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Hypochlorite-Induced Peroxidation of Egg Yolk Phosphatidylcholine is Mediated by Hydroperoxides

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Using a chemiluminescent method, the consumption of $HOCl/OCl^-$ was investigated during interaction with liposomes prepared from dimyristoylphosphatidylcholine (DMPC) or egg yolk phosphatidylcholine (EYPC). The concentration of $HOCl/OCl^-$ decreased with time in the suspension of EYPC that contain unsaturated lipids and did not change in DMPC liposome suspensions. $HOCl/OCl^-$ was consumed more rapidly in peroxidized EYPC. The amount of double bonds was lowered by 40% in peroxidized liposomes and decreased by approximately one-third under the action of $HOCl/OCl^-$ in both native and peroxidized EYPC samples. Second-order rate constants for the interaction between HOCl and phospholipid double bonds of 0.50 $M^{-1}s^{-1}$ were calculated for native EYPC on basis of the consumption of $HOCl/OCl^-$ or from the decrease in concentration of double bonds. In peroxidized EYPC this reaction constant was similar as determined following changes in double bonds. It is concluded that the consumption of $HOCl/OCl^-$ increased in peroxidized liposomes due to additional reactions with lipid peroxidation products.

tert-Butyl hydroperoxide and cumene hydroperoxide, or organic peroxides or epoxides (cis-9,10-epoxystearic acid; cholesterol-5α,6α-epoxide; trans-2,3-epoxy-butane; cis-2,3-epoxy-butane) were incorporated into liposomes and investigated in respect to their ability (1) to increase the consumption of $HOCl/OCl^-$ in DMPC liposomes, (2) to generate a non-enhanced chemiluminescence with $HOCl/OCl^-$ and (3) to evoke an accumulation of lipid peroxidation products (TBARS) in EYPC liposomes in the absence and presence of NaOCl. None of peroxides or epoxides tested showed any effect on the consumption of $HOCl/OCl^-$ or the generation of chemiluminescence. Nor increase of TBARS both in the absence or presence of $HOCl/OCl^-$. In contrast, tert-butyl hydroperoxide and cumene hydroperoxide increased the consumption of $HOCl/OCl^-$ in DMPC liposomes and mediated a higher accumulation of TBARS in EYPC liposomes in the presence of $HOCl/OCL^-$ over the control. These data suggest that lipid peroxidation in EYPC can be initiated by the reaction of $HOCl/OCL^-$ with organic hydroperoxides.

Keywords: Hypochlorite, Lipid peroxidation, Chemiluminescence, Hydroperoxides, Free radicals, TBA-reactive products

INTRODUCTION

Lipid peroxidation (LPO) has been implicated in development of a number of physiological and pathological processes.[1–3] Phagocytes, in particular, neutrophils may be responsible for induction of LPO reactions *in vivo*. It has been well documented that neutrophils are able to initiate LPO in

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liposomes and human blood plasma lipoproteins.[4–7] This can be attributed primarily to their ability to produce reactive oxygen species: superoxide, hydrogen peroxide, hypochlorous acid, hydroxyl radical, and singlet oxygen.[8]

However, superoxide and H_2O_2 are weak oxidants and apparently are unable to initiate LPO directly.[3,9] Hydroxyl radical is extremely reactive, but its involvement in LPO initiation by phagocytes is equivocal.[7,10–12] Free metal ions enhance the yield of peroxidation products in LPO initiated by activated phagocytes.[6,12] Hypochlorous acid (HOCl), or the hypochlorite anion (OCl^-), being added to the system or produced by myeloperoxidase in the presence of H_2O_2 and Cl^-, has been also shown to initiate LPO in liposomes[13–16] and human blood lipoproteins.[17–20] A number of LPO products is produced in this reactions, including primary products (diene conjugates and oxygen-containing species),[13–15,19,20] aldehydes,[13–20] and Schiff bases.[18]

Myeloperoxidase has been detected in human atherosclerotic lesions.[21] Its products are assumed to play a pivotal role in transforming low density lipoprotein (LDL) into atherogenic particles during the development of atherosclerosis. Low density lipoproteins modified by hypochlorous acid were found to be captured by mouse peritoneal macrophages in larger amounts than unmodified ones.[22,23] Another product of the myeloperoxidase-H_2O_2 system is the tyrosyl radical.[24] Exposure of LDL to L-tyrosine and activated human neutrophils caused lipid peroxidation in LDL.[25] Other oxidants derived from myeloperoxidase *in vitro* are hydroxyl radical[26] and singlet oxygen,[27] both being able to promote lipid peroxidation.[28,29]

We focused our attention on the ability of hypochlorous acid to promote lipid peroxidation. Lipid peroxidation products accumulate in artificial phospholipid vesicles[13–16,20] and in human blood lipoproteins[14,15,17–20] upon the action of exogenous hypochlorous acid or myeloperoxidase and H_2O_2 in Cl^- containing solutions. In other studies, no lipid peroxidation after the action of hypochlorous acid on lipid containing systems was defected.[23,30–32] Our results indicate, that the yield of lipid peroxidation products depends mainly on the concentration of hypochlorous acid and the concentration of target groups for $HOCl/OCl^-$.[13,14,16]

Regarding the mechanism of LPO initiation by $HOCl/OCl^-$, it has been shown that catalase, H_2O_2, $FeSO_4$, $FeCl_3$, and superoxide dismutase did not influence the accumulation of LPO products.[13] Obviously, none of the metal ions or reactive oxygen species ($O_2^{-\bullet}$, H_2O_2, $^1O_2$, $^\bullet OH$) possibly present in the liposome suspension or derived from reactions with $HOCl/OCl^-$ participate in the initiation of hypochlorite-induced LPO. It can be assumed that LPO is initiated as a result of a direct interaction of HOCl (or OCl^-) with either a double bond of the hydrocarbon chain in a phospholipid molecule (-HC=CH-) or some different reactive groups possibly formed in the lipid material.

In the former case, chlorohydrins are shown to be formed.[30,31,33] Epoxides derived from chlorohydrins under mildly alkaline conditions can be considered as new targets for hypochlorite followed by LPO initiation. Among other targets different oxygen-containing products of LPO may be of importance for further LPO activation by $HOCl/OCl^-$. We here address two questions: (1) is $HOCl/OCl^-$ capable to react with hydro-, dialkyl-, diacyl-, alkylacylperoxide and epoxide groups present in the lipid phase of liposomes, and (2) which of these reactions will initiate lipid peroxidation in systems containing unsaturated lipids?

To answer to these questions different compounds with hydroperoxide, peroxide, or epoxide groups were incorporated into liposomes composed of dimyristoylphosphatidylcholine (DMPC) or egg yolk phosphatidylcholine (EYPC). The consumption of $HOCl/OCl^-$, the accumulation of lipid peroxidation products, and non-enhanced chemiluminescence have been investigated in these liposomes. Only hydroperoxides seem to be responsible for the initiation of LPO by $HOCl/OCl^-$.

MATERIALS AND METHODS

Chemicals

Dimyristoylphosphatidylcholine (DMPC) and purified egg yolk phosphatidylcholine (EYPC) were purchased from Fluka, Switzerland. The approximate fatty acid composition of EYPC as given by the supplier was 16:0–33%, 18:0–14%, 18:1–30%, 18:2–14%, and 20:4–4%.

Tert-butyl hydroperoxide, cumene hydro peroxide, di-tert-butyl peroxide, tert-butyl perbenzoate, dibenzoyl peroxide were also purchased from Fluka, Switzerland. Cis-9,10-epoxystearic acid, cholesterol-5α,6α-epoxide, cis-2,3- epoxybutane, trans-2,3-epoxy-butane, n-butanol, isopropanol, chloroform, potassium iodide, iodine monobromide and sodium hypochlorite were obtained from Sigma, Germany. Luminol was from Boehringer, Mannheim, Germany. 2-Thiobarbituric acid (TBA) was from Serva, Germany. Butylated hydroxytoluene and a colour reagent for determination of hydroperoxides (Cat. Nr. 14106) were obtained from Merck, Germany.

Liposomes

Multilamellar liposomes were prepared immediately before the experiment from egg yolk phosphatidylcholine or dimyristoylphosphatidylcholine. An aliquot of phospholipids dissolved in chloroform was evaporated to dryness. Liposomes were prepared by dissolving the lipid film in 0.14 mol/l NaCl, 10 mmol/l phosphate (pH 7.4) and vortexing rigorously for 30 s. In some experiments, epoxides, hydroperoxides or peroxides were incorporated into liposomes by mixing their chloroform solutions with that of phospholipids before evaporation.

Autoxidation of Liposomes

Liposomes from egg yolk phosphatidylcholine (2 mg/ml) were stored at room temperature in open round-bottom flasks and shaken from time to time. Aliquots were assayed for peroxidation products after 72, 96, and 120 h. Table I shows the accumulation of hydroperoxides and TBARS in our liposome samples. The content of LPO products increases drastically during peroxidation.

Incubation of Liposomes with NaOCl

A stock solution of NaOCl was kept in the dark at 4°C. The hypochlorite concentration was determined spectrophotometrically at pH 12 using $\varepsilon_{290} = 350\ M^{-1}\ cm^{-1}$.[34] It was diluted with 0.14 mol/l NaCl, 10 mmol/l phosphate immediately prior to use and adjusted to pH 7.4. In a typical experiment liposomes (final concentration 2 mg/ml) were incubated with NaOCl at 37°C during 40 min (if not otherwise indicated). Control measurements showed that pH 7.4 did not change after the addition of NaOCl.

Consumption of Hypochlorous Acid

The consumption of hypochlorous acid in liposome samples was followed by addition of luminol after various incubation periods. An intense chemiluminescence flash results from the oxidation of luminol by hypochlorous acid.[35] Briefly, a liposome suspension was diluted with phosphate buffer to yield final concentrations of $HOCl/OCl^-$ between $2 \cdot 10^{-6}$ and $7 \cdot 10^{-5}$ mol/l. 950 µl of this diluted suspension was transferred into a polystyrene vial and placed in a luminometer, AutoLumat LB 953 (Berthold, Germany). Then 50 µl of luminol (final concentration $5 \cdot 10^{-5}$ mol/l) was injected. Photons were

TABLE I Accumulation of hydroperoxides and TBA-reactive products during autoxidation of EYPC liposomes

Time of autoxidation, h	Hydroperoxides, nmol/mg	TBARS, nmol/mg
0	0.40 ± 0.1	0.10 ± 0.01
72	10.1 ± 0.3	0.56 ± 0.05
96	29.3 ± 0.09	1.47 ± 0.05
120	58.2 ± 1.3	2.83 ± 0.06

counted over the next 10 seconds. More than 99% of light emission appeared during the first two seconds. The luminescence increases linearly with increasing concentration of $HOCl/OCl^-$.[35]

Loss of Double Bonds

A modified iodometric method[13] was used to quantify double bonds. Briefly, lipids were extracted with heptane/isopropanol (1:1). An aliquot of the heptane phase was evaporated under nitrogen to dryness. Then lipids were dissolved in 100 µl chloroform and 200 µl IBr solution (4 mg/ml in glacial acetic acid) and incubated at room temperature in the dark for 1 h. 200 ml KI solution (10%, w/v) was added. Immediately after that the concentration of I_3^- was determined spectrophotometrically using $\varepsilon_{350} = 2.64 \cdot 10^4\ M^{-1}cm^{-1}$.[36] In control experiments the same procedure was repeated without lipid. Differences of I_3^- values between control and sample measurements correspond to the amount of double bonds.

Assays for Peroxidation Products

In order to determine lipid hydroperoxides in EYPC liposomes, lipids were extracted with heptane/isopropanol (1:1). The heptane layer was completely evaporated and lipids were dissolved in 1 ml commercially available reagent for hydroperoxide determination. The concentration of hydroperoxide was determined as free iodine liberated.[37] TBARS were determined as given in ref. 38.

Non-enhanced Chemiluminescence of Liposomes

The intrinsic (non-enhanced) chemiluminescence was measured in some experiments with liposomes in order to detect the evolution of reactive species (probably free radicals) under the action of $HOCl/OCl^-$. Various compounds (final concentration 2 mmol/l) containing epoxide, hydroperoxide, or peroxide groups were incorporated into liposomes composed of DMPC (2.5 mmol/l) in these experiments. 50 µl NaOCl (final concentration 2.3 mmol/l) was added to 950 µl of the liposome suspension placed into the luminometer AutoLumat LB 953. Photons were counted during 10 s if not stated otherwise.

RESULTS

Consumption of Hypochlorous Acid in Native and Oxidized Lipids

The consumption of $HOCl/OCl^-$ was investigated during the course of its interaction with liposomes prepared from (1) saturated phospholipid DMPC, (2) egg yolk phosphatidylcholine (EYPC), and (3) EYPC preoxidized to different degrees. Figure 1 shows typical kinetic curves of the consumption of hypochlorous acid added to

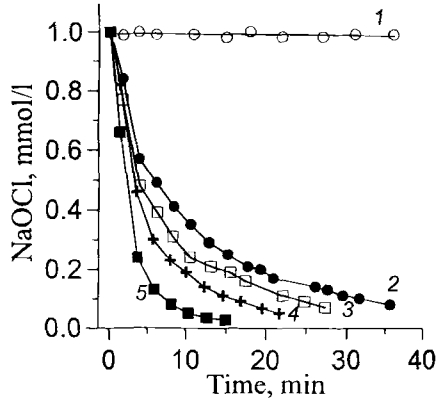

FIGURE 1 The kinetics of consumption of $HOCl/OCl^-$ during incubation with liposomes composed of DMPC (2.5 mmol/l) (1) or EYPC (2 mg/ml) preoxidized for 0 (2), 72 (3), 96 (4), or 120 hours (5).

Liposomes were prepared in 0.14 mol/l NaCl, 10 mmol/l phosphate buffer, pH 7.4. The incubation of liposomes with NaOCl was at 37°C. The concentrations of $HOCl/OCl^-$ were calculated from chemiluminescence intensities arising after addition of luminol in the final concentration of 50 mmol/l. Measurements were made in triplicates. Standard deviation of the mean did not exceed 4%.

DMPC liposomes and EYPC liposomes preoxidized during different times. It is seen that hypochlorous acid did not virtually react in DMPC liposome suspensions (Figure 1, curve 1), while its concentration decreased with time in suspensions of EYPC that contain unsaturated lipids (Figure 1, curve 2). The concentration of olefinic double bonds was 3.4 mmol/l in this sample as determined by IBr reduction. The reaction between HOCl and olefinic double bonds results in formation of chlorohydrins. Assuming that only this reaction contributes to the consumption of $HOCl/OCl^-$ in native EYPC liposomes (Figure 1, curve 2) and considering a stoichiometry of 1:1 between the consumption of hypochlorous acid and the loss of double bonds in EYPC,[30] a second order rate constant of 0.47 $M^{-1}s^{-1}$ was calculated.

$HOCl/OCl^-$ is consumed more rapidly in peroxidized lipid. The rate of hypochlorite consumption increases with the degree of peroxidation in these samples (curves 3–5 in figure 1). Whereas 90% of $HOCl/OCl^-$ were consumed after 32 min in nonoxidized samples, this period was shortened to 7 min in EYPC peroxidized for 120 h.

The Loss of Double Bonds in Native and Oxidized Liposomes

To explain the increase in $HOCl/OCl^-$ consumption in peroxidized phospholipid micelles there are at least two theoretical possibilities: (1) an increased reaction rate of $HOCl/OCl^-$ with double bonds in oxidized liposomes due to a partial degradation of the lipid phase under peroxidation and (2) the reaction of HOCl or OCl^- with some products of LPO.

To check the first possibility, we measured changes in the concentration of double bonds under the action of $HOCl/OCl^-$ in nonoxidized and peroxidized liposomes (Figure 2). The amount of double bonds was lowered by approximately 40% in liposomes peroxidized for 120 h. The addition of hypochlorous acid brought about

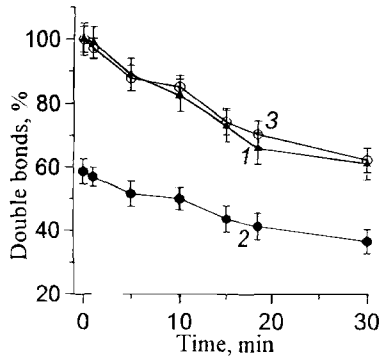

FIGURE 2 Kinetics of the loss of double bonds in native (1) and oxidized for 120 h (2 and 3) EYPC liposomes after the addition of NaOCl (1 mmol/l).

Incubation conditions were as in Figure 1. In curves 1 and 2 the amounts of double bonds are given as a percentage of their initial amount in non-oxidized liposomes, in curve 3 as a percentage of the initial amount of double bonds in oxidized vesicles. Data are given as the means and standard deviations (n = 4).

a loss of approximately one-third of these double bonds in both native and peroxidized samples. An important point is that the absolute amount of double bonds removed by $HOCl/OCl^-$ in peroxidized liposomes was lower than in nonoxidized micelles. These observations do not support the assumption that double bonds react more readily with hypochlorite in peroxidized liposomes.

Moreover, second-order rate constants for the reaction between HOCl and olefinic double bonds have been calculated also from data in figure 2. It is 0.50 $M^{-1}s^{-1}$ for nonoxidized EYPC, corresponding well to the second-order rate constant of this sample determined on the basis of the consumption of $HOCl/OCl^-$ (see above). A rate constant of 0.52 $M^{-1}s^{-1}$ is found in EYPC peroxidized for 120 h. Both reaction rate constants have been calculated using initial concentrations of double bonds of 3.4 mmol/l or 2.0 mmol/l in native or peroxidized liposomes, respectively. Therefore, the chlorohydrin formation occurs with the same rate in both native and peroxidized egg yolk phosphatidylcholine.

These results favor the hypothesis about a reaction of $HOCl/OCl^-$ with some LPO products.

Consumption of Hypochlorous Acid in DMPC Liposomes Containing Epoxides, Organic Peroxides and Hydroperoxides

A wide variety of primary and secondary products of lipid peroxidation has been described in the literature including hydroperoxides, diacylperoxides, alkylacylperoxides, dialkylperoxides, epoxides,[3] and various aldehydes, among which TBARS are frequently used as an indicator of lipid peroxidation.[39] The question is which of these compounds would react with $HOCl/OCl^-$ when being incorporated into lipid membrane layer. We measured the consumption of $HOCl/OCl^-$ in DMPC liposomes into which different synthetic peroxides and epoxides had been incorporated. Since pure DMPC liposomes were shown not to interact with $HOCl/OCl^-$ (Figure 1, curve 1), all the consumption of $HOCl/OCl^-$ in the liposomes may be attributed to peroxides or epoxides added exogenously.

The compounds used in these experiments are presented in Table II, and the results obtained are shown in figure 3. The concentration of $HOCl/OCl^-$ decreases with time only in those DMPC liposome suspensions that contain hydroperoxides (Figure 3, a and b) and does not change in liposomes containing peroxides (Figure 3, d–f), or cis-9,10-epoxystearic acid (Figure 3, c). Cholesterol-5α,6α-epoxide, cis-2,3-epoxybutane, trans-2,3-epoxybutane were also inert against $HOCl/OCl^-$ (Table II).

$HOCl/OCl^-$ was consumed more rapidly in the reaction with tert-butyl hydroperoxide than with cumene hydroperoxide. The reaction mechanism between $HOCl/OCl^-$ and both hydroperoxides seems to be complicated. It was impossible to fit data from figures 3a and 3b to a second-order reaction. Moreover, hypochlorous acid is nearly completely exhausted in all these experiments. Using 0.5 mmol/l tert-butyl hydroperoxide, a final value of 0.5 mmol/l $HOCl/OCl^-$ should be expected in the case of a second-order reaction. Likely, $HOCl/OCl^-$ reacts not only with the hydroperoxide group but also with some products formed after the initial attack on hydroperoxides.

Chemiluminescence Associated with the Reactions of Hypochlorite with Peroxides and Epoxides

Intrinsic (low-level, non-enhanced) chemiluminescence (CL) has been observed in oxidative reactions of organic substrates mediated by free

TABLE II Characterization of organic hydroperoxides, peroxides and epoxides to react with hypochlorous acid and to initiate the lipid peroxidation reaction

Compound	Consumption of $HOCl/OCl^-$,mmol/l[a],	Light intensity[b] arb. units	Accumulation of TBARS, nmol/mg[c]
tert-Butyl hydroperoxide	0.98 – 0.01	141,000 ± 5,000	0.14 ± 0.01
Cumene hydroperoxide	0.95 ± 0.01	7,050 ± 420	0.07 ± 0.01
Di-tert-butyl peroxide	0	360 ± 90	0
tert-Butyl perbenzoate	0	490 ± 80	0.01
Dibenzoyl peroxide	0	400 ± 50	0.005
cis-9,10-Epoxystearic acid	0	420 ± 90	0.01
Cholesterol-5α,6α-epoxide	0	450 ± 80	0.005
trans-2,3-Epoxy-butane	0	470 ± 60	0
cis-2,3-Epoxy-butane	0	490 ± 70	0
Control	0	450 ± 70	0

[a] The consumption of $HOCl/OCl^-$ (1 mmol/l) in its reaction with hydroperoxides, peroxides or epoxides (all 1 mmol/l) incorporated into DMPC liposomes during 10 min is given. All other experimental conditions are indicated in Figure 3.

[b] Photons were counted during 10 s after the addition of 50 µl NaOCl (2.3 mmol/l, final concentration) to 950 µl DMPC (2.5 mmol/l) liposomes containing various peroxides or epoxides (2 mmol/l). All other conditions are the same as given in Figure 1. Data are given as means and S.D. (n = 4).

[c] Effects of hydroperoxides, peroxides or epoxides (all 0.5 mmol/l) on an additional accumulation of TBARS in lipid peroxidation in EYPC liposomes induced by hypochlorous acid. Other experimental conditions are given in Figure 4.

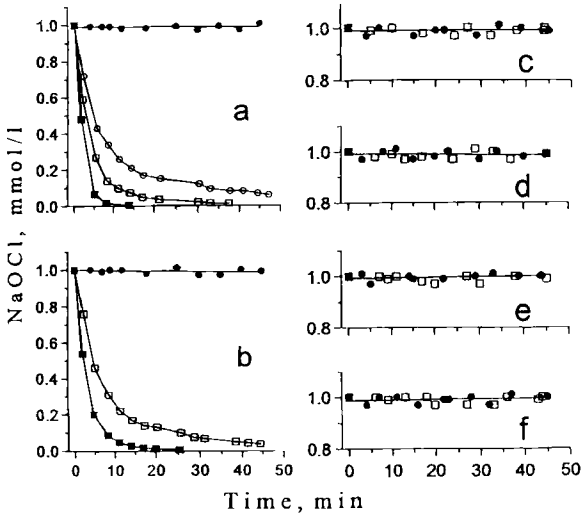

FIGURE 3 Consumption of HOCl/OCl⁻ in suspensions of DMPC liposomes.

The liposomes (2.5 mmol/l) contained: (a) tert-butyl hydroperoxide; (b) cumene hydroperoxide; (c) cis-9,10-epoxystearic acid; (d) di-tert-butyl peroxide; (e) tert-butyl perbenzoate; or (f) dibenzoyl peroxide. The concentration of hydroperoxides, peroxides and epoxides (mmol/l): (●) - 0; (○) - 0.5; (□) - 1.0; (■) - 2.0. Experimental conditions as in Figure 1.

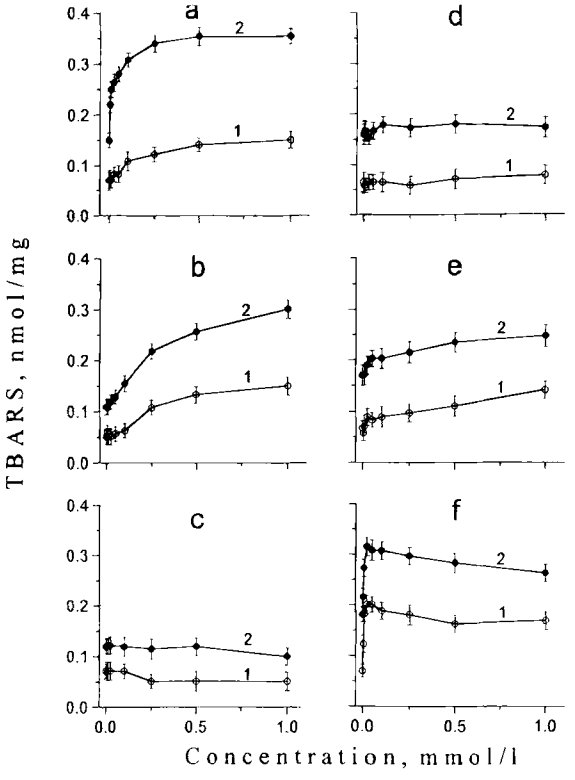

FIGURE 4 The accumulation of TBARS in EYPC liposomes (2 mg/ml) after incubation during 40 min at 37°C without (1) or with (2) HOCl/OCl⁻ (final concentration 50 μmol/l).

Compounds incorporated were tert-butyl hydroperoxide (a), cumene hydroperoxide (b), cis-9,10-epoxystearic acid (c), di-tert-butyl peroxide (d), tert-butyl perbenzoate (e), or dibenzoyl peroxide (f). Incubation conditions as in???

radicals.[1,2] Decomposition of organic hydroperoxides and lipid hydroperoxides[1,3] in the presence of transition metal ions is also accompanied by light emission. Although CL is an indirect method to detect free radicals, it seemed worthwhile to measure CL in reaction of HOCl/OCl⁻ with DMPC liposomes containing different peroxides and epoxides. Column 4 in Table II shows the cumulative CL during the first 10 s after the addition of NaOCl to the liposome suspensions. Only samples containing tert-butyl hydroperoxide or cumene hydroperoxide yielded any light emission.

Effects of Various Compounds on Initiation of Lipid Peroxidation by Hypochlorous Acid

The accumulation of TBARS was investigated in EYPC liposome samples containing hydroperoxide, epoxide, or peroxide groups. In Figure 4, curves 1 and 2 demonstrate the accumulation of TBARS in liposomes incubated during 40 min at 37°C without and with 50 μmol/l NaOCl, respectively. In addition to cis-9,10-epoxystearic acid, three other epoxides have been studied: cholesterol-5α,6α-epoxide, cis- and trans-2,3-epoxybutane. The results obtained with all these compounds were quite similar, and only data for cis-9,10-epoxystearic acid are given in Figure 4.

The background concentration of TBARS in EYPC liposomes not containing any added compounds and incubated without NaOCl varied within 0.05–0.07 nmol/mg lipid. After incubation with NaOCl (final concentration 50 μmol/l) this value increased to 0.12–0.17 nmol/mg. This NaOCl concentration was chosen in respect to the pronounced dependence of lipid peroxidation products in EYPC on the concentration of HOCl/OCl⁻.[13] At this concentration the effect of

NaOCl on accumulation of TBARS in native EYPC without added hydroperoxides is small in comparison to a NaOCl concentration of 1 mmol/l.[13]

In the presence of organic hydroperoxides (Figure 4a and b) the concentration of TBARS increased with growing concentration of hydroperoxides. It should be pointed out that an increase in TBARS was produced by hydroperoxides even in the absence of NaOCl (curves 1 in figure 4a and b). Tert-butyl hydroperoxide is known to contribute to the formation of red pigments in the reaction with thiobarbituric acid.[40] However, the effect of hydroperoxides was several times higher in the presence of $HOCl/OCl^-$. Therefore, both hydroperoxides alone and hydroperoxides in combination with $HOCl/OCl^-$ seem to contribute to additional production of TBARS.

Both tert-butyl perbenzoate (Figure 4e) and dibenzoyl peroxide (Figure 4f) cause also an increase in TBARS in the presence or absence of $HOCl/OCl^-$. However, there are no indications for a more pronounced effect of $HOCl/OCl^-$. All other compounds incorporated in EYPC liposomes did not produce any increase in TBARS both in the absence (Curves 1 in figure 4c, d) and presence of $HOCl/OCl^-$ (Curves 2 in figure 4c, d),

There is a continuous increase in the difference of TBARS concentrations with growing concentrations of tert-butyl hydroperoxide incorporated into EYPC liposomes up to approximately 50 µmol/l (Figure 4a). Already 5 µmol/l tert-butyl hydroperoxide have a pronounced effect on the additional accumulation of TBARS. Considering a final concentration of 50 µmol/l for NaOCl in our experiments, these data are a further indication for the formation of TBARS in the result of a direct reaction between hydroperoxide groups and $HOCl/OCl^-$. Moreover, this additional formation of TBARS is completely inhibited by the free radical scavenger butylated hydroxytoluene[41] in these experiments (data not shown).

Data from experiments with cumene hydroperoxide (Figure 4b) are not so obvious. However, differences of TBARS values obtained from curves 2 and 1 in Figure 4b rise more slowly at concentrations of cumene hydroperoxide higher than approximately 50 µmol/l.

DISCUSSION

It is known that lipid peroxidation products are accumulated in artificial phospholipid vesicles[13–16,20] and in human blood lipoproteins[14,15,17–20] upon the action of exogenous hypochlorous acid or myeloperoxidase and H_2O_2 in Cl^- containing solutions. Diene conjugates, lipid hydroperoxides, TBARS, Schiff bases have been shown to be formed in hypochlorite-induced LPO.[13–20] Apparently, free radicals are involved in hypochlorite-induced lipid peroxidation because two well-known scavengers of free radicals—α-tocopherol and butylated hydroxytoluene—completely inhibited the accumulation of TBARS.[13]

The lipid peroxidation reaction is apparently initiated as result of a direct reaction between hydroperoxides and $HOCl/OCl^-$. This conclusion is mainly based on effects of tert-butyl and cumene hydroperoxides on the consumption of $HOCl/OCl^-$, the appearance of a non-enhanced chemiluminescence in DMPC liposomes, and on the additional accumulation of TBARS in EYPC liposomes. To better demonstrate the interaction between these hydroperoxides and $HOCl/OCl^-$ (consumption of $HOCl/OCl^-$ and non-enhanced chemiluminescence) unphysiological high concentrations of reactants have been used. Because of the pronounced dependence of lipid peroxidation products in EYPC on the concentration of $HOCl/OCl^-$,[13] this reactant was used at 50 µmol/l in experiments to show an additional accumulation of TBARS (Fig. 4). Already 5–10 µmol/l hydroperoxides added had a pronounced effect on TBARS. These experimental conditions are not so far from a pathophysiological-relevant situation. 5–50 µmol/l $HOCl/OCl^-$ can be found under pathological conditions in body fluids.[42] $2 \cdot 10^6$ stimulated neutrophils per ml produce 100–140 µmol/l $HOCl/OCl^-$.[43,44]

Possible pathways of reactions of $HOCl/OCl^-$ in phospholipid samples are summarized in Figure 5. First of all, hypochlorous acid reacts with double bonds of phospholipids (pathway 1) to form chlorohydrins.[30,31,33] Epoxides can be derived from chlorohydrins under moderately alkaline conditions. Epoxides have been also shown to be produced in lipid peroxidation reactions[3,45], so that their accumulation may be used as a measure of efficiency of lipid peroxidation.[3] Thus, the involvement of epoxides in initiation of new lipid peroxidation reaction after the addition of hypochlorous acid seems rather probable. However, our experiments demonstrated that all epoxides used were unable to react with hypochlorous acid (Figure 4c, Table II). When added to liposomes, cis-9,10-epoxystearic acid and other epoxides did not increase the formation of TBARS upon the action of hypochlorous acid (Figure 4c, Table II). Therefore, the involvement of pathway 1 in initiation of lipid peroxidation in egg yolk phosphatidylcholine is unlikely.

A reaction of $HOCl/OCl^-$ with a minor component of the membrane suspension is hence a more plausible source of free radicals initiating the lipid peroxidation reaction. The possible role of Fe^{2+}, Fe^{3+}, H_2O_2, and $O_2^{\cdot -}$ in initiation of hypochlorous acid induced lipid peroxidation (pathways 5 and 6) was addressed previously.[13]

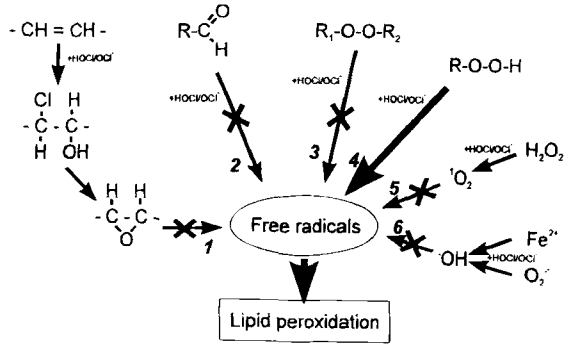

FIGURE 5 Hypothetical pathways for the formation of free radicals in the lipid phase of egg yolk phosphatidylcholine capable to initiate a lipid peroxidation (LPO) under the action of $HOCl/OCl^-$.

The addition of catalase and superoxide dismutase to the liposome suspension as well as Fe^{2+}, Fe^{3+}, H_2O_2 did not influence the amount of LPO products accumulated in liposomes under the action of $HOCl/OCl^-$.[13]

Hydrogen peroxide reacts with $HOCl/OCl^-$ to yield singlet oxygen that can be detected by a red chemiluminescence at 634 nm and 703 nm.[46] The reaction of tert-butyl or cumene hydroperoxides with $HOCl/OCl^-$ is also accompanied by a light emission. This luminescence exhibits other features than the so-called dimol emission of singlet oxygen.[47] Moreover, no singlet oxygen could be detected in the reaction between tert-butyl or cumene hydroperoxides with $HOCl/OCl^-$ using infrared luminescence at 1268 nm (unpublished results). From chemiluminescence data singlet oxygen can be excluded as a product in these reactions.

It may be concluded that the lipid peroxidation is initiated as a result of $HOCl/OCl^-$ interaction with minor components such as peroxidation products preexisting in the membrane phase. The fact that the consumption of $HOCl/OCl^-$ increases several times in oxidized liposomes (whereas the amount of double bonds is reduced) is in agreement with this proposal.

Various aldehydes including malondialdehyde (pathway 2) are shown to react with $HOCl/OCl^-$.[17,32] A dichlorinated product is derived from malondialdehyde and $HOCl/OCl^-$.[32] Free radicals seem not to be involved in reactions between aldehydes and $HOCl/OCl^-$. Moreover, the addition of hypochlorous acid to oxidized liposomes decreased the concentration of TBARS.[17,18,48]

Organic peroxides (pathway 3) are obviously not involved in both free radical formation and LPO initiation. Like in experiments with epoxides, there are no effects of these compounds.

Only tert-butyl and cumene hydroperoxides (pathway 4) were able (1) to increase the consumption of $HOCl/OCl^-$ in DMPC liposomes (Figure 3a,b), and (2) to initiate new peroxidation reactions in EYPC liposomes induced by hypochlorous acid.

The reaction mechanism between $HOCl/OCl^-$ and hydroperoxides remains unknown. The involvement of free radicals in this reaction is indicated by the effect of the free radical scavenger butylated hydroxytoluene. The additional formation of TBARS in EYPC liposomes with incorporated tert-butyl hydroperoxide is completely inhibited by butylated hydroxytoluene. Chemiluminescence was observed in the reaction of tert-butyl or cumene hydroperoxides with hypochlorous acid, whereas all other compounds investigated did not yield any light emission. Organic hydroperoxides, especially tert-butyl hydroperoxide, are widely used as a source of free radicals in organic chemistry.[49] On the other hand, free radicals may be formed as intermediates in the reaction of $HOCl/OCl^-$ with different organic compounds.[50–54]

There is apparent discrepancy between the concentration of NaOCl (50 μmol/l) used and the amount of TBARS (0.4–0.5 μmol/l) formed additionally in the presence of hydroperoxides in data from figure 4. It should be considered that most of hypochlorous acid added to EYPC reacts with double bonds to yield chlorohydrins. Furthermore, although TBARS can be easily detected, they are only a minor product in lipid peroxidation.

Regarding biological significance, it is of interest that the effect of hydroperoxides on the LPO initiation by $HOCl/OCl^-$ appears at rather low concentrations of hydroperoxides. For example, 2.5 nmol tert-butyl hydroperoxide induced a two-fold higher increase of TBARS in 1 mg egg yolk phosphatidylcholine in comparison to control samples. This concentration of hydroperoxides is comparable to the values found *in vivo*. Various tissues of animals or human beings contain from 0.4 to 19.6 nmol hydroperoxides per mg lipids.[3]

Hydroperoxides are regarded as primary products in autoxidation, metal-catalysed oxidation and other types lipid peroxidation.[4] They are also a product of the action of lipoxygenases.[55] An increase of the level of hydroperoxides in biological membranes and lipoproteins under these conditions would make these lipids more susceptible to new peroxidation reactions induced by hypochlorous acid.

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