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Iron (III) Stimulation of Lipid Hydroperoxide-Dependent Lipid Peroxidation

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In an experimental system where both Fe^{2+} autoxidation and generation of reactive oxygen species is negligible, the effect of $FeCl_2$ and $FeCl_3$ on the peroxidation of phosphatidylcholine (PC) liposomes containing different amounts of lipid hydroperoxides (LOOH) was studied; Fe^{2+} oxidation, oxygen consumption and oxidation index of the liposomes were measured. No peroxidation was observed at variable $FeCl_2/FeCl_3$ ratio when PC liposomes deprived of LOOH by triphenylphosphine treatment were utilized. By contrast, LOOH containing liposomes were peroxidized by $FeCl_2$. The $FeCl_2$ concentration at which Fe^{2+} oxidation was maximal, defined as critical Fe^{2+} concentration $[Fe^{2+}]^*$, depended on the LOOH concentration and not on the amount of PC liposomes in the assay. The LOOH-dependent lipid peroxidation was stimulated by $FeCl_3$ addition; the oxidized form of the metal increased the average length of radical chains, shifted to higher values the $[Fe^{2+}]^*$ and shortened the latent period. The iron chelator KSCN exerted effects opposite to those exerted by $FeCl_3$ addition. The experimental data obtained indicate that the kinetics of LOOH-dependent lipid peroxidation depends on the Fe^{2+}/Fe^{3+} ratio at each moment during the time course of lipid peroxidation. The results confirm that exogenously added $FeCl_3$ does not affect the LOOH-independent but the LOOH-dependent lipid peroxidation; and suggest that the Fe^{3+} endogenously generated exerts a major role in the control of the LOOH-dependent lipid peroxidation.

Keywords: Fe(III), Fe(II)/Fe(III) ratio, lipid hydroperoxide, lipid peroxidation, phosphatidylcholine liposome

Abbreviations: DPPC: dipalmitoyl phosphatidylcholine; $[Fe^{2+}]^*$: critical Fe^{2+} concentration; LOOH: lipid hydroperoxides; Mes: morpholinoethanesulphonic acid; PC: egg yolk phosphatidylcholine; TPP: triphenylphosphine.

INTRODUCTION

Lipid peroxidation is a complex system where the generation of the initiator molecule is followed by chain initiation, propagation, branching and termination reactions.[1–3] The experimental requirement for iron in lipid peroxidation was demonstrated in the original papers that described the phenomenon.[4–7] Many investigators have

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suggested that the rate and extent of lipid peroxidation depends on the simultaneous availability of Fe^{2+} and Fe^{3+} and on the ratio of these species in solution.[8–16] Whereas the role exerted by Fe^{2+} on primary lipid hydroperoxide (LOOH)-independent and secondary LOOH-dependent initiation and on the termination reactions is clear, the molecular mechanism responsible for the Fe^{3+} stimulation of lipid peroxidation is still obscure. It was proposed that the oxidized form of the metal may affect the Fe^{2+} catalyzed LOOH-independent initiation of lipid peroxidation by stimulating the generation of an ill defined oxygen derived initiator.[8–12,14–16]

However, the demonstration that liposomes chemically deprived of LOOH by treatment with triphenylphosphine (TPP), do not peroxidize when incubated in the presence of various ratios of Fe^{2+}/Fe^{3+},[17] proved, as other investigators suggested,[18–20] that the Fe^{3+} effect was not exerted on the LOOH-independent initiation of lipid peroxidation. Besides, our recent results[17] showing that Fe^{3+} enhances the peroxidation of liposomes supplemented with organic hydroperoxides, evidence suggest that the Fe^{3+} effect on lipid peroxidation is likely exerted on the LOOH-dependent lipid peroxidation.

In this report we present data confirming that the process stimulated by exogenously added $FeCl_3$ is the LOOH-dependent lipid peroxidation; our new findings also suggest a major role for the Fe^{3+} endogenously generated during the metal catalysis in determining the rate of lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Morpholineethanesulphonic acid (Mes), triphenylphosphine (TPP), dipalmitoyl phosphatidylcholine (DPPC), $FeCl_2$, $FeCl_3$ and all other chemicals, of the highest grade available, were purchased from Sigma Chemical Co. (St. Louis, MO. USA). Egg phosphatidylcholine was from Lipid Products (Redhill, U.K.) and 1,10-phenanthroline was from Merck (Darmstadt, Germany). All reagents were prepared in Chelex resin-treated distilled water[21] whose pH was brought to 6.5 with HCl.

Liposome Preparation

The standard sonicated egg yolk phosphatidylcholine liposomes (PC) were prepared as previously described.[22] The phospholipids (about 18 mg), dried under nitrogen, were added with 6 ml distilled water, vortex mixed for 10 min and stored at 4 °C for 1 h. This suspension was subsequently sonicated with a titanium probe sonicator (Labsonic 2000) for different times to obtain liposomes with a different oxidation index.[23] The vesicle dispersion was then transferred to a small-volume extrusion apparatus (produced by Avestin, Ottawa, Canada) which allowed the extrusion of large unilamellar vesicles through standard 19 mm polycarbonate filters with 0.1 mm pore size. Usually samples were subjected to 37 passes through two filters mounted in tandem in the mini-extruder, as recommended by Hope *et al.*[24] This procedure makes possible the preparation of large unilamellar vesicles (LUVETs) with an average diameter close to the pore size. Similar procedures were used to prepare the sonicated PC liposomes containing triphenylphosphine (PC-TPP). In the latter case 60 µl 10 mM TPP in chloroform were added to the phospholipid before drying.[25] PC-TPP liposomes, where TPP was removed before extrusion by 2,2,4-trimethylpentane extraction (liposome: 2,2,4-trimethylpentane 1:2), were also prepared. TPP removal was evaluated by analysis of the absorbance spectrum of the liposome suspension. The dipalmitoyl phosphatidylcholine liposomes (DPPC) were prepared by extrusion technique without prior sonolysis. All extrusion procedures were conducted at 20°C except for DPPC which was extruded at 50°C, 10°C above the gel-liquid

crystal transition temperature. The phospholipid content of the liposome suspensions was determined by the method of Marinetti.[26]

Fe^{2+} Determination

Measurement of Fe^{2+} concentration was made by the o-phenanthroline method according to Mahler and Elowe.[27] All assays were carried out in 1 ml 5 mM Mes buffer, pH 6.5. The concentrations of the components of the reaction mixtures and the incubation conditions are given in the table and figure legends. The reactions initiated by $FeCl_2$ addition, were incubated at room temperature. At the time stated, the reactions were stopped by the addition of 0.2 ml 25 mM 1,10 phenanthroline and A_{515} was immediately read. Fe^{3+} generated in a given time was determined as difference between the Fe^{2+} concentrations measured at the beginning and at the end of the interval considered.

Oxygen Consumption

Lipid peroxidation was measured by monitoring oxygen consumption with a Clark type electrode. Reaction mixtures containing 5 mM Mes buffer, pH 6.5 and the liposomes were continuously stirred in a 3 ml sealed chamber where the O_2 electrode was immersed via a sealed port. Additions were made via a resealable port on top of the chamber.

Oxidation Index

Lipid peroxidation was measured by determining the oxidation index of the liposomes.[23] The samples containing 100–150 mg phospholipid were extracted with 1 ml butan-1-ol. Phases were separated by centrifugation at 3000 rpm for 10 min and the 200–300 nm ultraviolet spectrum of the upper organic phase was recorded against appropriate blanks containing all reagents but liposomes. The oxidation index (A_{233nm}/A_{215nm}) was determined.

LOOH Determination

The LOOH content of the liposomes was determined with the thiocyanate method[28] as described by Cavallini *et al.*[29] The liposomes (150 mg phospholipid) were dissolved in 3 ml of glacial acetic acid/chloroform (3:2 v/v); 5 ml of 3.6% $FeSO_4$ (in 3.6% HCl) were added, followed after 30s by 0.25 ml of 20% KSCN. Determination of lipid hydroperoxide content was made using a calibration curve, obtained under the same conditions with cumene hydroperoxide as a standard.

RESULTS AND DISCUSSION

To verify the hypothesis that Fe^{3+} stimulates the LOOH-dependent but not the LOOH-independent lipid peroxidation, we utilized an experimental system where both Fe^{2+} autoxidation and production of species able to oxidize deoxyribose are negligible.[17] As already reported,[17] in this system sonicated PC-TPP liposomes, which do not contain LOOH, were not peroxidized by $FeCl_2$ addition both in the absence and presence of various Fe^{2+}/Fe^{3+} ratio (Fig. 1). Similar results were obtained when the experiments were conducted with PC-TPP liposomes in which TPP was removed after the treatment (results not shown); this confirms that the phenomenon observed was not due to the presence of residual unreacted TPP able to remove any newly generated LOOH. By contrast, the incubation with increasing concentrations of $FeCl_2$ of standard PC liposomes prepared by ultrasonic irradiation, which contain 35 nmol LOOH/mg phospholipid (PL), resulted in Fe^{2+} oxidation (Fig. 1A) and in the rise of the oxidation index of the liposomes (Fig. 1B). The pattern of Fe^{2+} oxidation by PC liposomes is biphasic: as $FeCl_2$ concentration is increased, at first Fe^{2+} oxidation increases, reaches a maximum and then decreases. This pattern is observed when Fe^{2+} oxidation (Figs. 1A and 4), oxidation index

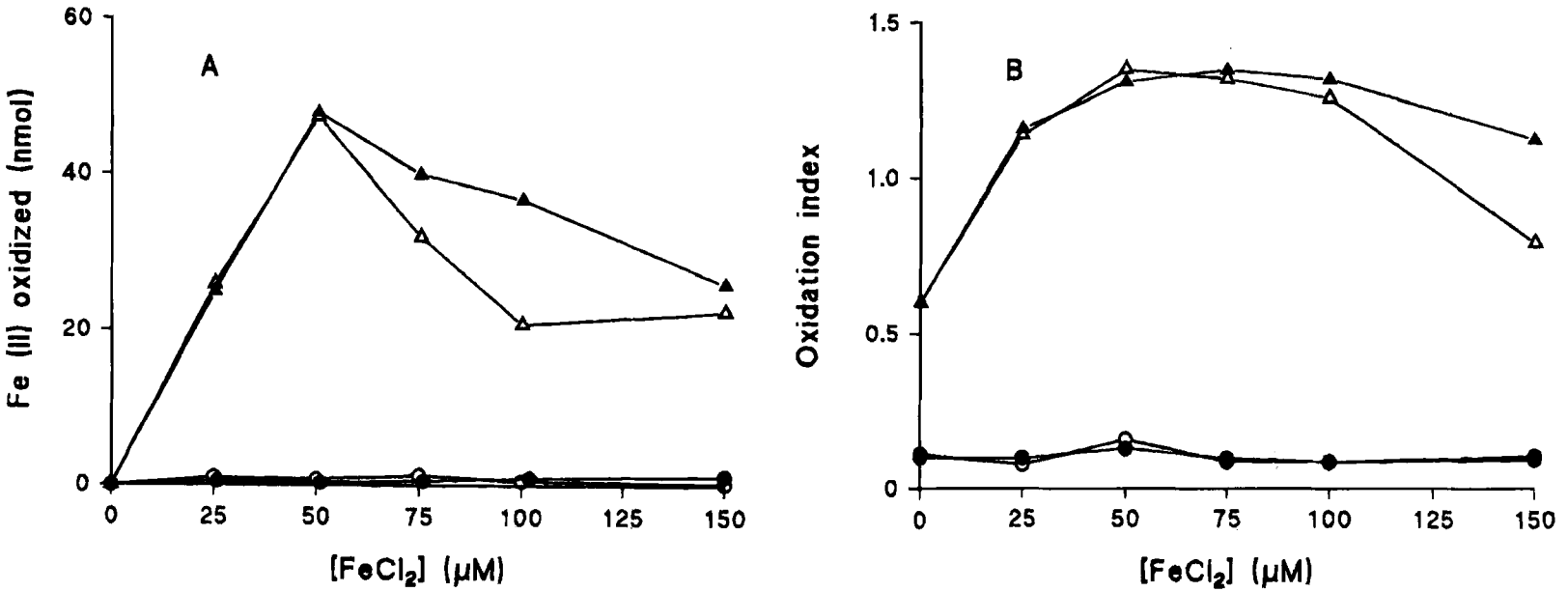

FIGURE 1 Effect of $FeCl_3$ on the peroxidation of PC and PC-TPP liposomes. Increasing concentrations of $FeCl_2$ were incubated with PC (100 μg PL, 3.5 nmol LOOH) (Δ), PC-TPP (150 μg PL, 10 nmol TPP) (○), in 5 mM Mes buffer, pH 6.5 in the absence (open symbols) and presence (closed symbols) of $FeCl_3$ 50 μM. The Fe^{2+} oxidized by the liposomes after 10 min incubation (panel A) and the oxidation index of the liposomes (panel B) were measured.

(Fig. 1B), O_2 consumption (Fig. 2A), LOOH, thiobarbituric reactive material[22] and conjugated diene generation (result not shown) are studied. $FeCl_3$ (50 μM) addition to the standard reaction mixtures caused an increased oxidation of Fe^{2+} by PC liposomes and an increase in their oxidation index (Fig. 1). The effect is mostly evident at high $FeCl_2$ concentrations; as a result of the concentration at which Fe^{2+} oxidation is maximal, defined as critical Fe^{2+} concentration $[Fe^{2+}]^*$ by Vladimirov's group,[30] is shifted to higher values.

To better characterize the Fe^{3+} effect on the LOOH-dependent lipid peroxidation, we studied the time course of the process. The continuous observation of lipid peroxidation was performed by measuring O_2 consumption. Figure 2A shows the time course of O_2 consumption obtained when PC liposomes were incubated with increasing concentrations of $FeCl_2$. At low $FeCl_2$ concentrations the curves were hyperbolic with similar initial rates but different final extents of O_2 consumption. When the liposomes were incubated with intermediate $FeCl_2$ concentrations the pattern of O_2 consumption became rather complex: after an initially rapid but limited hyperbolic phase, the curves became sigmoidal. By increasing $FeCl_2$ concentrations, the onset of the sigmoidal curve was delayed. At high $FeCl_2$ concentrations, the sigmoid curve started beyond the studied length of the reaction and thus only a small and rapid O_2 consumption was observed.[8–10,12,14–16] When the time course of O_2 consumption was conducted in the presence of $FeCl_3$ (50 μM) (Fig. 2B) patterns similar to those obtained in its absence were observed; however, the transition from hyperbolic to complex kinetics took place at higher $FeCl_2$ concentrations. The delayed addition of $FeCl_3$ (50 μM) to reaction mixtures containing inhibitory $FeCl_2$ concentrations resulted in the resumption of O_2 consumption (Fig. 1A line g *versus* line e).

These results, obtained in conditions where both the LOOH content of lipid substrate and the generation of oxygen derived initiator were carefully controlled,[17] provide experimental data that substantiate our preliminary work[17] and support the hypothesis that added Fe^{3+} stimulates the peroxidation of LOOH containing liposomes.

In the papers suggesting the involvement of Fe^{3+} in the stimulation of the LOOH-independent lipid peroxidation, both a biphasic pattern of lipid peroxidation *versus* $FeCl_2$ concentration and a lag phase were described. The biphasic pattern was taken as a proof to support the importance of the different Fe^{2+}/Fe^{3+} ratio in the stimulation of lipid peroxidation.[9,12,14–16] However, in the various experimental systems chosen to obtain the permissive Fe^{2+}/Fe^{3+} ratio, the Fe^{2+} concentration in the assay was modified. It was decreased by oxidation,[12] or increased by reduction of Fe^{3+},[14] or added in variable amount in the presence of Fe^{3+} to have a constant concentration of iron.[16] The lack of control assays, where $FeCl_2$ concentration was varied in the absence of $FeCl_3$, made the authors unaware that the standard high Fe^{2+} concentration they used (200 μM) favored the termination reactions. The lag phase in the standard assay[8–10,16] and its decrease by the addition of Fe^{3+}, either free[10] or complexed to ADP[8] and to nitrilotriacetic acid,[10] was also reported. However, although the Fe^{2+} concentrations utilized were high (100 μM[8,10] and 200 μM[9,16]), the likely occurrence of a termination phase was not recognized and confounded with a lack of initiation. This, together with no measurement of the LOOH content of the lipid substrates, led the authors to reject the possibility that the phenomenon they observed might be exerted on the LOOH-dependent lipid peroxidation.

Borg and Schaich[19] proposed that Fe^{3+} enhancement of lipid peroxidation was due to the inhibition of the Fe^{2+}-dependent termination reactions. To verify their hypothesis we studied the effect of $FeCl_3$ on the stoichiometry between O_2 consumed and Fe^{2+} oxidized by PC liposomes after 10 min incubation, here defined as average length of radical chain. In the absence of $FeCl_3$ the average length of radical chain was maximal at the lowest iron concentration tested; the increase

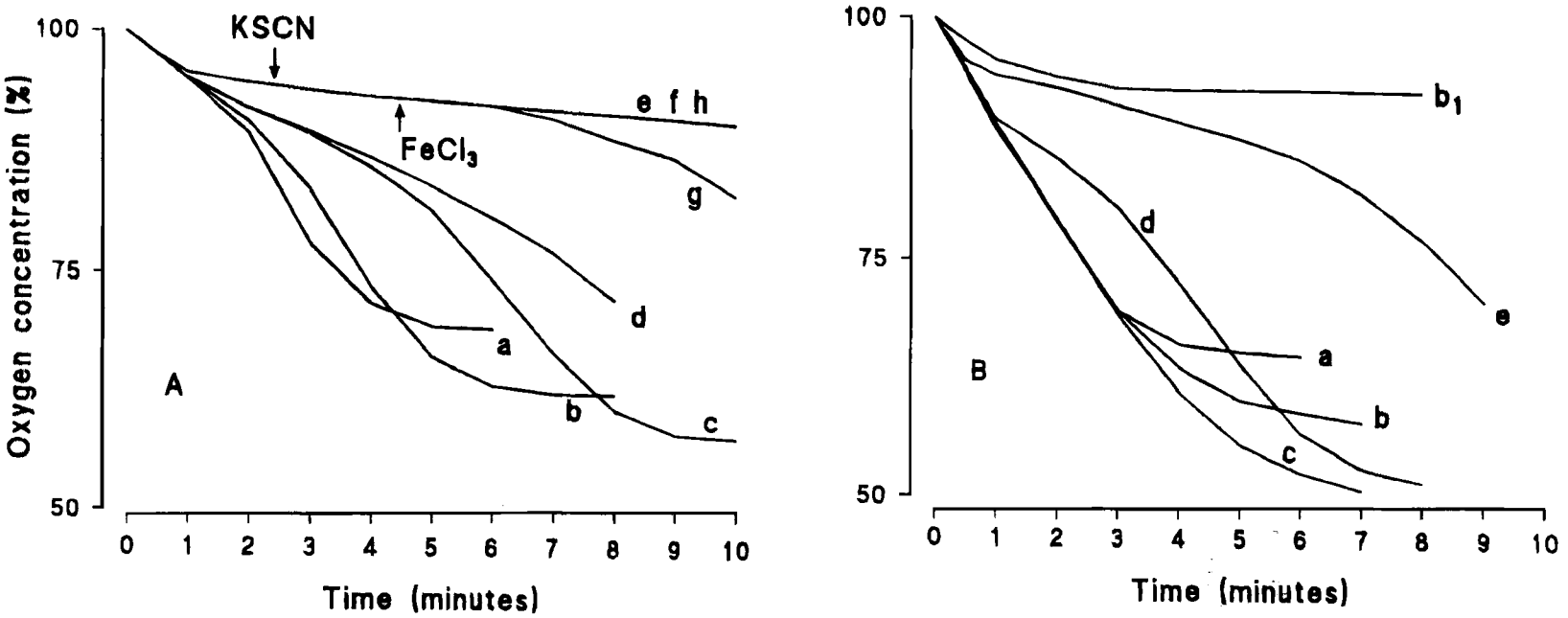

FIGURE 2 Effect of $FeCl_3$ and KSCN on the time course of O_2 consumption by PC liposomes. PC liposomes (446 μg PL) were incubated with increasing concentrations of $FeCl_2$ (a 50, b 75, c 100, d 125, e 150 μM $FeCl_2$, b_1 75 μM $FeCl_2$ + 400 μM KSCN, f 150 μM $FeCl_2$ + 200 μM KSCN, g 150 μM $FeCl_2$ + 50 μM $FeCl_3$, h 150 μM $FeCl_2$ + 200 μM KSCN + 50 μM $FeCl_3$ at the times) in 5 mM Mes buffer, pH 6.5. The reactions were conducted in the absence (panel A and line b_1 in panel B) or presence of 50 μM $FeCl_3$ (panel B).

in the concentration of $FeCl_2$ brought about the shortening of the chains (Fig. 3). $FeCl_3$ (50 μM) addition to the reaction mixtures enhanced the average length of chains of lipid peroxidation (Fig. 3). The data presented confirm that, in our experimental system, Fe^{3+} stimulates the LOOH-dependent lipid peroxidation apparently by increasing the efficiency of Fe^{2+} to exert a pro-oxidant effect.

According to Borg and Schaich,[19] the molecular mechanism responsible for the Fe^{3+} effect is the competition between the two oxidation forms of the metal for the membrane surface; in the presence of Fe^{3+}, Fe^{2+} concentration on the surface would decrease and this would result in a decreased reaction of Fe^{2+} with the chain-carrying organic radicals. Vladimirov's group[31] generalized this idea and suggested that the surface Fe^{2+} concentration was the main parameter determining both kinetics and efficiency of Fe^{2+}-induced lipid peroxidation in membrane systems. As the demonstraion of this hypothesis could explain Fe^{3+} stimulation of lipid peroxidation, we set up experimental conditions to verify it. We altered the surface Fe^{2+} concentration by varying the liposome concentration as suggested

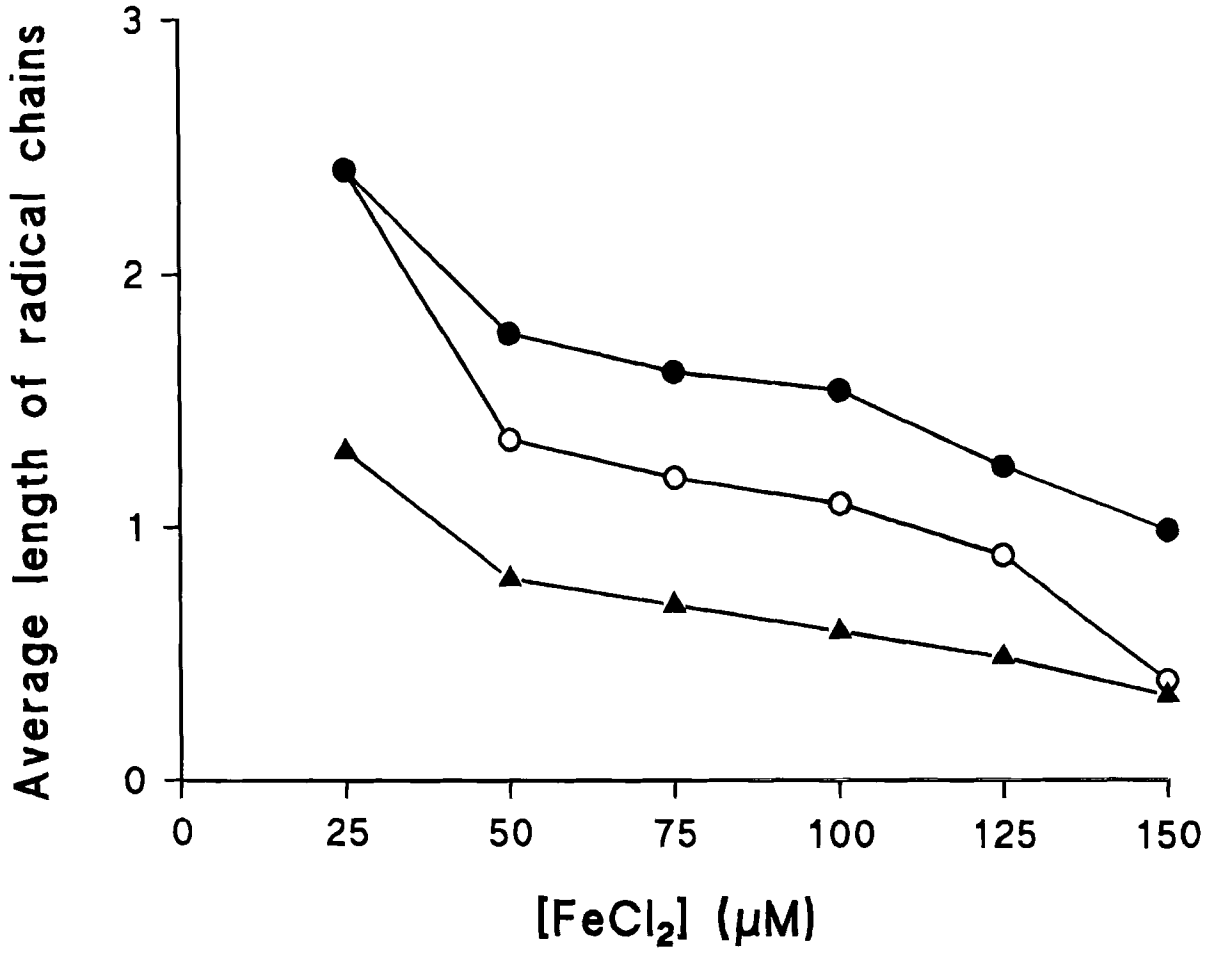

FIGURE 3 Effect of $FeCl_3$ and KSCN on the average length of radical chains. PC liposomes (430 μg PL) were incubated with increasing concentrations of $FeCl_2$ in the chamber of a Clark type electrode containing 3 ml of 5 mM Mes buffer, pH 6.5. The average length of radical chains was determined by dividing the nmol of oxygen consumed in 10 min by the nmol of Fe^{2+} oxidized in the same time interval in the absence (○) or presence of either 50 μM $FeCl_{3+}$ (●) or 200 μM KSCN (▲).

by Driomina *et al.*[31] We utilized two concentrations of LOOH—containing PC liposomes (101 and 202 μg PL containing 4.2 and 8.4 nmol LOOH respectively) and, as controls, two mixed populations composed of LOOH containing PC liposomes (101 μg PL, 4.2 nmol LOOH) and either PC-TPP liposomes (101 μg PL, no LOOH) or DPPC liposomes (101 μg PL, no LOOH). Figure 4A shows that the concentration at which Fe^{2+} oxidation by liposomes was maximal did not depend on the amount of liposomes (101 *versus* 202 μg phospholipid) but on the LOOH concentration (4.2 *versus* 8.4 nmol/ml) in the assay.

This result, showing that the dilution of Fe^{2+} hypothetically adsorbed to the PC liposomes by the presence of either DPPC or PC-TPP liposomes does not favor lipid peroxidation, strongly challenges Vladimirov's group hypothesis that iron complexing agents, Fe^{3+} and other cations affect lipid peroxidation by decreasing Fe^{2+} ion concentration on the membrane surface.[31]

To gain some insight on the possible molecular mechanism of Fe^{3+} stimulation of lipid peroxidation, we further investigated the influence exerted by LOOH concentration on lipid peroxidation. PC liposomes (about 100 μg PL) with different LOOH contents, showed a similar biphasic pattern of Fe^{2+} oxidation but different critical Fe^{2+} concentrations $[Fe^{2+}]^*$. This demonstration of the relative nature of $[Fe^{2+}]^*$ and its dependence on the oxidation state of the substrate is, in our opinion, an important result both for the experimental approach to the study of lipid peroxidation and for the comprehension of the mechanism of lipid peroxidation *in vivo*. In fact, the evidence that the results obtained with different amounts (Fig. 4A), different batches (Fig. 4B), differently aged PC liposomes[22] are often not comparable, should render more cautious in the use of a fixed $FeCl_2$ concentration as catalyst of lipid peroxidation. Only a plot of peroxidation *versus* $FeCl_2$ concentration would allow one to select the desired experimental conditions and to reproduce them in different occasions. Besides, the demonstration that $[Fe^{2+}]^*$ is not absolute but relative to the LOOH content of the lipids, questions the assumption that the very low concentration of free iron in the cytoplasm always exerts a prooxidant effect *in vivo*. In order to attribute either a prooxidant or an antioxidant role to the metal an accurate determination of the concentration of LOOH in the different physiological and pathological conditions would be necessary.

The observation that high LOOH concentrations (Fig. 4B) and exogenously added $FeCl_3$ (Fig. 1A) exert the same effect on lipid peroxidation, namely they shift the $[Fe^{2+}]^*$ to higher concentrations, suggested a possible cause-effect mechanism relating these two parameters. The rapid reaction of Fe^{2+} with LOOH could cause the endogenous generation of Fe^{3+}; the concentration of this species in the assay would somehow be directly responsible for the $[Fe^{2+}]^*$ observed. To verify this hypothesis we tried to lower the concentration of the endogenously generated Fe^{3+} ion utilizing an iron chelator able to preferentially bind Fe^{3+} without affecting Fe^{2+} concentration. We used KSCN as the different orders of magnitude of its stability constants for Fe^{3+} (Log Ka 6.4) and Fe^{2+} (Log Ka 0.95) assured a preferential binding of the oxidized form of the metal and a modest effect on Fe^{2+} concentration; chelators like EDTA and desferrioxamine were not utilized as they strongly bind Fe^{2+} and cause its rapid autoxidation.[32–35] KSCN (200 μM) proved a) not to interfere with Fe^{2+} determination by the o-phenanthroline assay; b) not to affect Fe^{2+} autoxidation; c) not to influence Fe^{2+} oxidation by either cumene hydroperoxide or t-butyl hydroperoxide (results not shown).

When KSCN (200 μM) was added to the reaction mixtures containing standard PC liposomes, it inhibited Fe^{2+} oxidation shifting to a lower value the $[Fe^{2+}]^*$ (Fig. 4B). Besides, it strongly inhibited O_2 consumption (Fig. 2B line b_1 *versus* Fig. 2A line b) and decreased the average length of chains of lipid peroxidation (Fig. 3). KSCN (200 μM), *per se* did not affect the low O_2 consumption by PC liposomes observed at high

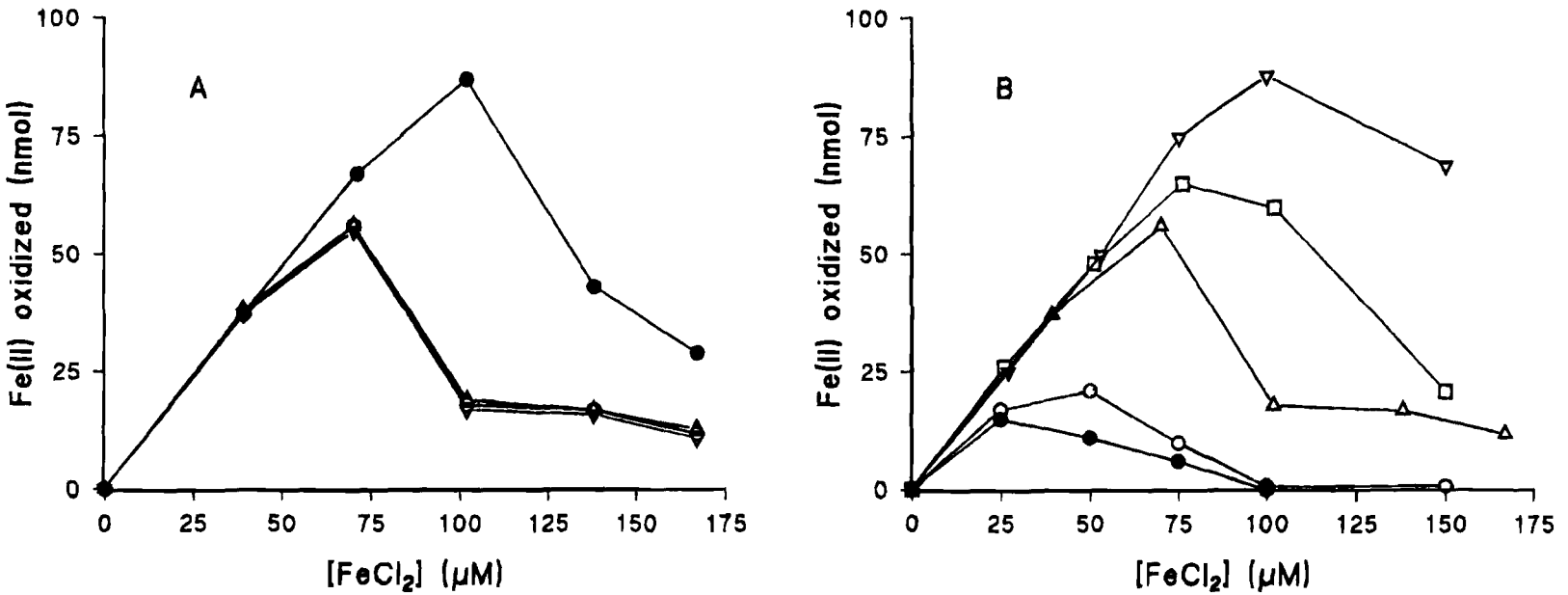

FIGURE 4 Effect of the amount of liposomes and of their LOOH content on Fe^{2+} oxidation. Liposomes were incubated with increasing concentrations of $FeCl_2$ in 5 mM Mes buffer, pH 6.5. The Fe^{2+} oxidized after 10 min incubation was measured. Panel **A.** (○) PC liposomes (101 μg PL, 4.2 nmol LOOH); (●) PC liposomes (202 μg PL, 8.4 nmol LOOH); (△) PC liposomes (101 μg PL, 4.2 nmol LOOH) and PC-TPP liposomes (101 μg PL, no LOOH); (▽) PC liposomes (101 μg PL, 4.2 nmol LOOH) and DPPC liposomes (101 μg PL, no LOOH). Panel **B.** PC liposomes: (○)102 μg PL, 2.9 nmol LOOH; (●)102 μg PL, 2.9 nmol LOOH + 200 μM KSCN; (△)101 μg PL, 4.2 nmol LOOH; (□)98 μg PL, 6.3 nmol LOOH; (▽)101 μg PL, 9.5 nmol LOOH.

$FeCl_2$ concentrations (Fig. 2A line f *versus* line e), but it prevented the O_2 consumption elicited by $FeCl_3$ addition (Fig. 2A line h *versus* line g). These results are in agreement with the proposed hypothesis, *i.e.* that Fe^{3+} endogenously generated is a factor controlling the kinetics and the efficiency of lipid peroxidation.

To understand the molecular mechanism responsible for the Fe^{3+} effect, we tried to find experimentally a parameter, related to Fe^{3+} concentration, able to describe the kinetics of lipid peroxidation. We monitored the time course of Fe^{3+} generation due to the oxidation of different $FeCl_2$ concentrations (75, 80, 85 and 90 μM) brought about by PC liposomes (168 μg PL, 7.5 nmol LOOH) (Figure 5A). The kinetics observed were different and comparable to those obtained when O_2 consumption was studied in similar experimental conditions (Fig. 2A). The rate of Fe^{2+} oxidation during the first minute after $FeCl_2$ addition (7.1, 7.3, 7.4 and 7.1 nmol Fe^{2+} oxidized/min), apparently, is not the parameter controlling the kinetics of lipid peroxidation. From the data obtained we calculated, at each minute since the beginning of the experiment, the ratio between the remaining Fe^{2+} and the generated Fe^{3+}; these ratios were plotted against the rate of Fe^{2+} oxidation during the next minute. The resulting curves presented in Figure 5B are very similar: all of them show that, when Fe^{3+} accumulates to give a Fe^{2+}/Fe^{3+} ratio ranging between 2:1 and 5:1, the rate of Fe^{2+} oxidation during the next minute is greatly enhanced.

In the same way we monitored the time course of Fe^{3+} generation due to the oxidation of a fixed amount of $FeCl_2$ (100 μM) by increasing concentration of PC liposomes (90, 120, 135, 150 μg PL, 17 nmol LOOH/mg PL). The initial rate of Fe^{3+} generation were 1.5, 2.0, 2.3 and 2.65 nmol Fe^{2+} oxidized/min. Although the patterns of the kinetics of Fe^{2+} oxidation greatly differ (Fig. 6A), the curves obtained by plotting the Fe^{2+}/Fe^{3+} ratio against the rate of Fe^{2+} oxidation during the next minute are similar (Fig. 6B). Also in these experimental conditions when Fe^{3+} accumulates to give a Fe^{2+}/Fe^{3+} ratio ranging between 2:1 and 5:1, the rate of Fe^{2+} oxidation during the next minute is greatly enhanced. These results suggest that the kinetics of lipid peroxidation depend on a critical Fe^{2+}/Fe^{3+} ratio: when this ratio is lower than the critical one, Fe^{2+} ions are prooxidants in all concentrations and at any time of incubation. When the ratio is higher than the critical value a lag phase associated to the prevailing of the termination reactions is observed; the length of the lag phase depends on the time occurring to approach the permissive Fe^{2+}/Fe^{3+} ratio. This finding suggests the importance not only of Fe^{2+} and LOOH but also of Fe^{3+} concentration in provoking *in vivo* lipid peroxidation and hints that both the total release of metal from storage molecules and its oxidation state are relevant for the stimulation of lipid peroxidation.

In their complex, our data confirm the pioneering work of many groups who described a stimulation of lipid peroxidation by Fe^{3+} and sustained the importance of this phenomenon. However, our experimental conditions allowed us to define that the target of Fe^{3+} action is not the LOOH-independent but the LOOH-dependent lipid peroxidation. The data presented confirm that Fe^{3+} enhances lipid peroxidation by shifting Fe^{2+} from serving as stoichiometric reactant in the termination reactions to being the repeatedly cycled catalyst of the chain branching and propagation reactions; however, they are not consistent with the hypothesis that Fe^{3+} exerts its effect by decreasing the Fe^{2+} ion concentration adsorbed to the membrane surface. In fact, they demonstrate that it is not the $[Fe^{2+}]^*_L$ (critical concentration of Fe^{2+} adsorbed to membrane lipid phase)[26] the species that controls the kinetics of lipid peroxidation. They evidence the importance of the Fe^{3+} endogenously generated in stimulating lipid peroxidation and indicate the Fe^{2+}/Fe^{3+} ratio as the parameter that better correlate with the phenomena observed. However, the data presented here do not provide elements to formulate an hypothesis on the molecular mechanism by which such a ratio exerts its action, a hypothesis that will

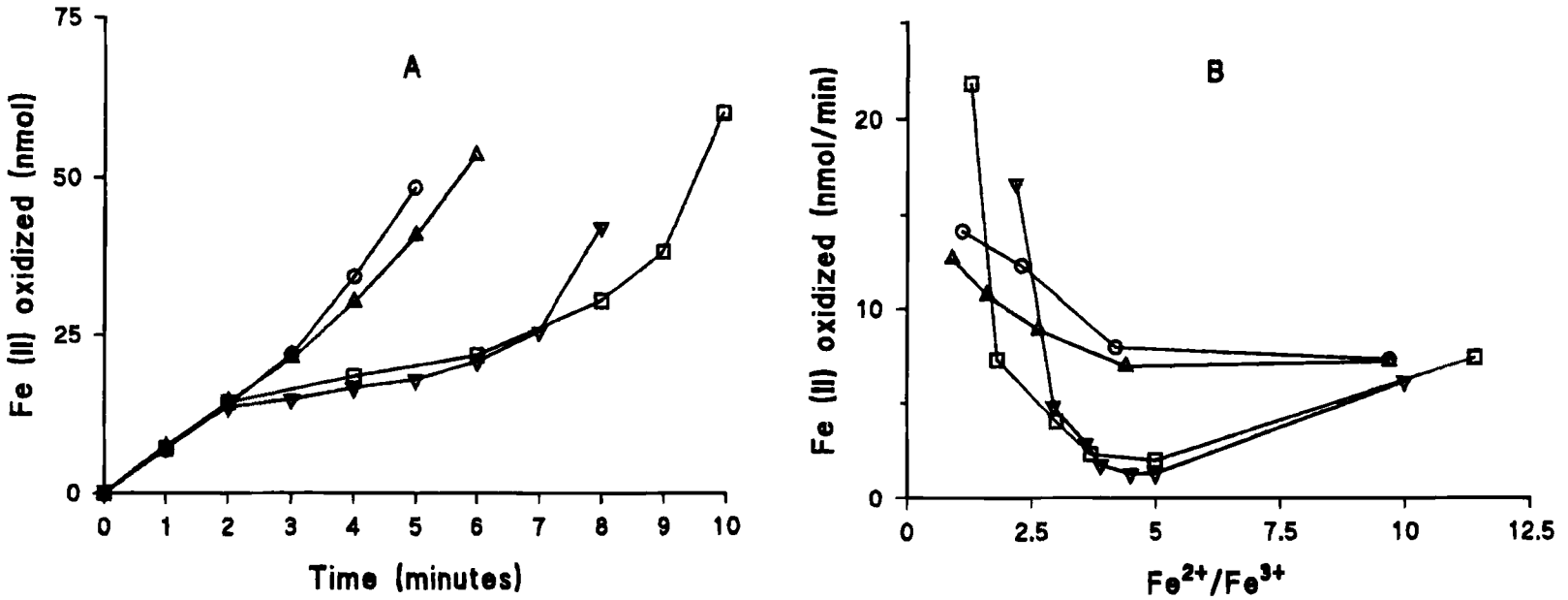

FIGURE 5 Effect of the ratio of Fe^{2+} and Fe^{3+} generated during Fe^{2+} oxidation by a fixed amount of PC liposomes on the rate of lipid peroxidation. The time course of oxidation of 75 (○), 80 (△), 85 (▽) and 90 (□) μM $FeCl_2$ by PC liposomes (168 μg PL) in 5 mM Mes buffer, pH 6.5 was measured (panel A). The data from panel A were analyzed and the rate of Fe^{2+} oxidation between successive minutes was plotted against the ratio of remaining Fe^{2+} and Fe^{3+} accumulated at the previous minute (panel B).

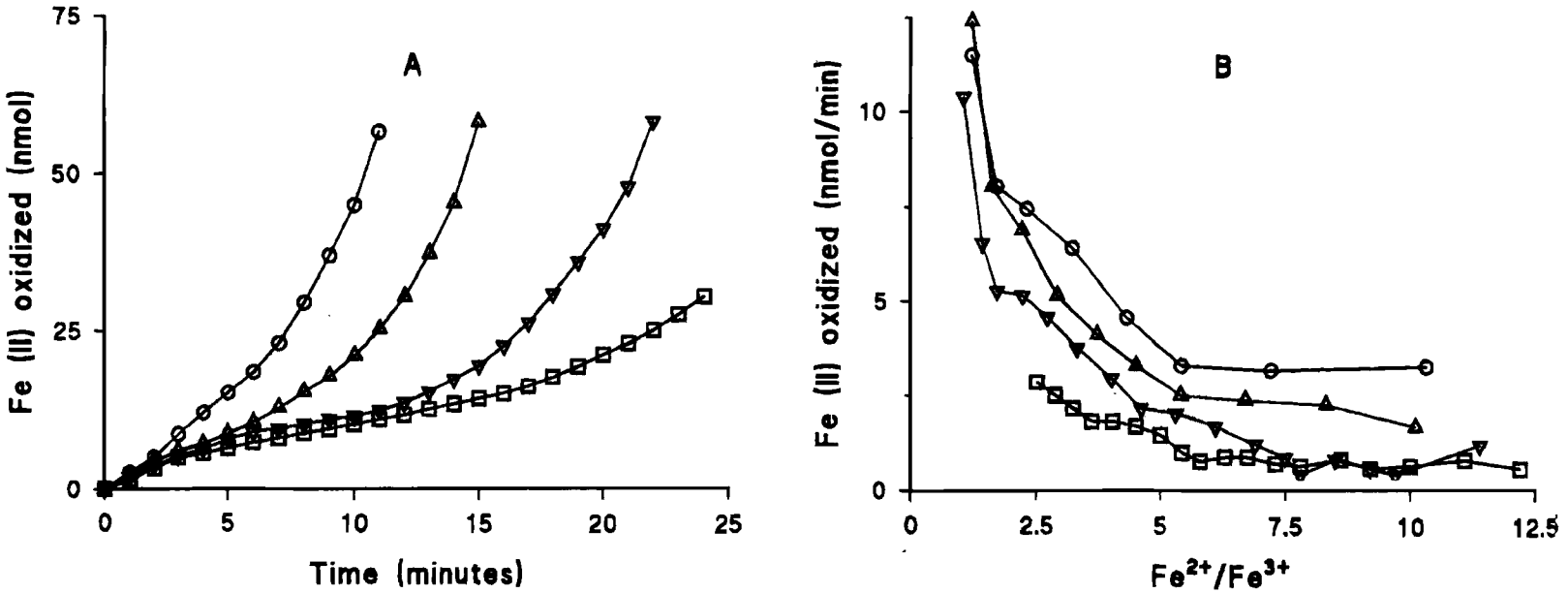

FIGURE 6 Effect of the ratio of Fe^{2+} and Fe^{3+} generated during Fe^{2+} oxidation by a variable amount of PC liposomes on the rate of lipid peroxidation. The time course of oxidation of 100 μM $FeCl_2$ by 90 (□), 120 (▽), 135 (△) and 150 (○) μg PC liposomes in 5 mM Mes buffer, pH 6.5 was measured (panel A). The data from panel A were analyzed and the rate of Fe^{2+} oxidation between successive minutes was plotted against the ratio of remaining Fe^{2+} and Fe^{3+} accumulated at the previous minute (panel B).

have to account for the data showing that other cations, besides Fe^{3+}, can stimulate lipid peroxidation.[20,31,36,37]

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