The Role of Fe^{3+} on Fe^{2+}-Dependent Lipid Peroxidation in Phospholipid Liposomes

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Fe^{2+}-dependent lipid peroxidation in phosphatidylcholine (PC) liposomes, assessed by thiobarbituric acid-reactive substances (TBARS) production, was stimulated in the presence of Fe^{3+} in a concentration-dependent manner. The rates of nitroblue tetrazolium (NBT) reduction and Fe^{2+} oxidation (Fe^{2+} disappearance and Fe^{3+} formation) were also enhanced by the addition of Fe^{3+} to the reaction mixture, and there is a good linear relationship between these parameters. These results suggest that the facilitation of reactive oxygen species (ROS) production *via* Fe^{2+} oxidation is closely related to the onset of the stimulatory effect of Fe^{3+} on Fe^{2+}-dependent lipid peroxidation. On the other hand, results using the liposomes containing various concentrations of endogenous lipid hydroperoxides (LOOH) indicated that endogenous LOOH is not directly involved in the onset of the Fe^{3+} stimulatory effect on Fe^{2+}-dependent TBARS production and ROS production. This hypothesis was further confirmed by the evidence that Fe^{2+}-dependent ROS production and Fe^{2+} oxidation of dipalmitoylphosphatidylcholine liposomes were also stimulated by the addition of Fe^{3+}. The results with several antioxidants and radical scavengers suggested that ROS related to Fe^{2+}-dependent lipid peroxidation and its stimulation by Fe^{3+} are ferrous–oxygen complexes rather than superoxide anion, hydrogen peroxide and hydroxyl radicals. Based on these results, we proposed a possible mechanism for the onset of the Fe^{3+} stimulation in Fe^{2+}-dependent lipid peroxidation.

Key words lipid peroxidation; ferric iron; reactive oxygen species; lipid hydroperoxide; phospholipid liposome

Lipid peroxidation in cell membrane phospholipids induced by reactive oxygen species (ROS) and/or free radicals leads to membrane damage and has been proposed to be a major mechanism for the onset of several pathological events *in vivo* including postischemic-reperfusion injury, cancer, Parkinson's disease, senile dementia and aging.[1)]

A number of studies have shown that transition metal ions catalyze many of the reactions involved in lipid peroxidation of the cellular membranes.[2—4)] Among them, Fe^{2+} is an effective catalyst of the production of a variety of potent oxidizing species which can initiate lipid peroxidation. Many investigators have reported that the rate and extent of lipid peroxidation depends on the simultaneous availability of Fe^{2+} and Fe^{3+}, and some iron-oxygen species, such as ferryl[5,6)] and perferryl.[7,8)] On the other hand, Aust and his co-workers demonstrated[9,10)] that a specific critical 1 : 1 ratio of Fe^{2+} to Fe^{3+} is required for maximal rate of Fe^{2+}-dependent lipid peroxidation. In these studies, they also proposed the formation of an Fe^{2+}–O_2–Fe^{3+} complex at early stage of the lipid peroixdation, although this compound can not be identified at present. These findings suggest that Fe^{3+} has an important role in the onset of Fe^{2+}-dependent lipid peroxidation, but the mechanism of iron-dependent lipid peroxidation has not yet been fully understood. In particular, the exact role of Fe^{3+} in Fe^{2+}-dependent lipid peroxidation is still unclear and controversial.

On the other hand, it has been well known[11)] that transition metal ions can also stimulate Fe^{2+}-dependent lipid peroxidation by the reductive cleavage of endogenous lipid hydroperoxide (LOOH) in membrane phospholipids to the corresponding alkoxyl radicals (LO·), and this is referred to as LOOH-dependent lipid peroxidation. Recently, Tadolini *et al.* have reported[12,13)] that LOOH-dependent lipid peroxidation, not LOOH-independent, is dependent on the Fe^{2+}/Fe^{3+} ratio, and that Fe^{3+} exerts a major influence on the control of the LOOH-dependent lipid peroxidation. These findings also strongly suggest that Fe^{3+} is an important key factor in the initiation and/or progress of Fe^{2+}-dependent lipid peroxidation, because membrane phospholipids generally contain small amounts of LOOH in the molecules. However, the molecular mechanism for the onset of the Fe^{3+} stimulatory effect in Fe^{2+}-dependent lipid peroxidation is still unclear. Therefore, it is important to verify this problem in order to understand the overall mechanism of iron-initiated lipid peroxidation. For this purpose, in the present study we particularly examined the effects of Fe^{3+} on Fe^{2+}-dependent lipid peroxidation in a liposomal system prepared from PC with different levels of endogenous LOOH in relation to Fe^{2+} oxidation and ROS production. In this report, we present evidence showing that the onset of the Fe^{3+} stimulatory effect in Fe^{2+}-dependent lipid peroxidation is mainly due to the facilitation of ROS production *via* Fe^{2+} oxidation and that endogenous LOOH is not directly involved in this Fe^{3+} stimulatory effect.

Experimental

Materials Phosphatidylcholine (PC, egg yolk), dipalmitoylphosphatidylcholine (DPPC), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 2-thiobarbituric acid, basophenanthroline disulfonic acid disodium salt (BPT), triphenylphosphine (TPP), 3(2)-*tert*-butyl-4-hydroxyanisole (BHA) and nitroblue tetrazolium (NBT) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Superoxide dismutase (SOD, bovine erythrocyte, 3500 U/mg protein) and catalase (bovine liver, 2900 U/mg protein) were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were of the highest quality commercially available.

Preparation of Phospholipid Liposomes A chloroform solution of PC (30 mg/ml) or DPPC (10 mg/ml) was evaporated to dryness under nitrogen gas. In preparation of TPP-treated liposomes, the phospholipid was mixed with various concentrations of TPP (10—500 μM) in chloroform as described in our previous paper,[14)] and then dried under nitrogen gas. After the residual solvent was completely removed under vacuum, the appropriate amounts of 10 mM Tris–HCl buffer (pH 7.4) were added, following by sonication with an Ultra Sonic Disrupter UR-200P (Tomy Seiko, Tokyo, Japan) until the dispersion became clear. This clear solution was centrifuged at 25000×g for 20 min, and then supernatant was used in the present study as

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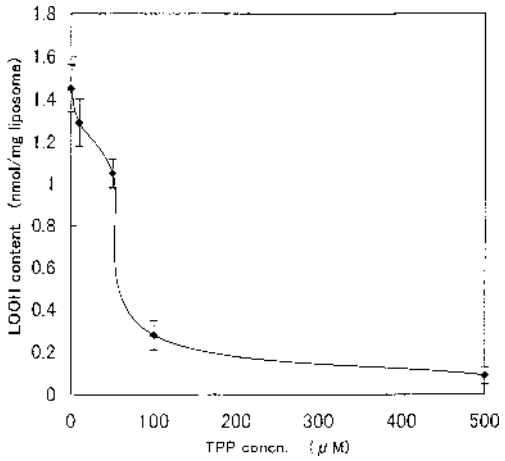

Fig. 1. Decrease in LOOH Content in PC Liposomes by Treatment with TPP

PC liposomes. The concentration of LOOH in the liposomes was determined by an iodometric method[15] using a calibration curve obtained with CumOOH as a standard, and the LOOH content in PC liposome was determined to be 1.45±0.11 nmol/mg phospholipid. The LOOH content in TPP-treated PC liposomes is presented in Fig. 1.

Lipid Peroxidation Phospholipid liposomes (0.2 mg/ml) were incubated with 100 μM $FeSO_4$ in 30 mM TES–NaOH buffer (pH 7.4) for 30 min at 37 °C in the absence and presence of $FeCl_3$, unless otherwise specified. In the scavenger experiments, PC liposomes were preincubated for 2 min at 37 °C in 30 mM TES–NaOH buffer (pH 7.4) with antioxidants or radical scavengers, then the reaction was started by the addition of $FeSO_4$ to the reaction mixture. The reaction was terminated by the addition of 1 mM EDTA and 5 mM BHA. The amount of the thiobarbituric acid-reactive substances (TBARS) was measured as previously reported,[16] and was represented as the absorbance at 530 nm (A_{530}).

Measurements of Ferrous and Ferric Ions The amount of Fe^{2+} was colorimetrically measured using BPT at 37 °C. The reaction was started by the addition of 100 μM $FeSO_4$ to the reaction mixture containing phsopholipid liposomes (0.2 mg/ml) and 30 mM TES–NaOH buffer (pH 7.4) in the absence and presence of $FeCl_3$. The total volume of the reaction mixture was 1 ml. The reaction was periodically terminated by the addition of 1.5 ml of 0.025% BPT (dissolved in 1.5 M sodium acetate). Then, 0.5 ml of 1 N HCl was added and the mixture was left for 10 min at room temperature. The amount of BPT-ferrous complex was calculated using the molar extinction coefficient of 22350 at 535 nm.[17] On the other hand, the formation of Fe^{3+} during the reaction was measured by recording the increase in the absorbance at 310 nm[18] after the addition of 100 μM $FeSO_4$ to the liposomes (0.2 mg/ml) in 30 mM TES–NaOH buffer (pH 7.4) in the absence and presence of $FeCl_3$, and the value was calculated by subtracting the value obtained with $FeCl_3$ alone from that obtained in the presence of both $FeSO_4$ and $FeCl_3$. The volume of the reaction mixture was 3 ml. In kinetic analysis of Fe^{2+} disappearance and Fe^{3+} formation, the absorbance at 535 and 310 nm was recorded until 1 min after the addition of $FeSO_4$, because these absorbance changes proceeded linearly up to 1 min in both cases.

Assay of ROS Generation The amount of ROS generated during the reaction was determined according to the NBT reduction method[19] as follows. Phospholipid liposomes (0.2 mg/ml) were incubated with 100 μM $FeSO_4$ in a reaction mixture containing 30 mM TES–NaOH buffer (pH 7.4) and 30 μM NBT in the absence and presence of $FeCl_3$ for 30 min at 37 °C. After termination of the reaction by the addition of 1 mM EDTA, the absorbance at 560 nm was measured, and the amount of reduced NBT formed was calculated using the molar extinction coefficient of 16500 $M^{-1}cm^{-1}$. The rate of NBT reduction was determined by recording the absorbance at 560 nm within 15 s after the addition of 100 μM $FeSO_4$.

Statistical Analysis Data were presented as the means±S.D. for three independent determinations, unless otherwise specified. To determine statistical significance between groups, the data were analyzed by an ANOVA Bonferroni's multiple *t*-test.

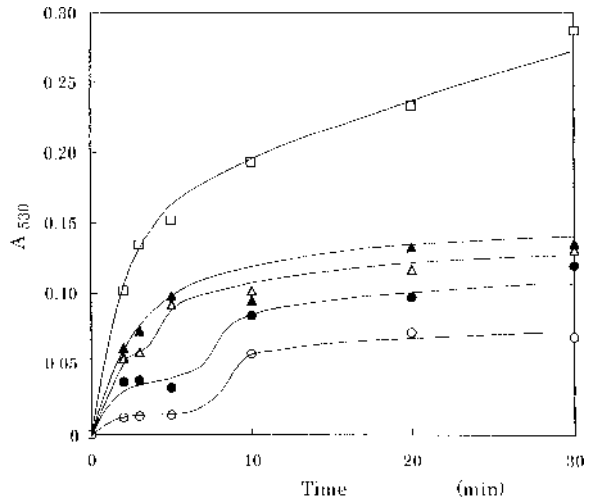

Fig. 2. Concentration Dependence of $FeCl_3$ on Fe^{2+}-Initiated TBARS Production

The incubation time was varied from 2 to 30 min. $FeCl_3$ concentration (μM): ○, 0; ●, 50; △, 100; ▲, 200; □, 400.

Results

Effects of Increasing Concentrations of $FeCl_3$ on Fe^{2+}-Dependent Lipid Peroxidation The time courses of Fe^{2+}-dependent lipid peroxidation in PC liposomes in the presence of various concentrations of $FeCl_3$ at pH 7.4 are presented in Fig. 2.

Incubation of PC liposomes with 100 μM $FeSO_4$ resulted in an increase in TBARS production after a short lag phase (about 5—7 min). This indicates that lipid peroxidation in the liposomes induced by Fe^{2+} is initiated after a few minutes of the addition of the salt, as described previously.[20,21] The addition of $FeCl_3$ to the system resulted in stimulation of TBARS production and shortening of the lag phase in a concentration-dependent manner. When the concentration of $FeCl_3$ was increased up to 200 μM, the lag phase in the TBARS production completely disappeared. On the other hand, $FeCl_3$ alone (400 μM) did not induce an appreciable production of TBARS, even after 30-min incubation (A_{530}= 0.015±0.005).

Effects of Fe^{3+} on Fe^{2+}-Dependent ROS Production When PC liposomes were incubated with 100 μM $FeSO_4$ for 30 min at 37 °C in the presence of 100 μM $FeCl_3$, the amount of reduced NBT increased from 65±3 to 177±2 nmol/mg liposome. In contrast, treatment of the liposomes with $FeCl_3$ alone under the same conditions did not lead to an appreciable increase of the formation of reduced NBT (3±0.2 nmol/mg liposome/30 min at 100 μM $FeCl_3$), indicating that Fe^{3+} itself has no ability to induce ROS production at pH 7.4. In addition, NBT reduction by the addition of 100 μM $FeSO_4$ was not observed in the system without the liposomes (A_{560}= 0.002±0.001), suggesting that ROS production associated with Fe^{2+} treatment proceeds on the membrane surface.

Figure 3 shows Fe^{2+}-induced NBT reduction as a function of the $FeCl_3$ concentration. As shown in the figure, the stimulation of Fe^{2+}-dependent NBT reduction by Fe^{3+} was dependent on the concentration of $FeCl_3$ in the reaction mixture, and there is a good liner correlation between the k_{NBT} value and the concentration of $FeCl_3$ tested (10—70 μM). This indicates that Fe^{2+}-dependent ROS production proceeds linearly,

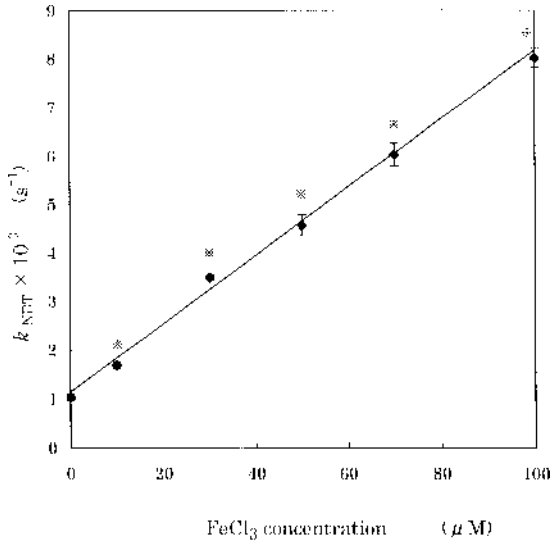

Fig. 3. Effects of Increasing Concentrations of $FeCl_3$ on the Rate of Reduced NBT Production

The concentration of $FeCl_3$ was varied from 10 to 100 μM. $*p<0.001$ *vs.* the system without $FeCl_3$. The k_{NBT} value represents the pseudo-first-order rate constant of NBT reduction.

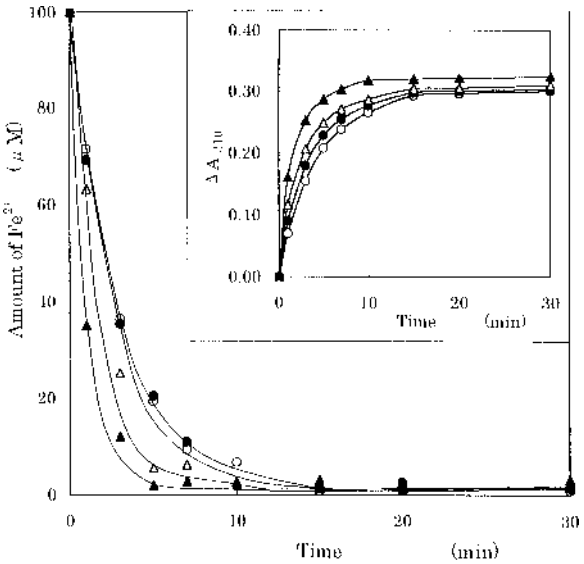

Fig. 4. Time Courses of Fe^{2+} Disappearance and Fe^{3+} Formation

$FeCl_3$ concentration (μM): ○, 0; ●, 10; △, 50; ▲, 100. The inset figure shows the time courses of Fe^{3+} formation in the presence of various concentrations of $FeCl_3$. The values (ΔA_{310}) are represented as the difference in A_{310} values of the systems with Fe^{2+}/Fe^{3+} and the system with Fe^{3+} alone in each concentration of Fe^{3+}.

depending on the concentration of $FeCl_3$.

Effect of Fe^{3+} on Fe^{2+} Oxidation The time courses of Fe^{2+} disappearance and Fe^{3+} formation after the addition of 100 μM $FeSO_4$ to PC liposomes in the presence of various concentrations of $FeCl_3$ are presented in Fig. 4.

The addition of $FeCl_3$ to the system resulted in the facilitation of Fe^{2+} disappearance and Fe^{3+} formation (ΔA_{310} value in the inset figure), depending on the salt concentration. In addition, it was found that there is a good linear relationship between the rates of Fe^{2+} disappearance and Fe^{3+} formation in the salt concentration range tested (Fig. 5).

Effects of Several Antioxidants or Radical Scavengers Next, we examined the effects of several antioxidants and

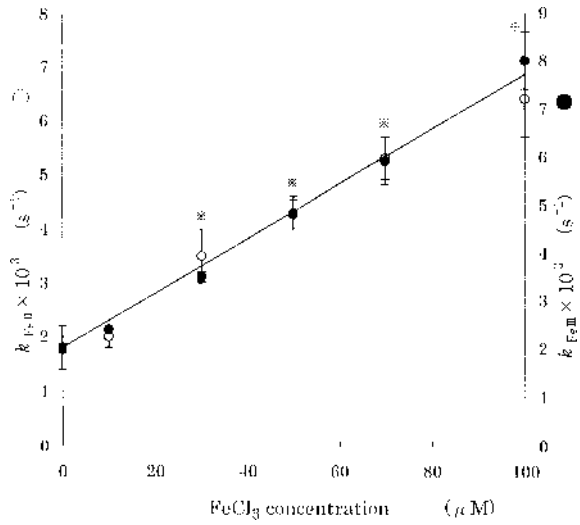

Fig. 5. Effect of $FeCl_3$ on the Rates of Fe^{2+} Disappearance and Fe^{3+} Formation

The concentration of $FeCl_3$ was varied from 10 to 100 μM. Symbols: ○, Fe^{2+} disappearance; ●, Fe^{3+} formation. The k_{FeII} and k_{FeIII} values represent the pseudo-first-order rate constants of Fe^{2+} disappearance and Fe^{3+} formation, respectively. $*p<0.001$ *vs.* the system without $FeCl_3$.

Table 1. Effect of Antioxidants and Radical Scavengers on Fe^{2+}-Dependent TBARS Production in the Absence and Presence of 100 μM $FeCl_3$

Scavenger	Concn.	A_{530}/30 min	
		Fe^{2+}	Fe^{2+}/Fe^{3+}
No addition	—	0.074±0.004	0.091±0.003
SOD	100 units/ml	0.065±0.003	0.085±0.001
Catalase	250 units/ml	0.067±0.001	0.097±0.004
Sodium benzoate	10 mM	0.073±0.003	0.094±0.002
Mannitol	50 mM	0.060±0.001	0.077±0.001
Dimethyl thiourea	1 mM	0.053±0.002	0.073±0.004
EDTA	1 mM	0.004±0.003	0.005±0.003

radical scavengers on Fe^{2+}-dependent TBARS production in the systems with and without Fe^{3+} in order to investigate ROS relating to the onset of the Fe^{3+} stimulatory effect.

As shown in Table 1, in both the systems, SOD, catalase and ·OH scavengers (sodium benzoate, mannitol and dimethylthiourea) did not inhibit the TBARS production, even at high concentrations. On the other hand, EDTA almost completely inhibited Fe^{2+}-dependent TBARS production, regardless of the absence or presence of Fe^{3+}. These results suggest that superoxide anion, H_2O_2 and ·OH are not directly related to the initiation of Fe^{2+}-dependent lipid peroxidation and its stimulation by Fe^{3+}.

Effects of Lipid Hydroperoxides on Fe^{3+} Stimulatory Effect As it is well known,[11] LOOH in membrane phospholipids are involved in the stimulation of metal-mediated lipid peroxidation to form the alkoxyl radicals (LO·) by the reductive cleavage of hydroperoxides. Therefore, we examined whether the endogenous LOOH in PC liposomes are directly involved in the onset of the stimulatory effect of Fe^{3+} in Fe^{2+}-dependent lipid peroxidation. In this experiment, we used TPP-treated PC liposomes, because TPP removes LOOH in the membrane phospholipids by reduction to the corresponding alcohols (Fig. 1).[14,22]

Table 2. Effects of LOOH on Fe^{2+}-Dependent TBARS and ROS Production, and Fe^{2+} Disappearance in the Absence and Presence of Fe^{3+}

LOOH content (nmol/mg liposome)	TBARS (A_{530}/30 min)		Amount of reduced NBT (nmol/mg/min liposome)		Amount of Fe^{2+} (μM/min)	
	Fe^{2+}	Fe^{2+}/Fe^{3+}	Fe^{2+}	Fe^{2+}/Fe^{3+}	Fe^{2+}	Fe^{2+}/Fe^{3+}
0.28	0.061±0.003	0.083±0.006	39.5±1.1	178.1±2.0	60.7±0.3	32.8±1.0
1.05	0.067±0.003	0.083±0.006	42.7±1.3	163.1±3.4	60.9±0.5	31.8±0.6
1.29	0.060±0.005	0.080±0.004	42.9±1.3	161.0±4.5	61.1±0.7	31.0±0.4
1.45	0.063±0.002	0.081±0.003	43.9±1.1	174.5±3.4	62.7±0.7	32.5±0.7

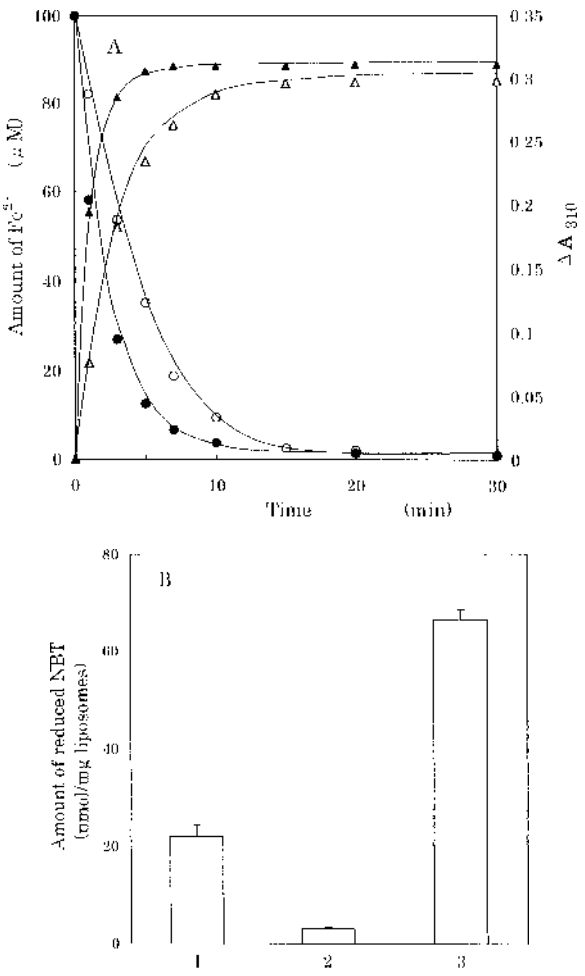

Fig. 6. Effect of $FeCl_3$ on Fe^{2+} Oxidation and Fe^{2+}-Dependent ROS Production in DPPC Liposomes

(A) Time courses of Fe^{2+} disappearance and Fe^{3+} formation. The concentration of $FeCl_3$ was 100 μM. Symbols: ○, ●, Fe^{2+} disappearance; △, ▲, Fe^{3+} formation. Open and closed symbols express systems with Fe^{2+} alone and Fe^{2+}/Fe^{3+}, respectively. Other experimental conditions are the same as those described in the legend to Fig. 4, (B) ROS production. 1, 100 μM $FeSO_4$ alone; 2, 100 μM $FeCl_3$ alone; 3, 100 μM $FeSO_4$ plus 100 μM $FeCl_3$.

As shown in Table 2, the extent of Fe^{2+}-dependent TBARS production in PC liposomes with various concentrations of LOOH in systems both with and without $FeCl_3$ were almost the same in the concentration range of LOOH from 1.45 to 0.28 nmol/mg phospholipid. Furthermore, LOOH variation in the liposomes did also not influence Fe^{2+} disappearance or ROS production regardless of the absence and presence of Fe^{3+}.

NBT Reduction and Fe^{2+} Oxidation in DPPC Liposomes Figure 6 shows the effect of $FeCl_3$ on Fe^{2+} oxidation and Fe^{2+}-dependent production of reduced NBT in liposomes prepared from DPPC which lacks unsaturated fatty acids in the molecule.

As shown in the figure, A and B, the addition of $FeCl_3$ to the liposomes resulted in the facilitation of Fe^{2+} disappearance and Fe^{3+} formation. In addition, Fe^{2+}-dependent ROS production in the liposomes was also markedly enhanced in the presence of Fe^{3+}.

Discussion

Fe^{2+}-dependent TBARS production in PC liposomes was markedly enhanced with the shortening of the lag phase in the presence of Fe^{3+} (Fig. 2), suggesting that lipid peroxidation is initiated after several minutes of the addition of Fe^{2+}. As shown in Figs. 4 and 5, the rates of Fe^{2+} disappearance and Fe^{3+} formation were accelerated by the addition of $FeCl_3$ to the system. And, there is a good linear relationship between these parameters in the concentration range of $FeCl_3$ tested, suggesting that Fe^{2+} oxidation is an important factor for the onset of the Fe^{3+} stimulatory effect, including the shortening of the lag phase at the early stage of lipid peroxidation.

Fe^{2+}-dependent NBT reduction was linearly accelerated depending on the concentration of $FeCl_3$ (Fig. 3), indicating that Fe^{2+}-dependent ROS production is stimulated under the co-presence of $FeCl_3$. Furthermore, ROS production in the presence of $FeCl_3$ proceeded depending on the rate of Fe^{2+} oxidation (Fig. 7). From these results, it is suggested that Fe^{2+}-dependent ROS production is closely related to Fe^{2+} oxidation at the early stage of lipid peroxidation, and that Fe^{3+} facilitates the ROS production *via* the Fe^{2+} oxidation process in Fe^{2+}-dependent lipid peroxidation.

The results with several antioxidants and radical scavengers suggest that $O_2^{\cdot-}$, H_2O_2 and $\cdot$OH are not directly involved in the initiation of Fe^{2+}-dependent lipid peroxidation, because SOD, catalase and $\cdot$OH scavengers (sodium benzoate, mannitol and dimethylthiourea) did not inhibit Fe^{2+}-dependent TBARS production (Table 1). No response of Fe^{2+}-dependent TBARS production against these radical scavengers was observed in the presence of Fe^{3+}. However, in contrast, EDTA completely inhibited Fe^{2+}-dependent TBARS production, regardless of the absence or presence of Fe^{3+}. In addition, the TBARS production in PC liposomes by Fe^{2+} addition was not observed under N_2 gas flow, and the A_{530} values of the systems without and with N_2 gas flow were 0.075±0.003 and 0.010±0.002, respectively. From these findings, we speculated that iron-oxygen complexes may act as the initiator of Fe^{2+}-dependent lipid peroxidation, and that Fe^{3+} promotes the production of iron-oxygen complexes such as ferryl ion[5,6] and perferryl ion,[7,8] although these

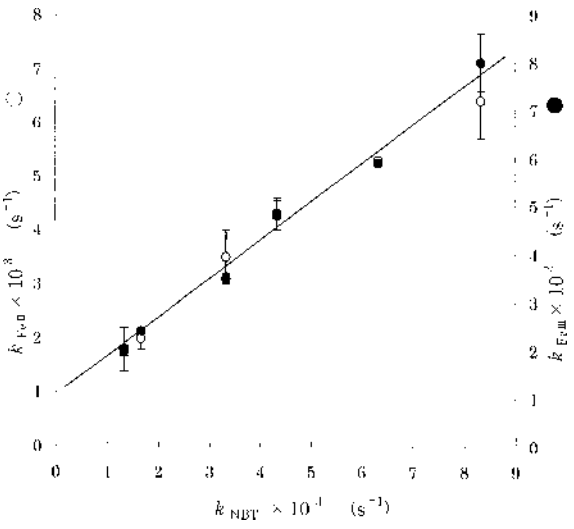

Fig. 7. Relationship between the Rates of Fe^{2+} Oxidation and NBT Reduction in the Presence of Various Concentrations of $FeCl_3$

Symbols: ○, Fe^{2+} disappearance; ●, Fe^{3+} formation. The data were obtained from Figs. 3 and 5.

complexes have not been isolated at present. Goddard and Sweeney[23] have also suggested that perferryl ion, formed during Fe^{2+} autooxidation, is the likely initiator of Fe^{2+}-dependent lipid peroxidation. In fact, our results indicate that there is a good linear relationship between the rates of NBT reduction and Fe^{2+} oxidation in the presence of various concentrations of $FeCl_3$ (Fig. 7).

Next, we used PC liposomes with different levels of LOOH to examine whether the endogenous LOOH in the liposomal membrane is directly involved in the Fe^{3+} stimulation mechanism of Fe^{2+}-dependent lipid peroxidation, because LO· and LOO· radicals are generated from LOOH by interaction with Fe^{2+} [11] and Fe^{3+},[24] respectively. Recently, Tadolini *et al.* have also reported[12,13] that Fe^{3+} plays a major role in the control of LOOH-dependent lipid peroxidation. However, the present results showed that Fe^{2+}-dependent TBARS production, regardless of the absence or presence of Fe^{3+}, is almost independent of the LOOH level (1.45 to 0.28 nmol LOOH/mg phospholipid) in the liposomes (Table 2). Similar phenomena were also observed in Fe^{2+} disappearance and ROS production. From these results, it is suggested that endogenous LOOH is not directly related to the onset of the Fe^{3+} stimulation effect in Fe^{2+}-dependent lipid peroxidation. This hypothesis was further confirmed in that Fe^{2+} disappearance and Fe^{3+} formation (Fig. 6A), and Fe^{2+}-dependent ROS production (Fig. 6B) in DPPC liposomes were also markedly enhanced by the addition of Fe^{3+}. Furthermore, these results suggest that Fe^{3+} facilitates ROS production *via* Fe^{2+} oxidation, because the stimulation of Fe^{2+} oxidation and Fe^{2+}-dependent ROS production by Fe^{3+} was not observed in the absence of DPPC. In addition, our present observation is different from that reported by Tadolini *et al.*,[12,13] who observed that Fe^{3+} controls LOOH-dependent lipid peroxidation. The exact reason for the difference in both observations is unclear at present.

Combining these findings, we concluded that the stimulatory effect of Fe^{3+} in Fe^{2+}-dependent lipid peroxidation is mainly due to the acceleration of ROS production, probably an iron-oxygen complex, *via* facilitation of the Fe^{2+} oxidation process at an early stage of lipid peroxidation, and that LOOH in the liposomal membrane is not directly involved in this mechanism. In addition, the present results give us an important clue for the mechanism of the Fe^{3+} stimulation and/or the role of Fe^{3+} in the iron-initiated lipid peroxidation.

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