Oxidation of Phosphatidylcholine Liposomes in Aqueous Dispersions Induced by Copper and Iron

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The oxidations of phosphatidylcholine liposomes in aqueous dispersions induced by copper and iron have been studied aiming specifically at elucidating the action of copper ion in the chain initiation of the oxidation of liposomal membranes. Both copper(II) and iron(III) ions induced steady oxidations and the addition of *t*-butyl hydroperoxide enhanced the rate of oxidation. The reduction of hydroperoxides present initially in the liposomes by triphenylphosphine markedly reduced the rate of oxidation. The rate of oxidation induced by copper(II) ion was proportional to the first power of phosphatidylcholine concentration and to the half power of both copper(II) ion and *t*-butyl hydroperoxide concentrations, suggesting that the oxidation was initiated by the oxygen radicals formed in the decomposition of hydroperoxide by copper(II) and copper(I) ions. The anionic dipalmitoylphosphatidic acid added into the liposomes enhanced the rate of oxidation induced by metal ions, whereas the addition of cationic octadecylamine showed little effect on the rate of oxidation.

The oxidations of lipids have been accepted to be the important and probably the primary events in the deterioration of foods and oil and also in a progress of various pathological disorders including cancer and aging.[1] Such oxidations usually proceed by a free radical chain mechanism and its initial event is the generation of free radicals which induce the chain initiation. The transition metal ions, especially iron, are strongly implicated in the generation of free radicals both *in vitro* and *in vivo*,[2,3] but its detailed mechanisms and kinetics are not clearly understood. Transition metal ions may contribute to the chain initiation by a variety of mechanisms such as decomposition of hydroperoxides and/or hydrogen peroxide to give alkoxyl, peroxyl, and hydroxyl radicals and also activation of oxygen.[4] It has been also pointed out that the higher oxidation state of these metal ions plays an important role in the oxidation of biological molecules.[5,6]

Copper has received less attention than iron but it is known to be as effective or even more active than iron in stimulating the decomposition of hydrogen peroxide[7] and hydroperoxide,[8,9] causing DNA damage,[10] protein[11] or peptide modification[12] or hemolysis,[13] formation of fluorescent lipid complexes,[14] and oxidation of low density lipoproteins.[15] Although most copper in plasma is present within the protein ceruloplasmin,[16] some is bound to serum albumin and to amino acids such as histidine.[17] In any event, it is clear that transition metal ions are strongly involved in the pathophisiology of free radicals and it is important to elucidate the mechanisms and kinetics of initiation reaction and their consequences. We have previously studied the chain initiation in the oxidation of methyl linoleate micelles in aqueous dispersions induced by copper and iron and found that the main initiation reaction was the decomposition of hydroperoxide.[18] In this work, we have used soybean phosphatidylcholine (PC) liposomes in aqueous dispersions as a lipid substrate and aimed at elucidating the mechanism of chain initiation induced by copper and iron. The liposomal membranes were chosen as substrate since it is a good model for biological membranes, much more biologically relevant than micelles. Soybean PC contains high concentrations of linoleic acid residues and quite susceptible to oxidation. Its oxidation gives conjugated diene hydroperoxides quantitatively and hence its oxidation can be followed easily as a model of lipid.[19,20]

Experimental

Reagents. Commercial soybean L-α-phosphatidylcholine purchased from Sigma (St. Louis, MO) was purified with alumina and silica-gel columns. The liposomes were prepared as follows. PC and lipid-soluble additives, when required, were dissolved in methanol and the solution was placed into a pear-shaped flask. Methanol was removed by evacuation on a water aspirator using a rotary vacuum evaporator to obtain a thin film on the flask wall. An appropriate amount of 50 mM (1 M=1 mol dm^{-3}) Tris-HCl buffer (pH 7.4) aqueous solution was added and the film was slowly peeled off by shaking to obtain white, milky liposome suspensions.

Dipalmitoylphosphatidic acid (DPPA) and octadecylamine used as anionic and cationic additives respectively were purchased from Sigma (St. Louis, MO) and used as received. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) used as water-soluble and lipid-soluble radical initiators respectively were obtained from Wako Pure Chemical Co. (Osaka). Natural (2*R*,4′*R*, 8′*R*)-α-tocopherol was kindly supplied from Eisai Co., Ltd. (Tokyo). Commercial 2,6-di-*t*-butyl-4-methylphenol (BHT) was purchased from Seiko Chemical Co., Ltd. and used as received. Commercial *t*-butyl hydroperoxide was distilled under reduced pressure before use. Commercial hydrogen peroxide, iron(III) sulfate, and copper(II) chloride were used as received.

Methods. The oxidations of soybean PC liposomes were

performed in aqueous dispersions at 37°C. AAPH, iron(III) sulfate, copper(II) chloride, and hydroperoxide were added, when required, to the aqueous emulsions as aqueous solutions. The rate of oxidation was followed with an oxygen monitor equipped with a Clark-type oxygen electrode (YSI model 5300).

The rate of formation of PC hydroperoxide (PCOOH) was followed with an HPLC by a UV detector at 234 nm. Silica-gel column was used and methanol/40 mM phosphate buffer (9/1 by volume) was delivered as an eluent at 1 ml min^{-1}. To 100 μl of reaction solution was added 200 μl of chloroform/methanol (2/1 by volume) and the mixture was vortexed for 1 min, centrifuged 3 min at 12000 rpm, and then the chloroform layer was injected to the HPLC.

Results

Rates of Oxidations of Phosphatidylcholine Liposomes in Aqueous Dispersions. Both copper and iron induced the oxidations of PC liposomes in aqueous dispersions at 37°C in air. Figure 1 shows the typical results of the oxidations of soybean PC liposomes in aqueous dispersions induced by copper(II) (Cu^{II}) ion in the absence and presence of 1 mM *t*-butyl hydroperoxide or hydrogen peroxide. The soybean PC liposomes were oxidized spontaneously even in the absence of copper. In the absence of hydroperoxides but presence of 100 μM copper(II) ion, the PC hydroperoxides (PCOOH) were accumulated at a steady and constant rate. When 1 mM *t*-butyl hydroperoxide or hydrogen peroxide was added into the aqueous phase, the rate of oxidation was increased and phosphatidylcholine hydroperoxides were formed at a constant rate. As shown in Fig. 2, BHT suppressed the oxidation and produced an induction period, the length of which (tinh) was proportional to

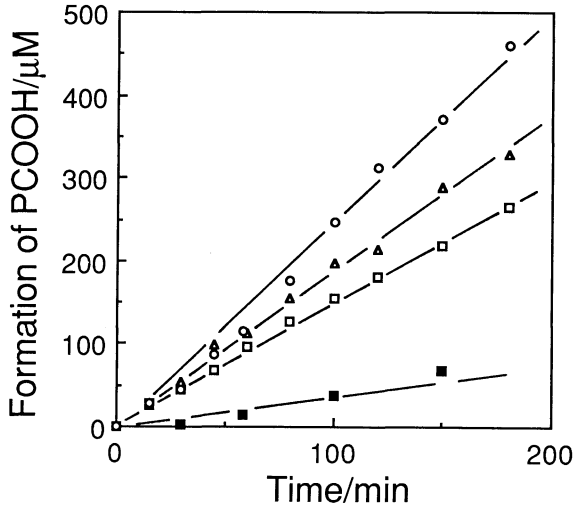

Fig. 1. Oxidations of soybean phosphatidylcholine liposomes in aqueous dispersions induced either spontaneously (■) or by 100 μM copper(II) ion in the absence (□) and presence of 1 mM BOOH (△) or 1 mM H_2O_2 (○). The formation of phosphatidylcholine hydroperoxide (PCOOH) was followed with an HPLC as described in Materials and Methods.

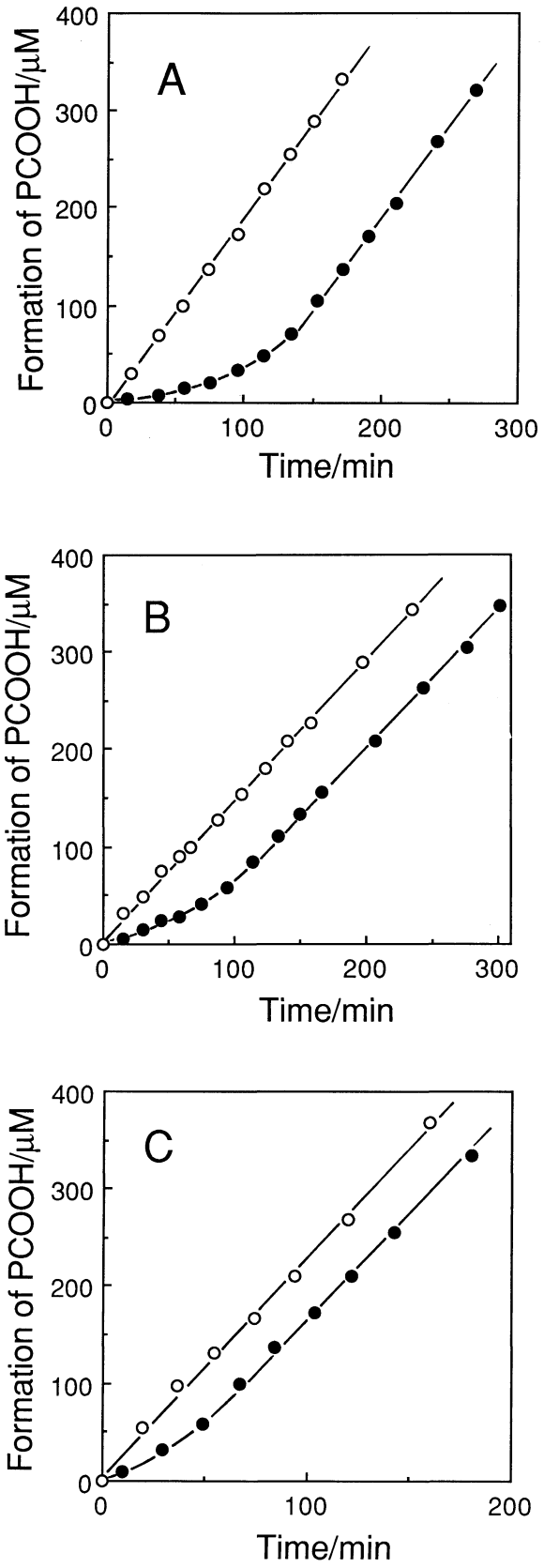

Fig. 2. Oxidation of 10.3 mM soybean phosphatidylcholine liposomes in aqueous dispersions induced by (A) 100 μM copper(II) chloride and 3 mM BOOH, (B) 2 mM AAPH or (C) 2 mM AMVN in the absence (○) and presence (●) of 1 μM BHT at 37°C in air.

BHT concentration (Fig. 3(A)). Furthermore, the rate of oxidation during the induction period was proportional to the reciprocal of BHT concentration (Fig. 3(B)).

Figure 4 shows the effects of concentrations of soybean PC, copper(II) ion, and *t*-butyl hydroperoxide on the rates of oxidations. The rate of oxidation was directly proportional to the concentration of PC and approximately to the half power of both copper(II) ion and *t*-butyl hydroperoxide concentrations.

Effects of Electric Charge on the Liposome Surface. Figure 5 shows the results of oxidations of PC liposomes induced by copper or iron in the absence and presence of *t*-butyl hydroperoxide or hydrogen peroxide. The rate of iron(III) ion induced oxidation was slow, but it pro-

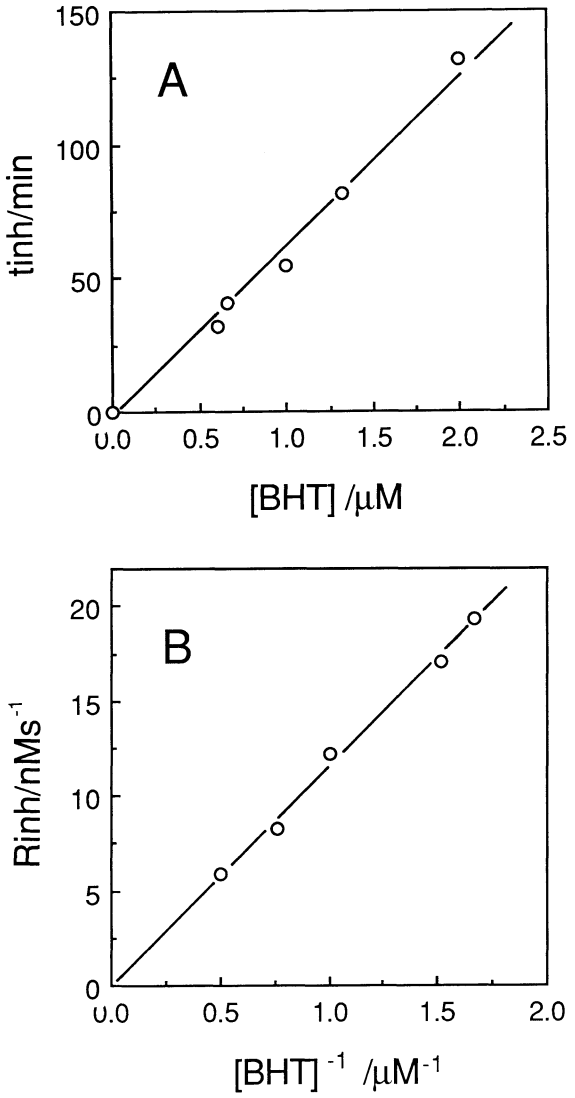

Fig. 3. (A) The length of induction period (t_{inh}) produced by 2,6-di-*t*-butyl-4-methylphenol (BHT) and (B) the rate of oxidation during the induction period (R_{inh}) in the oxidation of 10.3 mM soybean phosphatidylcholine liposomes induced by 100 μM copper(II) chloride and 3 mM *t*-butyl hydroperoxide at 37°C in air.

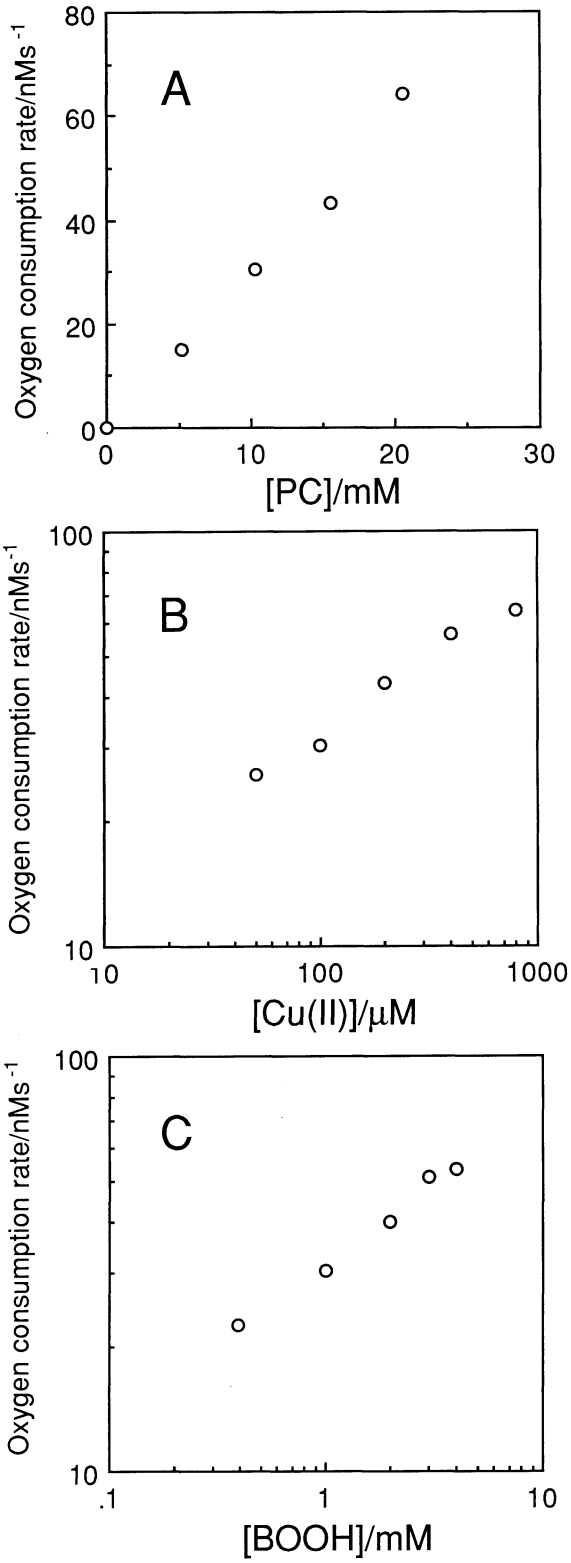

Fig. 4. Oxidations of phosphatidylcholine liposomes in aqueous dispersions induced by copper(II) ion at 37°C in air. (A): Effect of phosphatidylcholine concentration on the rate of oxidation induced by 100 μM copper(II) ion and 1 mM BOOH. (B): Effect of copper(II) ion concentration on the rate of oxidation of 10.3 mM phosphatidylcholine liposomes with 1 mM BOOH. (C): Effect of BOOH concentration on the rate of oxidation of 10.3 mM phosphatidylcholine liposomes with 100 μM copper(II) ion.

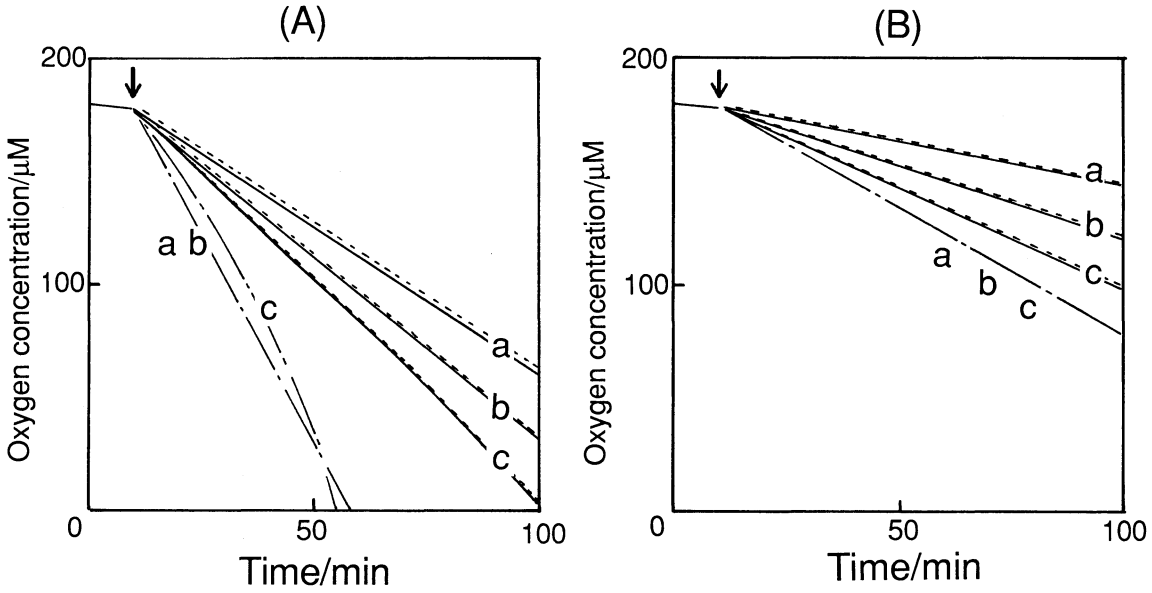

Fig. 5. Oxidations of 10.3 mM soybean phosphatidylcholine liposomes in aqueous dispersions induced by (A) 100 μM $CuCl_2$ or (B) 100 μM $Fe_2(SO_4)_3$ in the absence and presence of 1 mM *t*-butyl hydroperoxide (BOOH) or hydrogen peroxide (H_2O_2) in the absence (solid line) and presence of 0.1 mM dipalmitoylphosphatidic acid (half solid line) or 0.1 mM octadecylamine (broken line) at 37°C. Metal ions were added to the reaction mixture at the time indicated by an arrow. a: without BOOH or H_2O_2; b: with BOOH; c: with H_2O_2.

ceeded at a constant rate for a long period (Fig. 5(B)a). The oxidation induced by copper(II) ion also proceeded at a constant rate, faster than that induced by iron(III) ion (Fig. 5(A)a). When *t*-butyl hydroperoxide or hydrogen peroxide was added to the reaction system, the rate of oxidation induced by either copper(II) or iron(III) ion was enhanced (Fig. 5 b,c). Furthermore, as shown in Fig. 1 and 4, the rate of oxidation induced by copper(II) with hydrogen peroxide was faster than that with *t*-butyl hydroperoxide. Similar effects of the addition of hydroperoxide were observed for iron(III) ion-induced oxidations. In the presence of 0.1 mM DPPA, the rate of oxidation induced by either Cu^{II} or Fe^{III} was faster than that in the absence of DPPA, but the addition of *t*-butyl hydroperoxide or hydrogen peroxide into this system did not influence the rate of the oxidation (Fig. 5 half solid line). Such a marked accelerating effect of DPPA was not observed in the AAPH-induced oxidation. On the other hand, the rate of Cu^{II} or Fe^{III}-induced oxidation of 10.3 mM soybean PC liposomes containing 0.1 mM octadecylamine (1% of soybean PC) was similar to that in the absence of octadecylamine. The effect of addition of octadecylamine was also quite small in the presence of *t*-butyl hydroperoxide or hydrogen peroxide (Fig. 5 broken line).

Discussion

As shown above, both copper and iron induced the oxidations of soybean PC liposomes in aqueous dispersions even without addition of *t*-butyl hydroperoxide or hydrogen peroxide. The oxidation must be initiated by oxygen radicals formed by the decomposition of PC hydroperoxide (PCOOH) initially present in PC liposomes. In fact, the PC used in this study contained about 510 μM PCOOH (0.99% of PC) and accordingly it is calculated that about 102 μM hydroperoxide was present initially in the total aqueous suspensions. These hydroperoxides must be decomposed by copper or iron to give peroxyl and alkoxyl radicals which initiate the chain oxidation. It was found that, when PC was treated with triphenylphosphine Ph_3P/PCOOH=1.67/1.0 by mol/mol), the rate of oxidation induced by 100 μM copper(II) ion was reduced markedly from 24.3 nM s^{-1} to 0.83 nM s^{-1}, supporting the above assumption that the initiating radicals are formed by the decomposition of hydroperoxide contained in the PC. Recently, Thomas and Jackson[21] have found that the initiation of copper-dependent oxidation of low density lipoprotein requires the trace amounts of lipid hydroperoxides in the lipoprotein. We have also found that the rate of oxidation of methyl linoleate micelles is dependent on the concentration of its hydroperoxide within micelles.[18]

The oxidation induced by copper is assumed to proceed by a following scheme,[18] where ROOH is either lipid hydroperoxide, *t*-butyl hydroperoxide or hydrogen peroxide:

Chain initiation:

$$ROOH + Cu^{II} \xrightarrow{k_1} RO_2\cdot + Cu^{I} + H^{+} \quad (1)$$

$$ROOH + Cu^{I} \xrightarrow{k_2} RO\cdot + Cu^{II} + {}^{-}OH \quad (2)$$

$$RO_2\cdot + LH \longrightarrow ROOH + L\cdot \quad (3)$$

$$RO\cdot + LH \longrightarrow ROH + L\cdot \quad (4)$$

$$L\cdot + O_2 \longrightarrow LO_2\cdot \quad (5)$$

Chain propagation:

$$LO_2\cdot + LH \xrightarrow{k_p} LO_2H + L\cdot \quad (6)$$

$$L\cdot + O_2 \longrightarrow LO_2\cdot \quad (7)$$

Chain termination:

$$2LO_2\cdot \xrightarrow{k_t} \text{non-radical products} \quad (8)$$

If we assume that the steady state holds and that the reaction 2 is much faster than the reaction 1, we obtain the equation 9,

$$-d[O_2]/dt = d[LOOH]/dt = k_p(k_1/2k_t)^{1/2} [ROOH]^{1/2}[Cu^{II}]^{1/2}[LH], \quad (9)$$

where LH and LOOH are PC and PC hydroperoxide and k_1, k_p, and k_t are the rate constants for reactions 1,6, and 8, respectively. Note that ROOH in Eq. 9 is the sum of lipid hydroperoxide, *t*-butyl hydroperoxide and hydrogen peroxide, if any. If BHT is present, the chain termination is assumed to proceed by a following scheme:

$$LO_2\cdot + IH \xrightarrow{k_{inh}} LOOH + I\cdot \quad (10)$$

$$LO_2\cdot + I\cdot \longrightarrow LOOI \quad (11)$$

The rate of oxidation during the induction period (R_{inh}) and the length of induction period (t_{inh}) are expressed by Eqs. 12 and 13, respectively,

$$R_{inh} = k_1k_p[LH][ROOH][Cu^{II}]/n\,k_{inh}[IH] \quad (12)$$

$$t_{inh} = n[IH]/k_1[ROOH][Cu^{II}] \quad (13)$$

where IH is an inhibitor and k_{inh} and the constant n are the rate constant for reaction 10 and the stoichiometric number of peroxyl radicals trapped by each inhibitor molecule. Table 1 shows the relation between the rate of formation of LOOH and that of consumption of oxygen, which shows good agreement between them. This result and Fig. 1 suggest that the steady state holds in the oxidations of phosphatidylcholine liposomes induced by copper(II) ion at least at the initial stage. The rate of chain initiation (R_i) was calculated from the data in Fig. 3(A) to be 0.505 nM s^{-1}, if we assume the number of n for BHT is 2.[22] From this rate of chain initiation and the rates of oxidation shown in Fig. 2(A), the kinetic chain length (the number of chain propagation which takes place per one initiation, that is, the rate of oxidation/R_i)[23] is calculated as 8.5 and 63 during and after the induction period respectively in the oxidation of soybean PC liposomes induced by 100 μM $CuCl_2$ and 3 mM *t*-butyl hydroperoxide.

Table 1. The Rates of Oxidation of Phosphatidylcholine Liposomes Induced by Copper(II) Ion or Azo Initiator

Initiation system	Rate of oxidation/nM s^{-1}	
	d[PCOOH]/d*t*	−d[O_2]/d*t*
Control	7.8	7.4
AAPH (2 mM)	23.5	30.5
AMVN (2 mM)	31.2	35.9
Cu(II) (100 μM) [A]	24.3	21.5
[A]+BOOH (1 mM)	30.8	26.8
[A]+BOOH (3 mM)	32.3	34.7
[A]+H_2O_2 (1 mM)	44.0	37.6

Note. The oxidation of 10.3 mM phosphatidylcholine liposome induced by several initiation systems were performed at 37°C. Control shows the spontaneous oxidation at 37°C.

As predicted from the Eqs. 9 and 12, it was found experimentally that the rate of oxidation was proportional to the first power of the substrate concentration and to the half power of copper(II) ion and hydroperoxide concentrations (Fig. 4) and that the rate of oxidation during the induction period was proportional to the reciprocal of BHT concentration (Fig. 3(B)).

The higher rate of oxidation induced by hydrogen peroxide than that induced by *t*-butyl hydroperoxide (Figs. 1 and 5) is probably due to the higher rate of decomposition by metal ion of hydrogen peroxide than that of *t*-butyl hydroperoxide (unpublished results from this laboratory), and not to the higher activity of hydroxyl radical than *t*-butoxyl radical. The formation of *t*-butoxyl radical has been confirmed by its spin trapping.[18]

The electric charge of the liposome surface must affect the efficiency of coordination or binding of metal ions to the liposome and consequently the rate of decomposition of PC hydroperoxides in the liposomes. The anionic DPPA is assumed to facilitate this coordination and this must be the reason why DPPA increases the rate of copper and iron induced oxidation of soybean PC liposomes in the absence of added *t*-butyl hydroperoxide or hydrogen peroxide (Fig. 5). The generation of active radicals in a very proximity of target molecules must also enhance the efficiency of chain initiation.[24] Under these circumstances, little effect of addition of hydroperoxide or hydrogen peroxide is observed (Fig. 5). When 10 times as much DPPA (about 1 mM) was added to soybean PC liposomes, substantially the same results were observed. In contrast to this observation, Viani et al.[25] found that DPPA exerted a strong inhibitory effect on Fe-induced peroxidation of arachidonic acid inserted into liposomal dipalmitoylphosphatidylcholine vesicles. The cationic octadecylamine added to the PC liposome

did not affect the rate of oxidation and, as predicted, the addition of *t*-butyl hydroperoxide and hydrogen peroxide increased the rate of oxidation (Fig. 5).

In conclusion, this study shows that, as observed in the oxidation of methyl linoleate micelles,[18] copper can act as a strong oxidant for phospholipid liposomal membranes and that the binding of copper to the target site greatly enhances its deleterious effect. Such site-specific binding of the metal ion may not be always a prerequisite, but, as shown above, the active oxygen radicals formed in the aqueous phase may also attack the target molecules in the liposomal membranes and induce their damage.

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