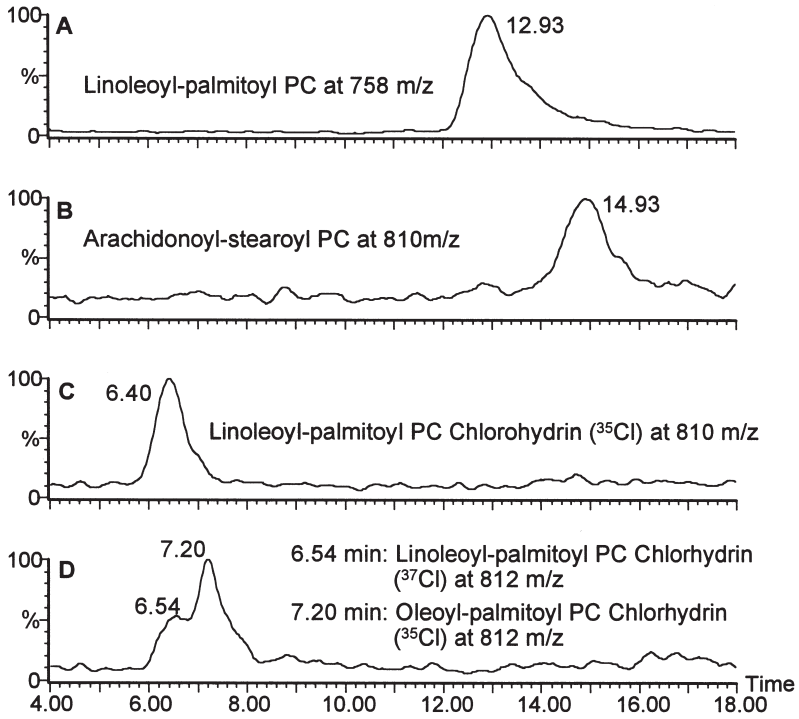

Figure 5. Extracted ion chromatograms of native and HOCl-modified PCs from LDL.

LDL was incubated with 355 μmol/l HOCl in phosphate-buffered saline (PBS), pH 6.0, at 37°C. One experiment representative of three similar ones is shown. All chromatograms are normalised to their largest peak. A: Native linoleoyl-palmitoyl PC of 0.5 mg/ml LDL (758 *m/z*). B: Native arachidonoyl-stearoyl PC of 0.5 mg/ml LDL (810 *m/z*). C: Mass chromatogram at 810 *m/z* showing depletion of the arachidonoyl-stearoyl PC after 30 min of 355 μmol/l HOCl treatment and appearance of a new species at 6.4 min corresponding to linoleoyl palmitoyl PC + HOCl containing the ^{35}Cl-isotope (758 + 52 *m/z*). D: Mass chromatogram at 812 *m/z*. The peak at 7.20 min represents the HOCl adduct of oleoyl-palmitoyl PC containing the ^{35}Cl isotope and the peak at 6.54 min the HOCl adduct of linoleoyl-palmitoyl PC containing the ^{37}Cl isotope [36].

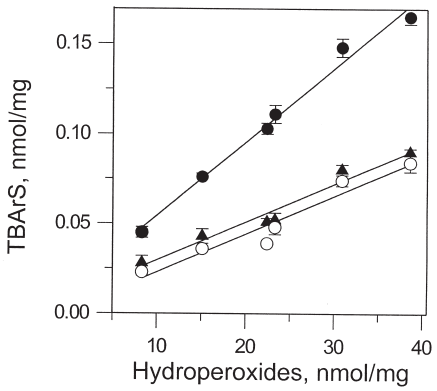

Figure 6. Dependence of TBArS formation induced by the MPO/H_2O_2/Cl^- system on the concentration of linoleic acid hydroperoxides.

The concentration of hydroperoxide groups was adjusted by incorporation of linoleic acid hydroperoxide into phosphatidylcholine liposomes. Liposomes (l, s 2 mg/ml total lipid concentration) were incubated with 3.6 μg/ml MPO and H_2O_2 (50 μmol/l, a second addition of H_2O_2 was made after 15 min) at 25°C for 36 min in PBS, pH 6.0. Some experiments (s) were performed in the absence of chloride. Initial values (m) of TBArS are also given. All data are given as means and S.D., n = 4 [37].

able [38, 39]. On the other hand, considering the reaction of lipid hydroperoxides and hypochlorous acid as a redox reaction, it would be expected that hypochlorous acid is reduced to Cl^- while hydroperoxides are oxidised to peroxy radicals.

In the case of LDL and other lipoproteins, initiating free radicals may be derived from the protein moiety as well. HOCl is able to produce radicals from tyrosine [40–42] and lysine [43, 28] side chains of proteins. In the

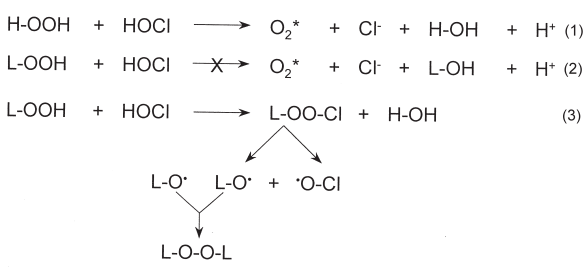

Figure 7. Hypothetical scheme for the formation of alkoxy radicals by the reaction of lipid hydroperoxides with HOCl [38].

case of lysine this involves reaction with the amino group to give chloramines, which can subsequently break down into a chlorine atom and a nitrogen centred radical [43], while MPO may produce a tyrosyl radical from tyrosine [41].

OXIDATION OF CHOLESTEROL

As with fatty acids, chlorohydrin formation is not the only possible reaction of cholesterol with HOCl. Several papers describe the formation of a number of oxysterols by treatment of cholesterol with HOCl. Van den Berg *et al.* [32] identified 4-hydroxycholesterol as a major product besides several other hydroxy- and keto-derivatives in smaller amounts. In a comprehensive study Momynaliev *et al.* [44] were able to isolate eighteen fractions and identified eleven oxidised species of cholesterol by gas chromatography-mass spectrometry. The reaction of cholesterol with HOCl in various systems (egg phosphatidylcholine liposomes, LDL, and aqueous colloidal dispersion of cholesterol) resulted in the same products. The mechanism of these oxidation reactions is unknown.

REACTION OF THE HEAD GROUP OF PHOSPHOLIPIDS

In some lipids there may be other targets of HOCl besides C=C double bonds. In phosphatidyl-ethanolamine, the predominant phospholipid of *Escherichia coli*, there is a primary amino group available as a competitive reactive group besides the double bonds of unsaturated fatty acids. According to Carr *et al.* [45] chloramines are the much preferred products and chlorohydrins were formed in substantial amounts only when HOCl was in excess of the amount required to convert the amine to the di-chloramine. Interestingly the reactions of a second hypohalous acid, HOBr, were also studied. HOBr can be formed from bromide

ions, which occur at much lower levels *in vivo* than chloride. At all concentrations, bromoamines and bromohydrins were formed concurrently from HOBr, indicating a greater reactivity with unsaturated fatty acids than HOCl.

BIOLOGICAL PROPERTIES OF CHLOROHYDRINS

Not only biochemical, but also biophysical effects, might be expected to arise from oxidised or chlorinated lipids. Winterbourn *et al.* [29] proposed that chlorohydrins, if formed in cell membranes, could cause disruption to membrane structure, since they are more polar than the parent fatty acids. Therefore they might be significant in neutrophil-mediated cytotoxicity. Vissers *et al.* [46] investigated membrane changes associated with lysis of red blood cells by HOCl. They observed both chlorohydrin formation of membrane lipids and modification of membrane proteins, concluding that HOCl-mediated damage to the lipid bilayer or to the membrane proteins sets the cells on a path toward eventual lysis. According to Vissers *et al.* [47] the modification of membrane proteins occurs at lower doses of HOCl than chlorohydrin formation of phospholipids and cholesterol. When pre-formed fatty acid and cholesterol chlorohydrins were incubated with erythrocytes lysis also occurred [48]. 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 (6 α-chloro-5 β-cholestane-3 β,5-diol) [34].

Results consistent with a perturbation of bilayer packing have been obtained by Drobnies [49]. CTP-phosphocholine cytidyltransferase, an integral membrane enzyme which catalyses a regulatory step in mammalian biosynthesis of phosphocholine, is activated by hypochlorite-oxidised phosphatidylcholines [49].

Heinecke *et al.* [30] suspected that cholesterol chlorohydrins might mediate powerful biological effects in the artery wall, since other oxygenated sterols are cytotoxic and mutagenic and are potent regulators of cholesterol homeostasis in cultured mammalian cells. Cholesterol chlorohydrin formation by reagent HOCl has been observed in various cell types including erythrocytes, neutrophils and mammary carcinoma cells [33].

CONCLUSION

For the situation *in vivo* it might be proposed that the type of reaction occurring between HOCl and lipids would very much depend on the circumstances, e.g. the pH and the presence of preformed hydroperoxides. These are likely to be sufficiently available to make peroxidation by HOCl, as well as chlorohydrin formation, a viable possibility.

With mounting evidence for the presence of myeloperoxidase in atherosclerotic lesions, any biological effects of phospholipid chlorohydrins can be expected to contribute significantly to the pathology of atherosclerosis, and similarly to other inflammatory diseases.

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