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Physicochemical Damage to Liposomal Membrane Induced by Iron- or Copper-Mediated Upid Peroxidation

Daxian Zhang[*] Tatsuji Yasuda[†]

Yingyan Yu[‡] Shigeru Okada[**]

[*]Okayama University,

[†]Okayama University,

[‡]Hospital of He Bei Medical College,

[**]Okayama University,

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Daxian Zhang, Tatsuji Yasuda, Yingyan Yu, and Shigeru Okada

Abstract

A carboxyfluorescein (CF)-enveloping soybean phosphatidylcholine liposome was used as a model of physicochemical damage of biomembranes. The liposomes were exposed to a metal-chelate complex [2 mM of ferric nitrilotriacetate (FeNTA) or cupric nitrilotriacetate (CuNTA)] plus a reductant (2 mM of ascorbate or various concentrations of reduced glutathione), and CF release from damaged liposomal membranes and the generation of thiobarbituric acid-reactive substances (TBARS) were measured. In the presence of a reducing agent, both FeNTA and CuNTA stimulated markedly CF release and an increase in the TBARS level, while in the absence of a reducing agent both of the chelate complexes showed little CF release and TBARS. The effects of H2O2 addition to the reaction system containing liposome with FeNTA or CuNTA plus ascorbate were also examined. The CF release was slightly increased by the addition of a smaller dose (0.5 mM) of H2O2 and it was inhibited by 8 mM of H2O2. A similar result was obtained in the TBARS test. These results suggest that FeNTA- or CuNTA-mediated lipid peroxidation can damage liposomal membranes physicochemically, and the redox reaction of the chelated metal itself is more important than a Fenton-type reaction in the process.

KEYWORDS: lipid peroxidation, liposome, metal-chelate complex, physicochemical damage

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Physicochemical Damage to Liposomal Membrane Induced by Iron- or Copper-Mediated Lipid Peroxidation

DAXIAN ZHANG*, TATSUJI YASUDA[a], YINGYAN YU[b] AND SHIGERU OKADA

Department of Pathology and [a]Institute of Cellular and Molecular Biology, Okayama University Medical School, Okayama 700, Japan and [b]Department of Pathology, 2nd Affiliated Hospital of He Bei Medical College. He Bei, the People's Republic of China

A carboxyfluorescein (CF)-enveloping soybean phosphatidylcholine liposome was used as a model of physicochemical damage of biomembranes. The liposomes were exposed to a metal-chelate complex [2 mM of ferric nitrilotriacetate (FeNTA) or cupric nitrilotriacetate (CuNTA)] plus a reductant (2 mM of ascorbate or various concentrations of reduced glutathione), and CF release from damaged liposomal membranes and the generation of thiobarbituric acid-reactive substances (TBARS) were measured. In the presence of a reducing agent, both FeNTA and CuNTA stimulated markedly CF release and an increase in the TBARS level, while in the absence of a reducing agent both of the chelate complexes showed little CF release and TBARS. The effects of H_2O_2 addition to the reaction system containing liposome with FeNTA or CuNTA plus ascorbate were also examined. The CF release was slightly increased by the addition of a smaller dose (0.5 mM) of H_2O_2 and it was inhibited by 8 mM of H_2O_2. A similar result was obtained in the TBARS test. These results suggest that FeNTA- or CuNTA-medited lipid peroxidation can damage liposomal membranes physicochemically, and the redox reaction of the chelated metal itself is more important than a Fenton-type reaction in the process.

Key words: lipid peroxidation, liposome, metal-chelate complex, physicochemical damage

There is increasing evidence which suggests that the transition metals directly or indirectly play an important role in pathological and toxicological events involving free radical reactions. They efficiently catalyze redox reactions for the vast majority of biomolecules, especially for unsaturated fatty acids (1-3). Since iron and copper are the two most predominant transition metals *in vivo*, their importance in free radical-induced tissue injury have been emphasized (2, 4).

It is known that lipid peroxidation *in vitro* is dependent on the free radical initiator and also the type of lipid, lipid composition and the structure of the model lipid membrane (5-8). In general, the lipid bilayer structure of the liposome is considered to be a better model than the lipid micelle or emulsion for studying injury of the cell membrane. Physicochemical changes such as penetrance or integrity of membranes by free radical attack seem to reflect the process of cell injury more directly than biochemical parameters (9, 10).

We used a carboxyfluorescein (CF)-enveloping liposome as a model of physicochemical biomembrane damage. The degree of the model membrane damage is dependent on the amount of lipid peroxidation (9). The liposomes were incubated with ferric nitrilotriacetate (FeNTA) or cupric nitrilotriacetate (CuNTA) and a reductant. These complexes are known to function as free radical initiators *in vivo* and *in vitro* (11, 12). Hydrogen peroxide, which generates free radicals in the presence of transition metals via the Fenton reaction was also examined in this reaction system. We observed CF release from liposomes and a parallel increment in the thiobarbituric acid reactive substances (TBARS). The comparison between biochemical and physicochemical damage of liposomes caused by FeNTA- or CuNTA-mediated lipid peroxidation, and the possible mechanism responsible for this oxidative damage are described in this report.

* To whom correspondence should be addressed.

Materials and Methods

Chemicals. Soybean phosphatidylcholine (SPC) was furnished by Nippon Shoji Co. (Osaka, Japan). Cholesterol and dipalmitoylphosphatidic acid (DPPA) were from Sigma Chemical Co. (St. Louis, MO, USA) and carboxyfluorescein (CF) was from Eastman Kodak Co. (Rochester, NY, USA). Desferrioxamine mesylate was obtained from Ciba-Geigy Co. (Basel, Switzerland). Catalase (from bovine liver, 5,000-9,000 units/mg) and all other reagents were purchased from Wako Pure Chemicals Co. (Osaka, Japan).

Liposome preparation. One micromole each of SPC and cholesterol, and 0.1 μmol of DPPA were dissolved in 1 ml of a mixture of chloroform and methanol (1:1 v/v). The organic solvent was removed by evacuation using a rotary vacuum evaporator to obtain a thin film on the flask wall. After dripping 200 μl of distilled water into the flask, the film was slowly shaken off to obtain a white, milky liposome suspension. The suspension was sonicated, and unilamellar and/or oligolamellar liposomal membranes were obtained. After freeze-drying, 100 μl of 0.05 M CF dissolved in water was added to the liposomal membranes, placed aside for more than 2 h, shaken gently, and washed with 0.05 M Hepes-saline buffer (HSB, pH 7.3) three times followed by centrifugation at 15,000 $\times g$ for 20 min. The resulting pellet was resuspended in 1 ml of HSB for further analysis.

Experimental conditions. FeNTA or CuNTA with a reductant were used as the radical initiator. The reductant was sodium L-ascorbate and the reduced form of glutathione (GSH). FeNTA was prepared by mixing the ferric nitrate solution with a nitrilotriacetic acid trisodium salt solution (1:4 molar ratio). The pH was adjusted with $NaHCO_3$ to 7.3 (11). CuNTA was prepared by using cupric sulfate in place of ferric nitrate in the preparation of FeNTA (12). FeNTA, CuNTA, reductants and H_2O_2 were adjusted to the desired concentrations with HSB. The various concentrations of H_2O_2 were added to the reaction system containing FeNTA or CuNTA plus ascorbate which generates the Fe^{2+} or Cu^{+} to produce a Fenton-type reaction. The HSB was used in place of metal-chelate complex as a negative control.

Analysis of liposome disruption. Fifty microliters of the liposome suspension containing 0.05 μM of SPC was added to 2 ml of radical initiator or control solution stirring at 20 ℃. The CF release from the liposomes was monitored by fluorospectrophotometry (Hitachi-F3010, Tokyo) at an excitation wavelength of 490 nm and emission wavelength of 520 nm. The reaction was stopped at 60 min by adding 50 μl of 5 % Triton X-100 to obtain 100 % release of CF. The specific marker release of each sample was calculated according to Yasuda's method (13): % specific marker release = (experimental release − spontaneous release) × 100/(total release − spontaneous release). The measurements were repeated at least three times.

TBARS determination. TBARS in the liposomes exposed to the radical initiator were measured by the method of Buege and Aust (14) with minor modifications. Briefly, 25 μl of the liposome suspension was added to 975 μl of radical initiator solution which contained 2 mM FeNTA or 2 mM CuNTA, with or without the reductant or H_2O_2, and incubated at 37 ℃ for the desired period. To terminate the incubation, 20 μl of 100 mM desferrioxamine mesylate was added to the iron-containing sample and 20 μl of 100 mM EDTA was added to the copper-containing sample. Twenty microliters of catalase (1 mg/1 ml) was added to each sample and incubated at 25 ℃ for 3 min. Two milliliters of solution containing 0.375 % thiobarbituric acid, 15 % trichloroacetic acid and 0.25 N HCl was added to each sample. The mixture was incubated at 100 ℃ for 10 min, cooled with ice, and the absorbance of chromogen was measured at 532 nm.

Results

Liposome disruption. Specific CF release from liposomes was measured after incubation with FeNTA or CuNTA plus ascorbate. The former released more CF than the latter at a concentration of 2 mM (Figs. 1 and 2). The dependence of FeNTA-induced CF release on the reductant dose was analyzed by incubating liposomes with 2 mM FeNTA and various concentrations of GSH for 60 min. CF release from liposomes was not observed when various concentrations of NaCl, which has no reducing capacity, was used in place of GSH (Fig. 3).

The effect of H_2O_2 on CF release was studied by adding different concentrations of H_2O_2 to a FeNTA or CuNTA plus ascorbate system. When 8 mM of H_2O_2 was added to the reaction system, a marked inhibition of both initiation and propagation of the chain reaction was

seen. However, these effects were not seen when 0.5 mM H_2O_2 was added (Fig. 4). Similar results were also observed when CuNTA was used in place of FeNTA (data not shown). The pH was maintained constant throughout these experiments.

TBARS. The increase in TBARS was associated with CF release in the time course of incubation. A high level of TBARS was generated in liposomes incubated

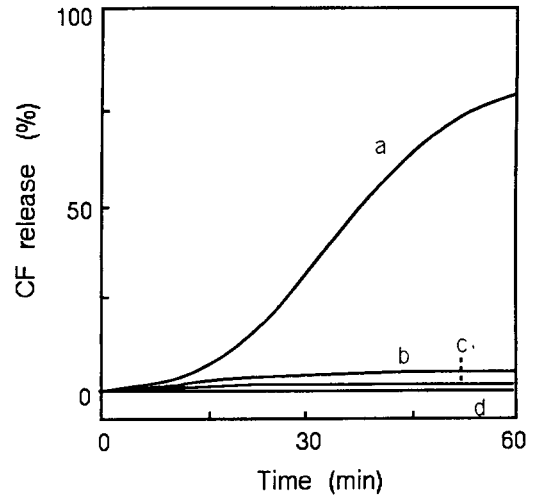

Fig. 1 CF release from liposomes damaged by FeNTA and ascorbate. The liposomes were incubated with (a) 2 mM FeNTA and 2 mM ascorbate, (b) 2 mM FeNTA, (c) 2 mM ascorbate, and (d) liposomes alone.

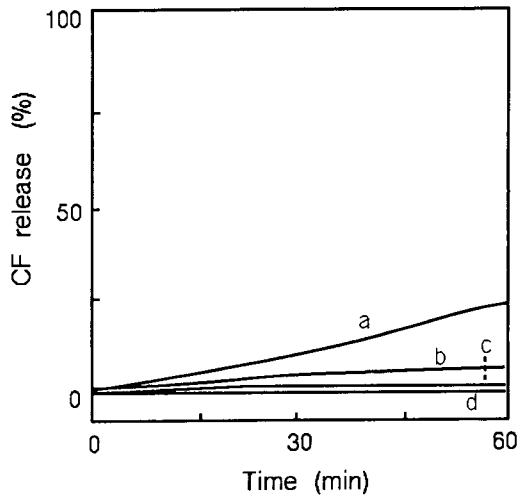

Fig. 2 CF release from liposomes damaged by CuNTA and ascorbate. The liposomes were incubated with (a) 2 mM CuNTA and 2 mM ascorbate, (b) 2 mM CuNTA, (c) 2 mM ascorbate, and (d) liposomes alone.

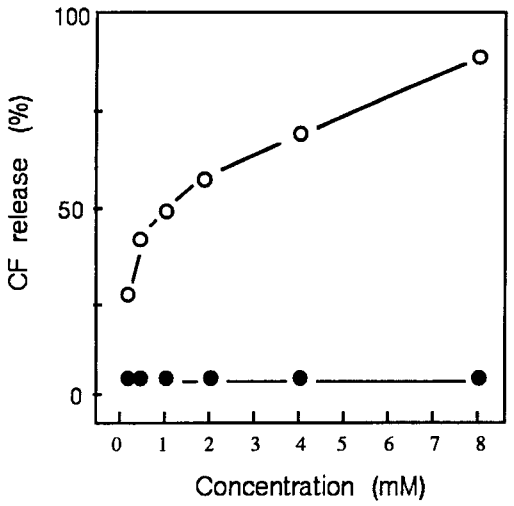

Fig. 3 FeNTA-induced CF release from damaged liposomes which was dependent on the dose of the reductant. Liposomes were incubated with (○) 2 mM FeNT and various concentrations of reduced glutathione; with (●) 2 mM FeNTA and various concentrations of NaCl as a control.

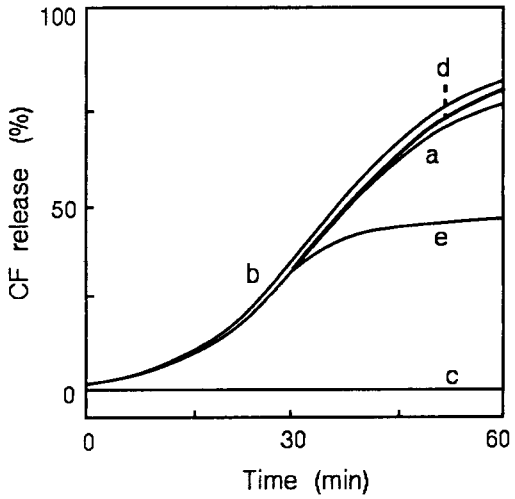

Fig. 4 Effects of the addition of H_2O_2 on CF release from liposomes damaged by FeNTA and ascorbate-catalyzed lipid peroxidation. Liposomes were incubated with 2 mM FeNTA and 2 mM ascorbate. (a) no H_2O_2 added; (b) 0.5 mM H_2O_2 was added at time 0 min; (c) 8 mM H_2O_2 was added at time 0 min; (d) 0.5 mM H_2O_2 was added at time 30 min; and (e) 8 mM H_2O_2 was added at time 30 min.

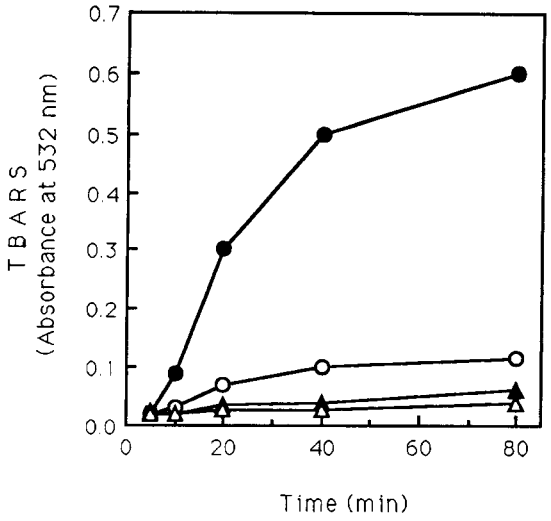

Fig. 5 Thiobarbitaric acid-reactive substance (TBARS) generated in liposomes caused by FeNTA and ascarbate-catalyzed lipid peroidation. Liposomes incubated with (●) 2mM FeNTA and 2mM ascorbate. (○) 2mM FeNTA, (▲) 2mM ascorbate, and (△) liposomes alone.

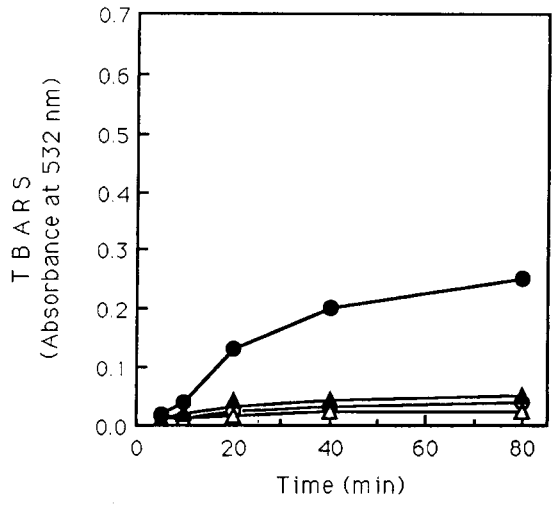

Fig. 6 TBARS generated in liposomes by CuNTA and ascorbate-catalyzed lipid peroxidation. Liposomes incubated with (●) 2mM CuNTA and 2mM ascorbate, (○) 2mM CuNTA, (▲) 2mM ascorbate, and (△) liposomes alone.

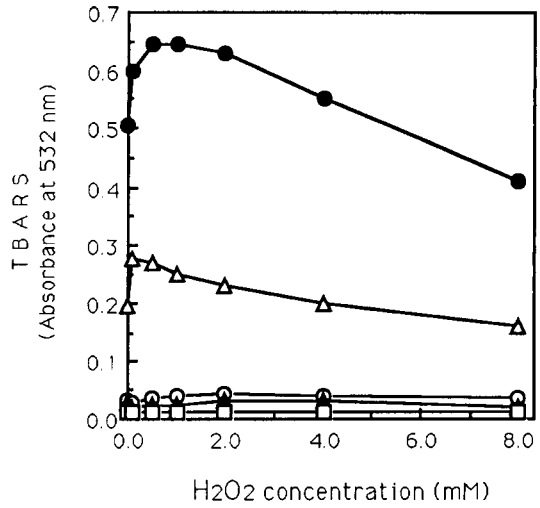

Fig. 7 The effects of H_2O_2 addition on TBARS generation from liposomes by FeNTA- or CuNTA-induced lipid peroxidation. Liposomes were incubated for 60min in the presence of various concentrations of H_2O_2 with (●) 2mM FeNTA and 2mM ascorbate, (○) 2mM FeNTA, (△) 2mM CuNTA and 2mM ascorbate, (▲) 2mM CuNTA, and (□) liposomes alone.

with FeNTA plus ascorbate or CuNTA plus ascorbate, but TBARS were not markedly generated in liposomes with metal-chelate complex alone or ascorbate alone (Figs. 5 and 6). The effects of H_2O_2 addition on TBARS production are shown in Fig. 7. When higher concentrations of H_2O_2 were added to the reaction system containing liposomes with FeNTA or CuNTA plus ascorbate, TBARS production was inhibited, although a stimulative effect was seen when low concentrations of H_2O_2 were added (Fig. 7). The data presented are the mean value of three or more experiments, and the intra-assay variation was less than 3%.

Discussion

Iron-catalyzed lipid peroxidation is generally thought to be mediated by hydroxyl radicals via a Fenton reaction catalyzed by ferrous ions (15). Ferric ions also can decompose hydrogen peroxide to generate hydroperoxyl radicals, but this reaction is considerably slower than a Fenton reaction (16, 17). Recently, it was hypothesized that lipid peroxidation may be induced by a cycle of iron oxidation and reduction under existing oxidants as O_2, $O_2^{\cdot-}$ and H_2O_2 or by a Fe(II)-Fe(III)-oxidant complex (2, 18). In addition, copper is generally thought to behave analogously to iron in terms of redox reactions.

In the present experimental system, both TBARS (a

biochemical indicator of membrane damage) and CF release (a physicochemical one) were increased when the liposomes were exposed to the FeNTA or CuNTA complex in the presence of a reductant (Figs. 1, 2, 4-6). The physicochemical membrane damage was also observed when GSH was used as a reductant, and CF release was dependent upon the GSH dose in the presence of FeNTA (Fig. 3). This fact suggests that the physicochemical damage of membrane by metal-chelate complex-induced lipid peroxidation was related to the reducing capacity of the system. A similar result has been reported by Hamazaki (11) who showed that elevation of TBARS by FeNTA-induced lipid peroxidation in linoleate micelles was dependent on the reductant. Similar effects of FeNTA or CuNTA have also been seen in *in vivo* experiments (19-21). These results fit well with the clinical observation that administration of ascorbic acid to patients with iron-overload has proved to be toxic unless given in conjunction with desferrioxamine mesylate (22).

In the present study, high concentrations of H_2O_2 inhibited both the initiation and propagation of the radical chain reaction in physicochemical membrane damage caused by metal chelate complex-mediated lipid peroxidation (Fig. 4). This indicates that membrane damage by a Fenton-type reaction was difficult to demonstrate using the present system, although we were unable to detect any membrane damage with chelated ferrous ion because it is oxidized quickly in an aqueous solution at physiological pH (11). It has been reported that the presence of excess H_2O_2 may serve as a scavenger for $^{\cdot}OH$ according to the following reaction: $H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + HO_2$ (23). TBARS has been shown to be inhibited by the presence of excess H_2O_2 in $FeCl_3$-induced lipid peroxidation (24) and also adriamycin-Fe^{+}_{3}-induced degradation of deoxyribose (25). This suggests that the mechanism in these reports may be the same as our finding.

Our results described above indicate that FeNTA- and CuNTA-mediated lipid peroxidation can certainly cause physicochemical damage to the lipid bilayer of membranes, and that a redox reaction between the chelated metal and reductant seems to play a more important role than Fenton-type reactions in the process.

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