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LIPID PEROXIDATION. DEFINITION OF EXPERIMENTAL CONDITIONS FOR SELECTIVE STUDY OF THE PROPAGATION AND TERMINATION PHASES

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To find experimental conditions to selectively study the propagation phase of lipoperoxidation we studied the lipoperoxidation, catalyzed by $FeCl_2$, of liposomes in a buffering condition where Fe^{2+} autoxidation and oxygen active species generation does not occur. Liposomes from egg yolk phosphatidylcholine, prepared by vortex mixing, do not oxidize Fe^{2+}; on the contrary they oxidize Fe^{2+} when prepared by ultrasonic irradiation. Dimyristoyl phosphatidylcholine liposomes prepared by ultrasonic irradiation do not oxidize Fe^{2+}. During sonication polyunsaturated fatty acid residues autoxidize and lipid hydroperoxides (LOOH) are generated. Only when LOOH are present in the liposimes Fe^{2+} oxidizes and its rate of oxidation depends on the amount of LOOH in the assay. The reaction results in the generation of both LOOH and thiobarbituric acid reactive material (TBAR); it is inhibited by butylated hydroxytoluene and has a acidic pH optimum; it is not inhibited by catalase and $OH^{\cdot}$ scavengers. The reaction studied, thus, appears to be the chain branching and propagation phase of lipoperoxidation. When we studied the dependence of Fe^{2+} oxidation, LOOH and TBAR generation on $FeCl_2$ concentration, we observed that at high $FeCl_2$ concentrations the termination phase of lipoperoxidation was prevalent. Thus, by selecting the appropriate $FeCl_2$ concentration the proposed experimental system allows study of either the propagation or the termination phase of lipoperoxidation.

KEY WORDS: Lipoperoxidation, propagation phase, termination phase.

Free radicals able to abstract hydrogen atoms from unsaturated fatty acids (LH) can initiate lipid peroxidatin. After initiation, a rapid propagation phase involving oxygen, follows with formation of peroxyl radicals ($LO_2^{\cdot}$). These further react with lipid and give rise to stable lipid hydroperoxide (LOOH).

$$O_2 + 3Fe^{2+} \xrightarrow{2H^+} OH^{\cdot} + OH^- + 3Fe^{3+} \quad (1)$$

$$OH^{\cdot} + LH \longrightarrow L^{\cdot} + H_2O \quad (2)$$

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \quad (3)$$

$$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (4)$$

Lipid hydroperoxide can decompose to form alkoxyl ($LO^{\cdot}$) and peroxyl ($LO_2^{\cdot}$) radicals which in turn can further propagate lipid peroxidation by chain branching

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$$LOOH + Fe^{2+} \longrightarrow LO^{\cdot} + OH^{-} + Fe^{3+} \quad (5)$$

$$LOOH + Fe^{3+} \longrightarrow LO_2^{\cdot} + H^{+} + Fe^{2+} \quad (6)$$

Thus iron salts can stimulate lipid peroxidatin both by directly reacting with molecular O_2 to produce the initiator hydroxyl radical (eqn. 1) and by decomposing lipid hydroperoxides, to form organic radical propagators (eqns. 5,6).

In experimental conditions normally used, the iron salt addition to membranes causes the simultaneous generation of these two types of radicals. Fe^{2+} in fact readily autoxidizes both in phosphate buffer[1,2] and in other moderately basic buffering conditions.[3,4] It has also been shown that lipid preparations contain traces of lipid peroxides which may react with Fe^{2+}.[5] This renders the study and the comprehension of the molecular mechanism of the different phases of lipoperoxidation rather difficult. We have recently studied the mechanism of Fe^{2+} autoxidation[4,6,7] in Good-type buffers[8] and we have shown that Fe^{2+} is very stable at acidic pH. The study of lipoperoxidation in an experimental condition where Fe^{2+} autoxidation and thus free oxygen radical production is absent could provide a suitable experimental system to selectively study the propagation phase of lipoperoxidation. In this paper we have verified this possibility and showed that, besides propagation, also termination of lipoperoxidation can be profitably studied.

MATERIALS AND METHODS

2-Morpholinoethanesulfonic acid (MES), ATP, ADP, AMP, dimyristoyl phosphatidylcholine (DML) and all other chemicals, of the highest grade available were from Sigma Chemical Co. (St. Louis MO. U.S.A.). 1,10-phenanthroline was from Merck (Darmstadt, Germany). Egg yolk phosphatidylcholine (EYL) was from Lipid Products (Redhill U.K.). All reagents were prepared in Chelex resin-treated distilled water.

Lipid preparation

A phospholipid suspension was prepared by adding 10 ml of water to 30 mg phosphatidylcholine dried under nitrogen. The solutin was vortex mixed for 10 min and stored at 4 °C for 1 h before use. This suspension was subsequently sonicated with a probe sonicator for different time spans at 4 °C under a nitrogen atmosphere. The vesicle dispersion was then centrifuged to remove any probe particles and large aggregates. The phospholipid content of the supernatant was determined by its phosphorus content by the method of Marinetti.[9]

Sample preparation

All incubations were carried out in 5 mM MES buffer, pH 6.5. The 1 ml reaction mixture contained 0.15 mg phosphatidylcholine, different compounds and $FeCl_2$ as indicated in figures and tables. The reactions, initiated by iron addition, were incubated at room temperature for the time stated.

Fe^{2+} oxidation

Fe^{2+} determination was made by the o-phenanthroline method according to Mahler and Elowe.[10] At the time stated the reactions under test were stopped by addition of 0.2 ml of 25 mM 1,10-phenanthroline and A_{515} was immediately read. The amount of Fe^{2+} oxidized was expressed as difference from the absorbance measured at time zero (ΔA_{515}).

Thiobarbituric acid-reactivity

Peroxidation of phospholipid polyunsaturated fatty acids was determined by the thiobarbituric acid-reactive materials according to Beuge and Aust.[11] At the time stated, the reactions under test were stopped by the addition of 1.5 ml of 1% thiobarbituric acid containing 10 μl of 2% butylated hydroxytoluene (BHT) and of 1.5 ml of 20% glacial acetic acid pH 3.5. The tubes were heated for 10 min at 100° C to develop the colour. Resulting chromogens were measured at 532 nm against appropriate blanks.

Lipid hydroperoxide determination

Peroxidation of phospholipid polyunsaturated fatty acids was also evaluated by the determination of the lipid hydroperoxide content. At the time stated the reactions were added with 10 μl of 2% BHT in ethanol. Lipid hydroperoxides, extracted as described by Cavallini *et al.*[12] were measured on 1 ml aliquots with the thiocyanate method[13].

RESULTS

Effect of composition and method of preparation of phosphatidylcholine liposomes

When EYL liposomes, prepared by ultrasonic irradiation, are incubated with 100 μM $FeCl_2$ in 5 mM MES buffer, pH 6.5, Fe^{2+} is oxidized as seen in Table I. Fe^{2+} oxidation depends on the amount of sonicated EYL liposomes in the reaction mixture. No Fe^{2+}

TABLE I

Effect of composition and method of preparation of phosphatidylcholine liposomes on Fe^{2+} oxidation. 100 μM $FeCl_2$ was incubated for 5 min in 5 mM MES buffer, pH 6.5 in the presence of different types of liposomes. Fe^{2+} oxidation was determined as described in the materials and methods section.

	Fe^{2+} oxidation ΔA_{515}
Control	0.002
+ EYL sonic. (30 μg)	0.141
+ EYL sonic. (75 μg)	0.352
+ EYL sonic. (150 μg)	0.798
+ EYL sonic. (150 μg) + catalase (100 μg)	0.758
+ DML sonic. (150 μg)	0.008
+ EYL vort. (150 μg)	0.002
+ EYL vort. (75 μg) + 100 μl H_2O sonic.	0.008
+ EYL vort. (75 μg) + EYL sonic. (75 μg)	0.348
+ EYL vort. (75 μg) + DML sonic. (75 μg)	0.006

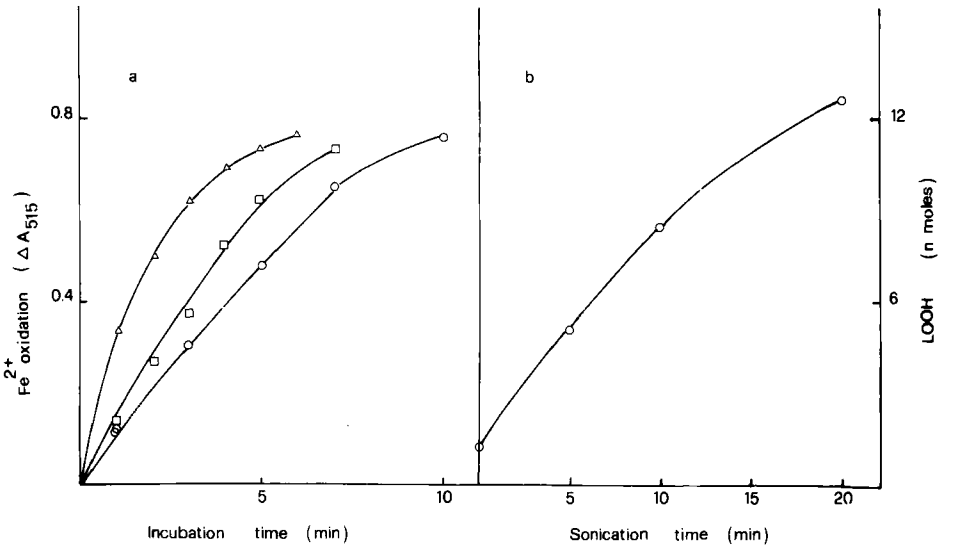

FIGURE 1 Effect of the length of ultrasonic irradiation of EYL liposomes on Fe^{2+} oxidation and LOOH content of liposomes. (a) The time course of oxidation of 100 μM $FeCl_2$ in 5 mM MES buffer, pH 6.5 by EYL liposomes was determined. The liposomes were prepared by 5 min (○), 10 min (□) and 20 min (△) ultrasonic irradiation. (b) The LOOH content of EYL liposomes was determined after different time span of ultrasonic irradiation.

oxidation is observed when $FeCl_2$ is incubated in the presence either of EYL liposomes prepared by vortex mixing or of DML liposomes prepared by sonication. In the presence of these types of liposomes no LOOH and TBAR are formed following $FeCl_2$ addition (results not shown). When $FeCl_2$ is incubated in the presence of both vortex mixed and sonicated EYL liposomes the amount of Fe^{2+} oxidized is only related to the amount of sonicated liposomes in the reaction mixture. Ultrasonic irradiation of water is known to generate hydrogen peroxide.[14] We have thus added to a reaction mixture containing vortex mixed EYL liposomes and $FeCl_2$, a volume of sonicated water comparable to that of sonicated samples. An instantaneous but rather limited Fe^{2+} oxidation was obtained which however did not differ from that obtained in the absence of vesicles. The rate of Fe^{2+} oxidation in the presence of sonicated EYL liposomes is also directly dependent on the length both of ultrasonic irradiation utilized during liposome preparation (Figure 1a) and of storage of sonicated liposomes (results not shown). EYL liposomes prepared by ultrasonic irradiation contain LOOH, their content is related to the length of sonication (Figure 1b) and to the storage of liposomes (results not shown). Sonicated EYL liposomes contain also TBAR whose content increases upon storage of the liposomes.

Effect of $FeCl_2$ concentration

When EYL liposomes prepared by 10 min sonication are incubated with increasing concentrations of $FeCl_2$, a biphasic pattern of oxidation of Fe^{2+} is observed. As the $FeCl_2$ concentration is increased, at first Fe^{2+} oxidation increases, reaches a platou and then decreases (Figure 2a). The concentration at which Fe^{2+} oxidation is maximal is dependent on the concentration of vesicles (Figure 2a), on the length of sonication

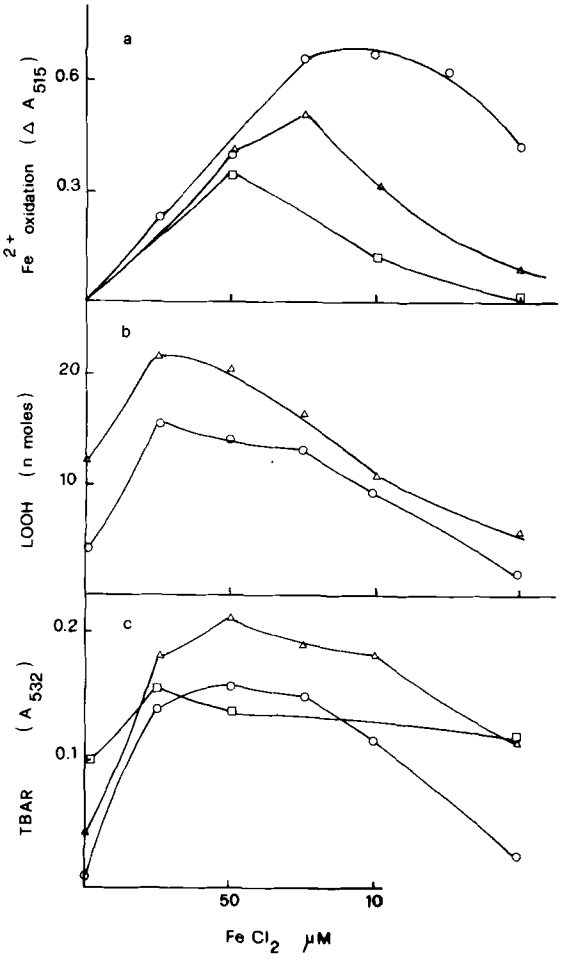

FIGURE 2 Fe^{2+} oxidation, LOOH and TBAR generation, as a function of increasing $FeCl_2$ concentration, in EYL liposomes prepared by ultrasonic irradiation. (a) The oxidation of $FeCl_2$ was determined, after 5 min incubation in 5 mM MES buffer, pH 6.5 containing 30 μg (□), 75 μg (△) and 150 μg (○) of EYL liposomes (b) The LOOH content of 75 μg of EYL liposomes was determined, after 5 min incubation in 5 mM MES buffer, pH 6.5, containing $FeCl_2$. The liposomes were prepared by 5 min (○) and 20 min (△) ultrasonic irradiation. (c) The TBAR content of 75 μg of EYL liposomes was determined after 5 min incubation in 5 mM MES buffer, pH 6.5 containing $FeCl_2$. The liposomes prepared by 5 min ultrasonic irradiation were treated either immediately (○) or 9 (△) and 18 (□) days after preparation.

and storage of the liposomes (results not shown). An increase of any of these parameters can shift the Fe^{2+} oxidation curve to the right. As shown in Figure 2b,c incubation of EYL liposomes with increasing concentrations of $FeCl_2$ causes also a biphasic pattern of formation of LOOH and TBAR. The $FeCl_2$ concentration at which these formations are maximal is lower than that required for maximal Fe^{2+} oxidation. The length of sonicatin and of storage also affects the pattern of dependence of LOOH and TBAR formation on $FeCl_2$ concentration.

TABLE II

Effect of different compounds on Fe^{2+} oxidation and thiobarbituric acid reactivity. Sonicated EYL liposomes were incubated in 5 mM MES buffer, pH 6.5 with 50 μM $FeCl_2$ for 5 min. Final reaction concentrations are shown.

	Fe^{2+} oxidation		Thiobarbituric acid reactivity	
	ΔA_{515}	% Inib.	A_{532}	% Inib.
Control (pH 6.5)	0.404	–	0.174	–
+ Mannitol (10 mM)	0.376	7	0.167	4
+ Thiourea (5 mM)	0.384	5	0.160	8
+ Formate (7.5 mM)	0.391	3	0.189	8
+ Benzoate (7.5 mM)	0.372	3	0.157	10
+ AMP (1 mM)	0.293	28	0.111	36
+ ADP (1 mM)	0.410	0	0.175	0
+ ATP (1 mM)	0.412	0	0.174	0
+ BHT (0.7 mM)	0.020	95	0	100
Control (pH 7)	0.263	35	0.120	32

Effects of different compounds

The occurrence of biphasic patterns of Fe^{2+} oxidation, LOOH and TBAR formation, observed when EYL sonicated liposomes are incubated in the presence of increasing concentration of $FeCl_2$, prompted us to investigate the effect of different compounds at a $FeCl_2$ concentration (50 μM) at which the propagation, but not the termination, phase seems to occur. In fact at 50 μM, Fe^{2+} is completely oxidized and a maximal production of LOOH and TBAR is observed. In the presence of 50 μM $FeCl_2$ of the many compounds tested only BHT exerts a strong inhibition both on Fe^{2+} oxidation and on TBAR generation. Some inhibition is also observed in the presence of AMP but not of ADP and ATP. $OH^{\cdot}$ scavengers such as mannitol, thiourea, formate and benzoate do not affect the reactions studied. When the reactions are conducted at pH 7 instead that 6.5 a decreased Fe^{2+} oxidation and TBAR formation is observed (Table II).

DISCUSSION

The aim of this study was to verify whether incubation of liposomes in a medium where Fe^{2+} autoxidation does not occur, might be an experimental system suitable to selectively study the chain branching and propagation phases of lipoperoxidation. Our results define experimental conditions which differentiate these two phases. In fact incubation of EYL liposomes, prepared by vortex mixing, with $FeCl_2$, in MES buffer pH 6.5 does not result in any Fe^{2+} oxidation and (results not shown) in any LOOH generation. $FeCl_2$ thus does not appear to be able to catalyze the synthesis of the initiator of lipid peroxidation in this experimental condition. By contrast EYL liposomes prepared by ultrasonic irradiation, when incubated in the same system, oxidize Fe^{2+} and consequently LOOH and TBAR are produced. Apparently ultrasonic irradiation of liposome suspension generates species which can react with Fe^{2+} and trigger lipoperoxidation. The irradiation of water with ultrasonic waves is known to produce chemical transformation of the liquid and substances dissolved in it[15]. The reactive species produced in water are, besides hydrated electrons and

hydrogen atoms, $OH^{\cdot}$, $O_2^{\cdot-}$ and ultimately H_2O_2.[14] In particular, H_2O_2, by reaction with Fe^{2+}, could initiate lipoperoxidation. However the mixing experiments an the addition to reaction mixture of catalase (Table I), indicate that production of the initiator is insufficient to cause lipoperoxidation. This is also confirmed by the low inhibition exerted by $OH^{\cdot}$ scavengers added to the reaction in the presence of low concentration of $FeCl_2$. As already mentioned, irradiation of water with ultrasonic waves caused degradation of substances dissolved in it. Experiments were conducted to discriminate which part of the phosphatidylcholine molecule could be the substrate responsible for the generation of degradation products able to react with Fe^{2+}. The results obtained with DML liposomes exclude the polar head and the hydrophobic saturated fatty acid residues. The compounds which by sonication of EYL liposomes generate the species reacting with $FeCl_2$ appear to be the polyunsaturated fatty acid residues. These molecules are known to be prone to autoxidation in the presence of oxygen during sonication.[16] Analysis of EYL liposomes prepared by ultrasonic irradiation shows the presence of LOOH and furthermore their content is proportional to the time span of sonication. The possibility that LOOH could be the substrate which reacts with Fe^{2+} and therefore that what we are observing are the chain branching and the propagation phases of lipoperoxidation, is supported by the experimental data. First of all we observe that there is a close relationship between the amount of LOOH in the rection mixture and the rate of Fe^{2+} oxidation; secondly the pH optimum for Fe^{2+} oxidation and TBAR formation in our experimental system agrees with the acidic pH optimum of the decomposition of LOOH by Fe^{2+};[17] it follows that the inhibition by BHT of the parameters measured agrees with its known ability to affect LOOH decomposition.[11]

As the experimental system appears suitable to selectively study the propagation phase of lipoperoxidation, we have studied the influence that a few compounds, known to affect lipoperoxidation, exert on it. Adenine-nucleotides, in the millimolar range, were reported to stimulate lipoperoxidation.[18,19] In our experimental conditions at low $FeCl_2$ concentration, di- and triphosphate adenine-nucleotides do not affect any of the parameters measured. By contrast the monophosphate form inhibits Fe^{2+} oxidation and TBAR generation and in no occasion a stimulation of lipoperoxidation was observed. The discrepancy with the results in the literature, as far as ADP and ATP are concerned, may be due to the inhibition of Fe^{2+} autoxidation exerted by these nucleotides.[7,20] In usual buffering conditions Fe^{2+} rapidly autoxidizes;[1-4] the presence of compounds which inhibit this reaction increases the availability of Fe^{2+} both for the generation of the initiator and for the catalysis of the propagation phase. In our experimental system Fe^{2+} is stable and the interference of these adenine-nucleodites on Fe^{2+} autoxidation can not affect the lipoperoxidation reactions. AMP, in Mops buffer, like the others adenine-nucleotides, inhibits Fe^{2+} autoxidation[7]. However in this experimental system it inhibits Fe^{2+} oxidation and TBAR generation. The data available do not allow to envisage a mechanism through which this may occur. A possibility might be that iron bound to AMP had a higher ability to catalyze the termination reaction which occurs in our experimental system. In fact, during the study of the dependence of the propagation phase on $FeCl_2$ concentration, we have observed that at higher $FeCl_2$ concentration a termination phase is increasingly prevailing. When $FeCl_2$ concentration is raised, at first Fe^{2+} oxidation still occurs, although not proportionally, whereas LOOH and TBAR generation is already quantitatively decreased; at higher Fe^{2+} concentration also Fe^{2+} oxidation is greatly lowered. It was shown in an early investigation that high $FeCl_2$ concentrations inhibit

lipoperoxidation.[21] The exact mechanism of antioxidant action of $FeCl_2$ is unknown. However a critical concentration of Fe^{2+} (60 μM), above which the termination reaction would prevail, was determined.[22] That critical concentration is comparable to the $FeCl_2$ concentration at which the shift from propagation to termation phases occurs in our experimental conditions. The experimental conditions that we have presented may thus provide a suitable system also to study the terminatin phase of lipoperoxidation.

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