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Biochimica et Biophysica Acta 1668 (2005) 145–155

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Antioxidant effect of bovine serum albumin on membrane lipid peroxidation induced by iron chelate and superoxide

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Received 14 January 2004; accepted 2 December 2004
Available online 24 December 2004

Abstract

Albumin is supposed to be the major antioxidant circulating in blood. This study examined the prevention of membrane lipid peroxidation by bovine serum albumin (BSA). Lipid peroxidation was induced by the exposing of enzymatically generated superoxide radicals to egg yolk phosphatidylcholine liposomes incorporating lipids with different charges in the presence of chelated iron catalysts. We used three kinds of Fe^{3+}-chelates, which initiated reactions that were dependent on membrane charge: Fe^{3+}-EDTA and Fe^{3+}-EGTA catalyzed peroxidation in positively and negatively charged liposomes, respectively, and Fe^{3+}-NTA, a renal carcinogen, catalyzed the reaction in liposomes of either charge. Fe^{3+}-chelates initiated more lipid peroxidation in liposomes with increased zeta potentials, followed by an increase of their availability for the initiation of the reaction at the membrane surface. BSA inhibits lipid peroxidation by preventing the interaction of iron chelate with membranes, followed by a decrease of its availability in a charge-dependent manner depending on the iron-chelate concentration: one is accompanied and the other is unaccompanied by a change in the membrane charge. The inhibitory effect of BSA in the former at high concentrations of iron chelate would be attributed to its electrostatic binding with oppositely charged membranes. The inhibitory effect in the latter at low concentrations of iron chelate would be caused by BSA binding with iron chelates and keeping them away from membrane surface where lipid peroxidation is initiated. Although these results warrant further in vivo investigation, it was concluded that BSA inhibits membrane lipid peroxidation by decreasing the availability of iron for the initiation of membrane lipid peroxidation, in addition to trapping active oxygens and free radicals.

Keywords: Serum albumin; Lipid peroxidation; Superoxide; Liposome; Membrane; Antioxidant; Free radical; Xanthine oxidase; Nitrilotriacetate; Iron

1. Introduction

Reactive oxygen species participate in the development of many pathologic events by causing oxidative deterioration of biological macromolecules such as DNA, proteins, and membrane lipids [1,2]. In particular, oxidation of membranes and lipoprotein lipids in circulation has been implicated in the pathogenesis of many vascular disorders, including atherosclerosis, diabetes, and hypertension [3,4], and there have been extensive studies on the initiation and prevention of lipid peroxidation.

Albumin is considered the major circulating antioxidant in the blood [5–7], which is exposed to continuous oxidative

Abbreviations: BSA, bovine serum albumin; aBSA, acetylated BSA; mBSA, methylated BSA; nBSA, native BSA; DCP, dicetylphosphate; EDTA, ethylenediamine tetraacetic acid; EGTA, bis(2-aminoethyl ether)-ethyleneglycol tetraacetic acid; EYPC, egg yolk phosphatidylcholine; HEPES, 2-hydroxyethylpiperazine-2-ethanesulfonic acid; NTA, nitrilotriacetic acid (nitrilotriacetate); PC-OOH, hydroperoxides of egg yolk phosphatidylcholine; poly-Glu, poly-L-glutamic acid; poly-Lys, poly-L-lysine; poly (Lys-Phe), poly-L-lysine-L-phenylalanine; SA, stearylamine; TBA, thiobarbituric acid; X, xanthine; XO, xanthine oxidase

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doi:10.1016/j.bbamem.2004.12.006

stress. In vitro experiments have shown that serum albumin protects human low-density lipoproteins from copper-mediated oxidation and red blood cells from hemolysis due to free radical membrane damage [8]. Further, it was reported that serum albumin protects cultured cells, such as renal tubular cells, macrophages, aortic endothelial cells, and lung fibroblasts, from oxygen radical damage [9,10]. Albumin protects cells mainly by directly scavenging reactive oxygen species by trapping them. Dean et al. [11] reported that bovine serum albumin (BSA) inhibited membrane lipid peroxidation of liposomes induced by lipid-soluble radicals less than that induced by water-soluble radicals, an effect that is probably due to lower scavenging efficiency for lipid-soluble radicals than for water-soluble radicals.

The superoxide anion radical ($O_2^{\cdot -}$) produced in aerobic organisms is widely held to be a major initiator of biological damage resulting in pathophysiological events associated with a variety of diseases [12,13]. The biological effectiveness of $O_2^{\cdot -}$ is usually explained by the formation of more-reactive species derived from it. A specific example is the oxidation of unsaturated lipids in membranes, an important event in pathophysiology, which cannot be initiated directly by $O_2^{\cdot -}$. The conversion of $O_2^{\cdot -}$ to more reactive intermediates requires the participation of metal catalysts, of which iron is the most important in biological systems [12–15].

Recently, we investigated the membrane lipid peroxidation in liposomes exposed to $O_2^{\cdot -}$ in the presence of chelated iron, and proposed that a Fenton-like reaction of iron-chelate with preformed lipid peroxides at the membrane surface triggers the initiation of lipid hydroperoxide (LOOH) formation [15,16]:

$$Fe^{3+} - \text{chelate} + O_2^{\cdot -} \rightarrow Fe^{2+} - \text{chelate} + O_2 \quad (1)$$

$$Fe^{2+} - \text{chelate} + \text{L} - \text{OOH} \rightarrow Fe^{3+} - \text{chelate} + \text{L} - \text{O}^{\cdot} \quad (2)$$

$$\text{L} - \text{O}^{\cdot} + \text{LH} \rightarrow \text{L} - \text{OH} + \text{L}^{\cdot} \quad (3)$$

$$\text{L}^{\cdot} + O_2 \rightarrow \text{L} - \text{OO}^{\cdot} \quad (4)$$

$$\text{L} - \text{OO}^{\cdot} + \text{LH} \rightarrow \text{L} - \text{OOH} + \text{L}^{\cdot} \quad (5)$$

where LH is the unoxidized lipid and L$^{\cdot}$, L-O$^{\cdot}$, and L-OO$^{\cdot}$; the lipid carbon-centered, alkoxyl, and peroxyl radicals, respectively.

This initiation system seems most likely to occur in blood circulation. Therefore, in this study, we used this lipid peroxidation system and investigated the inhibitory effect of BSA on it from the point of view of obstruction of the Fenton-like reaction that occurs at the membrane surface, and which differs from its well-known antioxidant effect due to trapping active oxygens and free radicals.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (EYPC) was obtained from Nippon Oil and Fats (Tokyo, Japan). The fatty acid composition (mol%) of EYPC was 33.1 palmitate, 1.2 palmitoleate, 11.6 stearate, 30.0 oleate, 15.5 linoleate, 3.3 arachidonate, and 5.3 docosahexaenoate. The commercial EYPC sample was contaminated with 0.3–0.4 mol% of hydroperoxide, as determined by the ferric–xylenol orange method [17]. Bovine serum albumin, fatty acid-free (native BSA:nBSA), carboxymethylated BSA (mBSA), xanthine oxidase (XO), stearyl amine (SA), dicetylphosphate (DCP), poly-L-lysine (poly-Lys) (m.w., 48100), poly-L-glutamic acid (poly-Glu) (m.w.,72500), and poly-L-lysine-L-phenylalanine (1:1) (poly (Lys-Phe)) (m.w., 35400) were purchased from Sigma Chemical (St. Louis, MO). Sodium ethylenediamine tetraacetate (EDTA-2Na), bis (2-aminoethyl ether) ethyleneglycol tetraacetic acid (EGTA), 2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES), xylenol orange, and $Fe(NO_3)_3$ were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Sodium nitrilotriacetate (NTA-2Na) and $FeSO_4$ were from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade. Acetylated BSA (aBSA) was prepared using a modification of the method of Basu et al. [18].

2.2. Preparation of liposomes

Liposomes were prepared as described previously [15]. Stock solutions of EYPC in chloroform with or without DCP or SA were evaporated under nitrogen. The resulting thin lipid films were dispersed in 10 mM HEPES buffer, pH 7.4, in a vortex mixer and subjected to ultrasonic irradiation in a Bransonic-12 sonic bath (Yamato Tokyo, Japan) at 40 °C for 5 min (charged liposomes) or 10 min (uncharged liposomes). When required, the EYPC was freed from contaminating hydroperoxides of EYPC (PC-OOH) by treatment with triphenylphosphine (TPP) [19] in chloroform just before the preparation of liposomes.

2.3. BSA acetylation and characterization of BSAs

nBSA was acetylated using acetic anhydride [18]. To 3 ml of 0.15 M NaCl solution of nBSA (15 mg/ml), 3 ml of sodium acetate-saturated solution was added, and then 40 μl of acetic anhydride was slowly added (2 μl/3 min) at room temperature. After incubation for 30 min, acetylated BSA was dialyzed using a hydrated dialysis membrane (Wako Chem., USA) at 4 °C in 0.15 M NaCl for 24 h and further in 10 mM HEPES buffer solution (pH 7.4) for 3 h. The protein concentration of the BSA preparation was determined using the Lowry method [20]. The net charge of the albumins was investigated by determining their migration times in capillary electrophoresis [21]. The migration times for

aBSA was 12.71±0.10 min (S.D., n=3), which was slightly but significantly long as compared with that of nBSA (12.21±0.02 min), indicating additional net negative charge. The amounts of carbonyl groups measured according to the dinitrophenylhydrazine method [22] were 119% higher in aBSA (0.166 mol carbonyl/mol BSA) than that in nBSA (0.139 mol carbonyl/mol BSA). Purchased mBSA prepared by Mandel and Hershey [23] was well confirmed as a net positively charged basic protein [24].

2.4. Assays of lipid peroxidation

Lipid peroxidation of EYPC liposomes was measured by following oxygen consumption [16]. The following conditions were used for Fe^{3+}-chelate-dependent lipid peroxidation initiated by $O_2^{\cdot -}$ in liposomes. The test systems consisted of liposomes containing 1 mM EYPC with or without DCP or SA in 3.6 ml of 10 mM HEPES buffer pH 7.4 at 37 °C. The oxidation was started by the addition of 10 μl Fe^{3+}-chelate (final concentrations 30 μM Fe^{3+} and 33 μM chelator), 10 μl xanthine (30 μM), and finally 10 μl XO (1 mU/ml) to 3.6 ml of liposome suspensions in 10 mM HEPES buffer (pH 7.4). The Fe^{3+}-chelates were prepared by mixing $Fe(NO_3)_3$ in H_2O or dilute HCl solution with the chelator (1.0 to 1.1 molar ratio). The rate of oxygen consumption associated with lipid peroxidation was measured with a Clark-type oxygen electrode, assuming an oxygen concentration of 217 nmol/ml in the initial incubation mixture at 37 °C.

The generation of $O_2^{\cdot -}$ in human granulocytes from normal blood samples during phagocytosis was reported to be 5–30 mU XO equivalent/ml [25]. In this study, the XO content (1 mU XO/ml) was decided considering the suitable condition for measurement of lipid peroxidation, under which O_2 consumption due to $O_2^{\cdot -}$ generation is negligible in comparison with that due to lipid peroxidation. The rate of $O_2^{\cdot -}$ generation by 1 mU XO/ml used in this experiment is equivalent to the rate (1 μM $O_2^{\cdot -}$/min) observed in our previous study that $O_2^{\cdot -}$ was responsible for apoptosis in rat vascular smooth muscle cells [26]. The thiobarbituric acid (TBA) method was used as described previously [16] to provide another assay for lipid peroxidation. The amount of TBA-reactive substances (TBARS) formed was expressed as equivalents of malondialdehyde (MDA). Other experimental details are described in figure legends.

2.5. Assay of binding of Fe^{3+}-NTA to BSA

Equilibrium dialysis experiments were performed with an Equilibrium Dialyzer (Sanplatec, Osaka, Japan) using a total volume of 13 ml of cells. A hydrated dialysis membrane was washed in deionized water and dried with N_2 gas. To one side of the membrane was added 7 ml of dialysate solution (50 mM HEPES–Tris in 10 mM NaCl buffer, pH 7.4) containing a certain concentration of Fe^{3+}-NTA solution. On the other side of the membrane, 7 ml of the sample (BSA or polypeptide) solution was dialyzed for 24 h. The volumes of the solutions on either side of the membrane were kept constant during the dialysis procedure. Aliquots from the chamber were removed and then free concentrations of Fe^{3+}-NTA were determined by the ferric–xylenol orange method [27]. The percentage binding of Fe^{3+}-NTA to the sample was calculated using the following equation:

$$B\ (\%) = (Abs_a - Abs_b)/Abs_a \times 100$$

where B (%) is the percentage of the added Fe^{3+}-NTA bound to the sample, and Abs_b and Abs_a are the absorbances at 560 nm of Fe^{3+}-NTA in the dialyzed solution with and without a sample, respectively.

2.6. Measurement of zeta potential of liposome membranes

The zeta potentials of the liposomes (1 mM EYPC with or without 0–0.2 mM DCP or SA) in 10 mM HEPES/10 mM NaCl buffer at pH 7.4 were measured electrophoretically in a NICOMP (model 380 apparatus, Particle Sizing Systems, Santa Barbara, California) at room temperature.

3. Results

Preliminary experiments showed that the complete system, made up of liposomes formed from 1 mM EYPC supplemented with 0.2 mM SA or 0.1 mM DCP, 10 mM HEPES buffer at pH 7.4, 1 mU/ml XO, 30 μM X, and Fe^{3+}-NTA (30 μM Fe^{3+}, 33 μM NTA), rapidly consumed oxygen, indicating the occurrence of lipid peroxidation (solid lines (1) in Fig. 1). Oxygen was not consumed if XO was excluded, but slightly consumed if Fe^{3+}-NTA was excluded, possibly due to the generation of $O_2^{\cdot}$ from oxygen by X-XO (broken lines in Fig. 1 (left)), indicating that lipid peroxidation was not induced directly by either $O_2^{\cdot -}$ or Fe^{3+}-NTA. The involvement $O_2^{\cdot -}$ in this process was confirmed by the complete inhibition of oxygen uptake by the addition of superoxide dismutase (100 U/ml) (data not shown). Similarly, oxygen consumption was completely inhibited if liposomes were prepared from EYPC pretreated with triphenylphosphine (TPP) (broken line (1) in Fig. 1 (right)), which reduces endogenous EYPC hydroperoxides (PC-OOH) to the corresponding alcohols (PC-OH) [19], indicating that the presence of PC-OOH was a prerequisite for the induction of lipid peroxidation.

Lines (2)–(4) in Fig. 1 show the time course of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in liposomes with positive and negative charges in the presence of various BSAs. Three types of BSAs, nBSA with a small net negative charge, negatively charged aBSA, and positively charged mBSA, were used. All BSAs inhibited the lipid peroxidation. The orders of their inhibitory effects were: aBSA>nBSA>mBSA in positively charged SA-EYPC liposomes, and mBSA>nBSA>aBSA in negatively

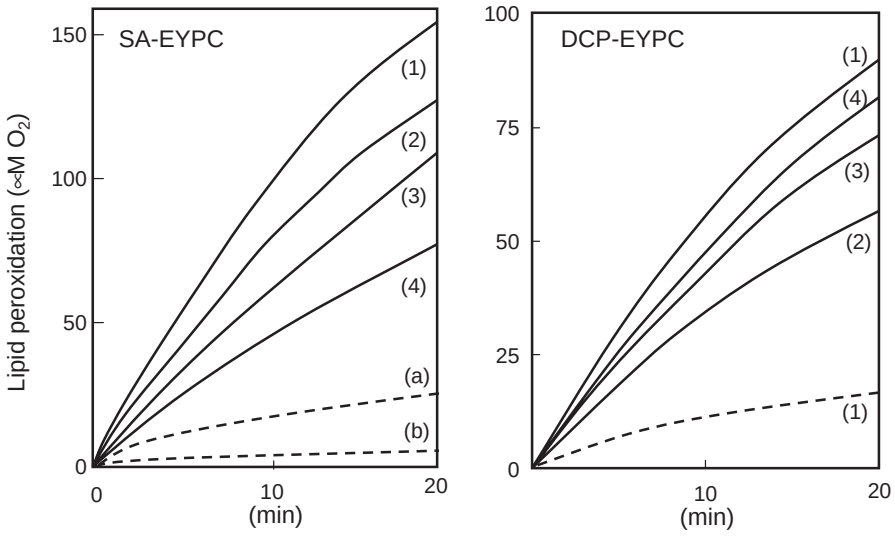

Fig. 1. Inhibition by BSAs of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in DCP-EYPC and SA-EYPC liposomes. (1) Control; (2) +mBSA; (3) +nBSA; (4) +aBSA. Broken lines in SA-EYPC liposome system indicate the oxygen consumption in the system that excluded only (a) Fe^{3+}-NTA or (b) X-XO from the control system (line (1)). The system of broken line (1) in DCP-EYPC liposome system is all the same to the control system (solid line (1)) but EYPC was pretreated with TPP before the preparation of liposomes. The concentration of BSAs added to the control system was 1.0 mg/ml. Concentrations of reagents in the control system were: 1 mM EYPC, 0.1 mM DCP or 0.2 mM SA, 10 mM HEPES buffer, pH 7.4, 1 mU/ml xanthine oxidase (XO), 30 μM xanthine (X), and iron chelate formed from 30 μM $Fe(NO_3)_3$ and 33 μM NTA. Incubation was at 37 °C, and the reaction started by the addition of XO.

charged DCP-EYPC liposomes. Same orders were observed by the assay of TBARS values (data not shown). Furthermore, nBSA more strongly inhibited TBARS formation in SA-EYPC liposomes (63% inhibition) than in DCP-EYPC liposomes (28% inhibition). These results indicate that the inhibitory effect of BSAs is higher in oppositely charged membranes.

Fe^{3+}-NTA, a renal carcinogen, has been reported to cause apoptosis associated with increase of lipid peroxide and 8-hydroxydeoxyguanosine levels in vivo [28,29] and in vitro [30]. In cultured cells treated with 20–100 μM Fe^{3+}-NTA, oxidative damage [31] and apoptotic [30], cytotoxic [32], and mutagenic [33] effects were observed. Therefore, we used 10–60μM Fe-NTA and examined its concentration dependency for the induction of X-XO-dependent lipid peroxidation in EYPC liposomes containing different amounts of positively charged SA (Fig. 2). The rate of lipid peroxidation increased with an increase in Fe^{3+}-NTA concentration. A decrease of the membranous SA concentration in EYPC liposomes lowered the rate of lipid peroxidation. The addition of nBSA (1 mg/ml) to SA-EYPC (0.2 mM/1 mM EYPC) liposomes slowed down the lipid peroxidation rate. The change in the lipid peroxidation rate in the presence of nBSA was similar to that of the rate depending on the reduction of SA concentration at concentrations of Fe^{3+}-NTA higher than 20 μM. On the contrary, the rate change was quite different from that at concentrations of Fe^{3+}-NTA lower than 10 μM, and the addition of nBSA almost completely inhibited the lipid peroxidation.

Before the antioxidant effect of BSA on the Fe^{3+}-chelate/$O_2^{\cdot-}$-dependent membrane lipid peroxidation at the membrane surface was examined in detail, we investigated the role of Fe^{3+}-chelate at the membrane surface, where a Fenton-like reaction initiates lipid peroxidation. The concentration of Fe^{3+}-chelate chosen was 30 μM, which was suitable for the investigation of inhibiting effect of BSA.

Fig. 3 shows the effect of the membrane charge on the rate of O_2 consumption indicative of lipid peroxidation by Fe^{3+}-chelates (Fe^{3+}-NTA, Fe^{3+}-EGTA, and Fe^{3+}-EDTA). Fe^{3+}-NTA catalyzed the lipid peroxidation both in positively

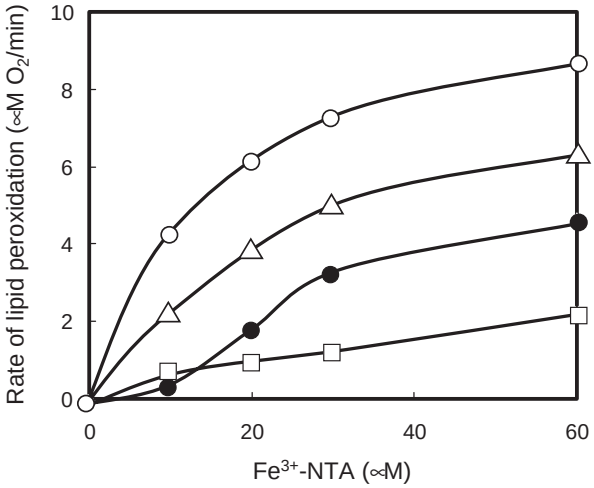

Fig. 2. Inhibitory effect of nBSA on the rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in the presence of various concentrations of Fe^{3+}-NTA in EYPC liposomes containing different amounts of SA. (○) SA-EYPC (0.2 mM/1.0 mM) liposomes; (△) SA-EYPC (0.05 mM/1.0 mM) liposomes; (□) EYPC (1.0 mM) liposomes; (●) SA-EYPC (0.2 mM/1.0 mM) liposomes+nBSA (1.0 mg/ml). The rate in each system was obtained after the subtraction of the value of blank, which was the system that only omitted Fe^{3+}-NTA from the control system.

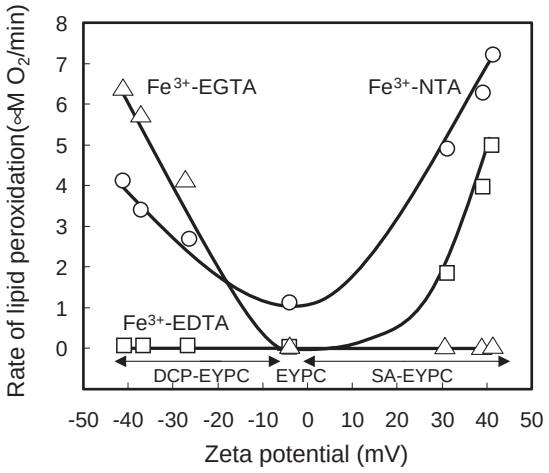

Fig. 3. Rates of Fe^{3+}-chelate/X-XO-dependent oxygen consumption indicative of lipid peroxidation in liposomes with various membrane charges. (○) Fe^{3+}-NTA; (△) Fe^{3+}-EGTA; (□) Fe^{3+}-EDTA. Liposomes were prepared from EYPC containing different amounts of DCP or SA. Concentrations of reagents for lipid peroxidation were: 1 mM EYPC, 0–0.2 mM DCP or SA, 10 mM HEPES buffer, pH 7.4, 1 mU/ml xanthine oxidase (XO), 30 μM xanthine (X), and iron chelate formed from 30 μM $Fe(NO_3)_3$ and 33 μM NTA, EDTA, or EGTA. Incubation was at 37 °C, and the reaction started by the addition of XO.

charged SA-EYPC liposomes and negatively charged DCP-EYPC liposomes, and the rates of lipid peroxidation increased with an increase in the zeta potential of liposomes. Our previous work revealed that the binding of Fe^{3+}-chelates with liposomes is indispensable for the initiation of $O_2^{\cdot-}$ driven lipid peroxidation [16]. Because of the good correlations between the abilities of Fe^{3+}-chelates to induce lipid peroxidation and electrostatic binding to membranes, we supposed that the increase of negative and positive zeta potentials of membranes causes more binding of Fe^{3+}-NTA to membranes, increasing its facilitating effect on lipid peroxidation. The initiation of lipid peroxidation by Fe^{3+}-EGTA and Fe^{3+}-EDTA was also dependent on the zeta potentials of the liposome membranes. The former and the latter catalyzed the lipid peroxidation in negatively charged DCP-EYPC liposomes and positively charged SA-EYPC liposomes, respectively, indicating that membrane charges of DCP-EYPC liposomes and SA-EYPC liposomes are closely associated with the electrostatic binding ability of oppositely charged Fe^{3+}-chelates, such as net positively charged Fe^{3+}-EGTA and net negatively charged Fe^{3+}-EDTA. Increased amounts of Fe^{3+}-chelates bound to the membrane would result in an increase of the availability of the Fe^{3+}-chelates required to initiate lipid peroxidation at the membrane surface.

As shown in Fig. 1, the inhibitory effect of BSA depending on membrane charge was suggested to be due to the neutralization of the membrane charge by electrostatic interaction with the membranes. Thus, we chose systems in which the charges of liposomal membranes and BSAs were opposite, and investigated the correlation between the effects of BSAs on the zeta potentials of liposomes and the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation. The rate of lipid peroxidation was obtained from the initial gradient of the curve in Fig. 1. As shown in Fig. 4, the rates of Fe^{3+}-NTA-dependent lipid peroxidation as a function of BSA concentration were well correlated to the changes in the zeta potentials of liposomes.

To determine the details of the ability of BSA to change the zeta potentials and lipid peroxidation, we compared the rates of lipid peroxidation in liposomes with same zeta potentials, which depend on the concentrations of BSAs added to the membranes and of charged molecules removed from the membranes. The rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in liposomes with different charges was plotted as a function of zeta potential of liposomes in the presence and absence of BSAs (Fig. 5). Under the same zeta potential conditions in systems in which the charge of the liposomes is opposite to that of the BSAs added, the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation were

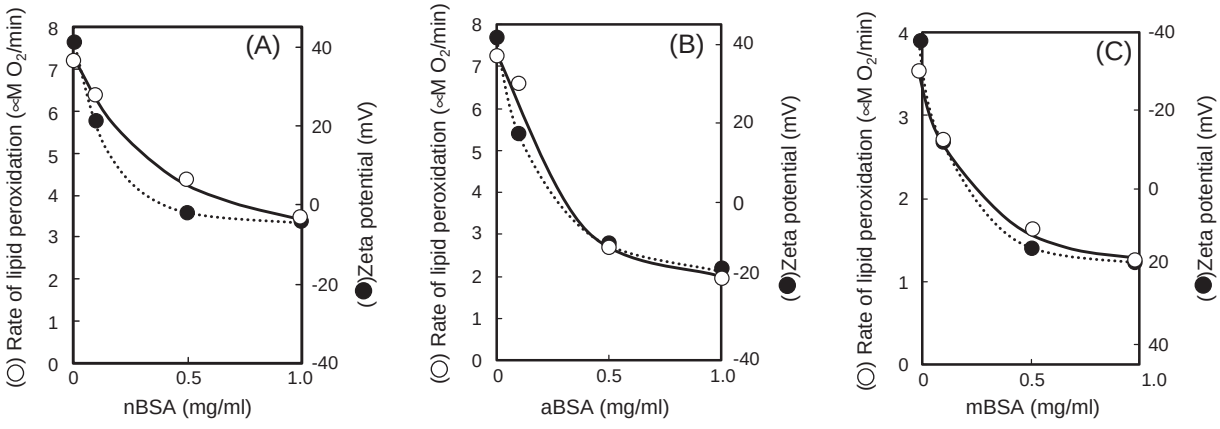

Fig. 4. The effects of the concentration of BSAs on the rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation and the zeta potential of EYPC liposomes with DCP or SA. Panel A: nBSA in SA-EYPC liposomes; panel B: aBSA in SA-EYPC liposomes; panel C: mBSA in DCP-EYPC liposomes. (○) Lipid peroxidation; (●) zeta potential. Concentrations of BSAs added to the DCP-EYPC (0.1 mM/1.0 mM) or SA-EYPC (0.2 mM/1.0 mM) liposomes were 0.1 mg/ml–1.0 mg/ml. Other experimental conditions were as shown in Fig. 3.

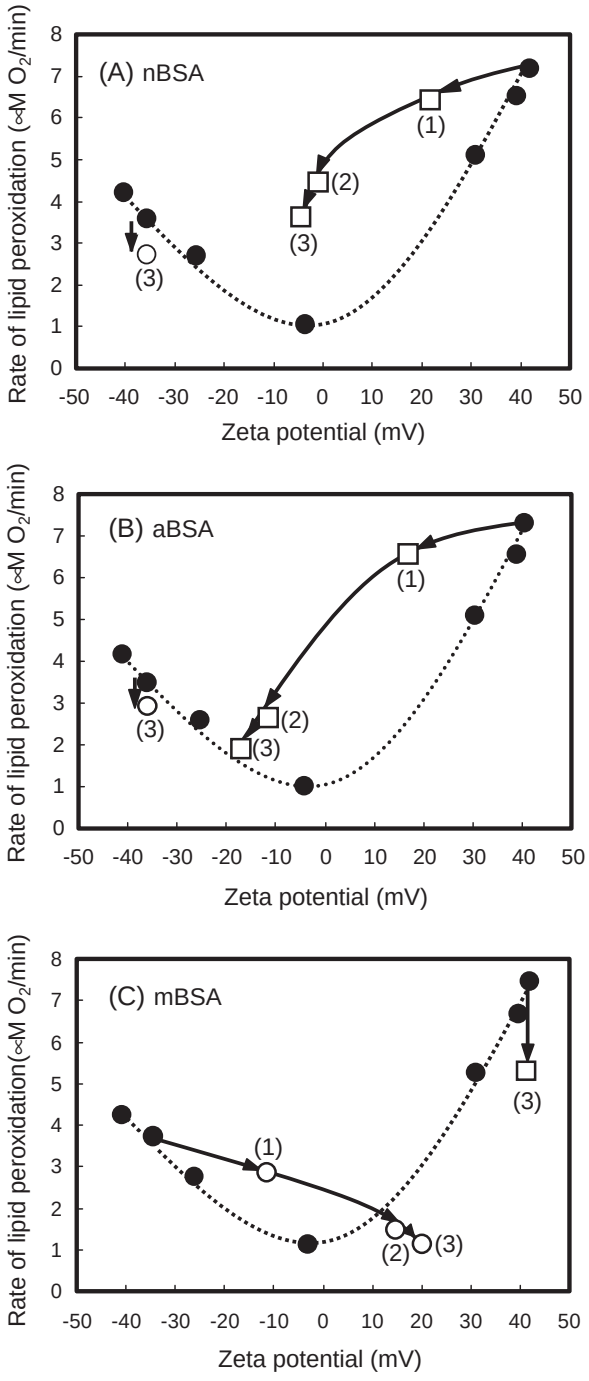

Fig. 5. The rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation as a function of zeta potential of liposomes with different charges in the presence or absence of nBSA, aBSA, or mBSA. Panel A: nBSA; panel B: aBSA; panel C: mBSA. (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC (0–0.2 mM/1.0 mM) and SA-EYPC (0–0.2 mM/1.0 mM) liposomes in the absence of BSAs. The concentrations of BSAs added to the liposomes are indicated by numbers: (1) 0.1 mg/ml; (2) 0.5 mg/ml; (3) 1.0 mg/ml. Experimental conditions were as shown in Fig. 3.

always higher in the presence of BSAs (□ in Fig. 5(A) and (B), ○ (1) in Fig. 5(C)) than in their absence (dotted lines in Fig. 5), except for the results shown in Fig. 5(C) (○ (2) and ○ (3)). The reductions of the rate of lipid peroxidation were larger for the addition of aBSA with a high net negative charge (□ in Fig. 5 (B)) than for addition of nBSA with a low net negative charge in SA-EYPC (0.2 mM/1 mM) liposomes (□ in Fig. 5(A)).

On the contrary, in the systems in which BSA was added to identically charged liposomes, Fe^{3+}-NTA/X-XO-dependent lipid peroxidation was inhibited without affecting the zeta potential: nBSA and aBSA with a net negative charge did not affect the zeta potential but weakly inhibited the lipid peroxidation in negatively charged DCP-EYPC (0.1 mM/1 mM) liposomes (○ (3), as shown in Fig. 5(A) and (B)), and mBSA with a net positive charge did not affect the zeta potential but did lower the rate of lipid peroxidation in positively charged SA-EYPC liposomes (□ (3) in Fig. 5(C)).

Fig. 6 shows the effects of mBSA and aBSA on the zeta potentials of liposomes and the rates of Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation. The addition of mBSA lowered both the negative value of the zeta potential and lipid peroxidation in DCP-EYPC liposomes, and their mBSA concentration-dependent decreases correlated well (Fig. 6 (A)). At the same zeta potential, the rates of lipid peroxidation in DCP-EYPC liposomes in the presence of mBSA (○ in Fig. 6 (B)) were always higher than those in their absence (dotted line in Fig. 6 (B)). Furthermore, Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was not induced in positively charged SA-EYPC liposomes (□ (1) in Fig. 6 (B)) but was induced in DCP-EYPC liposomes with a net positive charge by the addition of a high concentration of mBSA (0.5 mg, 1.0 mg/ml) (○ (3),○ (4) in Fig. 6(B)). On the contrary, Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was induced in negatively charged DCP-EYPC liposomes (dotted line in Fig. 6(B)) but was not induced in SA-EYPC liposomes with a net negative charge by the addition of a high concentration of aBSA (1.0 mg/ml) (□ (5) in Fig. 6(B)).

Fig. 7 shows the effects of aBSA and mBSA on the zeta potentials of liposomes and the rates of Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation. The addition of aBSA also concentration-dependently neutralized the charge of liposome membranes and lowered the lipid peroxidation rate in SA-EYPC liposomes (Fig. 7 (A)). However, in SA-EYPC liposomes, the rates of lipid peroxidation were always higher in the presence of aBSA (□ in Fig. 7(B)) than in the absence of it (dotted line) at the same zeta potential (Fig. 7 (B)). Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was not induced in negatively charged DCP-EYPC liposomes (○ (1) in Fig. 7(B)) but was induced in SA-EYPC liposomes with a net negative charge by the addition of a high concentration of aBSA (0.5 mg, 1.0 mg/ml) (□ (3), □ (4) in Fig. 7(B)). On the contrary, Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was induced in positively charged SA-EYPC liposomes (dotted line in Fig. 7(B)) but was not induced in DCP-EYPC liposomes with a net positive charge by

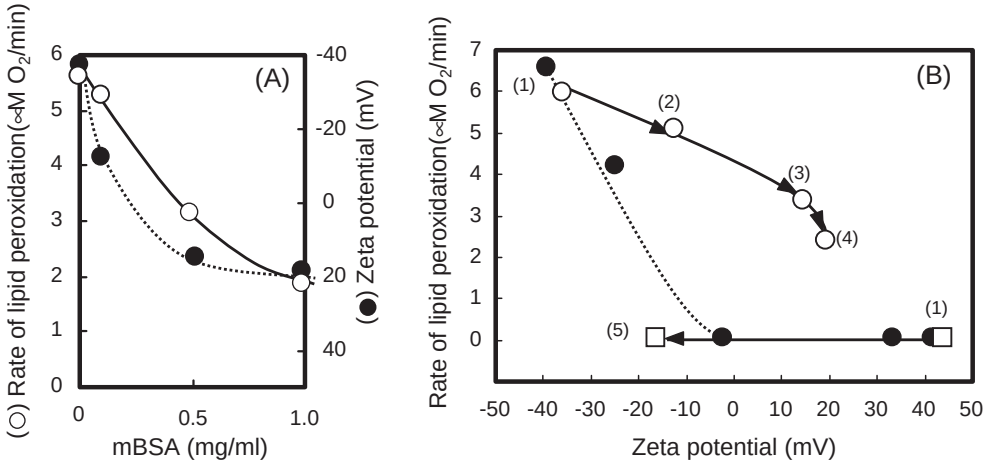

Fig. 6. The effects of the concentration of BSAs on the rate of Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation and the zeta potential of EYPC liposomes with DCP or SA. Panel A: (○) lipid peroxidation; (●) zeta potential. Concentrations of mBSA added to the SA-EYPC (0.2 mM/1.0 mM) liposomes were 0.1–1.0 mg/ml. Panel B: (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC or SA-EYPC liposomes in the absence of BSA. (1) control (no BSA); (2) 0.1 mg mBSA/ml; (3) 0.5 mg mBSA/ml; (4) 1.0 mg mBSA/ml; (5) 1.0 mg aBSA/ml. Other experimental conditions were as shown in Fig. 3.

the addition of a high concentration of mBSA (1.0 mg/ml) (○ (5) in Fig. 7(B)).

The effects of polypeptides, which were used instead of positively charged mBSA and negatively charged aBSA to simplify the experimental system, on the zeta potentials and the rates of lipid peroxidation were investigated further. As shown in Fig. 8(A), the addition of positively charged poly-Lys inhibited Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation in DCP-EYPC liposomes (△), and the addition of negatively charged poly-Glu inhibited Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation in SA-EYPC liposomes (□). Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was not induced in positively charged SA-EYPC liposomes (dotted line) but was induced in DCP-EYPC liposomes with a net positive charge by the addition of poly-Lys (△). Similarly, Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was not induced in negatively charged DCP-EYPC liposomes (dotted line) but was induced in SA-EYPC liposomes with a net negative charge by the addition of poly-Glu (□). Thus, the inhibition of lipid peroxidation by polypeptides as well as by BSAs with charges opposite those of the membrane was associated with neutralization of the membrane charge.

We further investigated the effects of positively charged copolypeptides with a hydrophobic residue, poly (Lys-Phe), on the zeta potentials and the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in EYPC and DCP-EYPC liposomes. As shown in Fig. 8(B), poly (Lys-Phe) behaved similarly to the charged molecule SA in the membranes, and

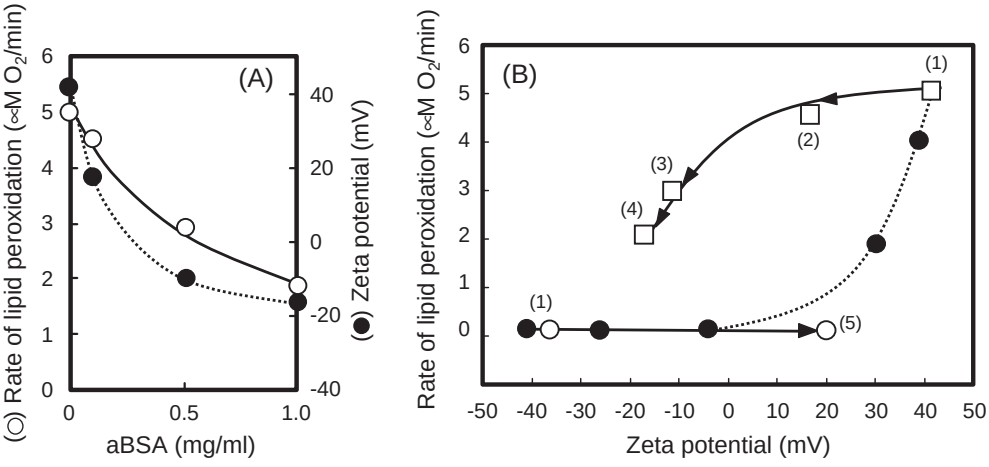

Fig. 7. The effects of the concentration of BSAs on the rate of Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation and the zeta potentials of EYPC liposomes with DCP or SA. Panel A: (○) lipid peroxidation; (●) zeta potential. Concentrations of aBSA added to DCP-EYPC (0.1 mM/1.0 mM) liposomes were 0.1–1.0 mg/ml. Panel B: (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC and SA-EYPC liposomes in the absence of BSAs. (1) control (no BSA); (2) 0.1 mg aBSA/ml; (3) 0.5 mg aBSA/ml; (4) 1.0 mg aBSA/ml; (5) 1.0 mg mBSA/ml. Other experimental conditions were as shown in Fig. 3.

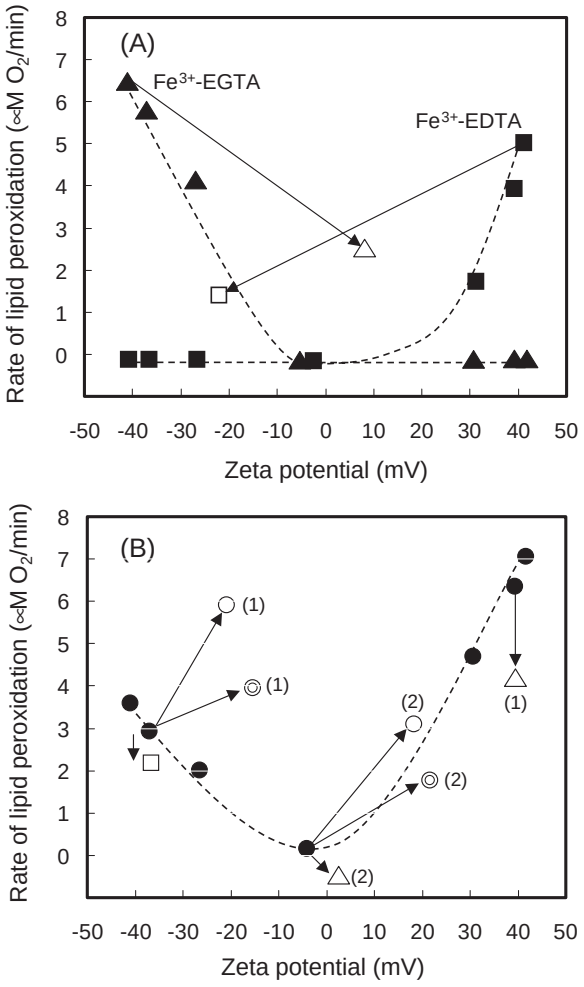

Fig. 8. The effects of polypeptides on the rate of Fe^{3+}-chelate/X-XO-dependent lipid peroxidation and the zeta potentials of EYPC liposomes with DCP or SA. Panel A: Fe^{3+}-EGTA/X-XO-system in the presence (△) or absence (▲) of poly-Lys; Fe^{3+}-EDTA/X-XO-system in the presence (□) or absence (■) of poly-Glu. Concentrations of poly-Lys and poly-Glu were 645 μg/ml (5 mM) and 38.7 μg/ml (0.3 mM), respectively. Panel B: Fe^{3+}-NTA/X-XO-system. (●) EYPC liposomes with different concentrations of SA or DCP in the absence of polypeptides. (○) Poly (Lys-Phe) incorporated into the membrane of the DCP-EYPC(0.1 mM/1.0 mM) or EYPC (1 mM) liposomes. (○) Poly (Lys-Phe) added to the solution of DCP-EYPC or EYPC liposomes, (△) poly-Lys added to the solution of SA-EYPC(0.1 mM/1.0 mM) or EYPC (1 mM) liposomes, and (□) poly-Glu added to the solution of DCP-EYPC (0.1 mM/1.0 mM) liposomes. Concentrations of poly (Lys-Phe) and poly-Glu were 17.8 μg/ml and 645 μg/ml, respectively. Concentrations of poly-Lys were 645 μg/ml and 6.45 μg/ml in SA-EYPC (0.1 mM/1.0 mM) liposomes and EYPC (1 mM) liposomes, respectively. Other experimental conditions were as shown in Fig. 3.

increased the lipid peroxidation and neutralized the zeta potential of DCP-EYPC liposomes (○ (1), ⊙ (1)). In EYPC liposomes, poly (Lys-Phe) also increased the lipid peroxidation and zeta potential (○ (2), ⊙ (2)), but the homopolypeptide poly-Lys decreased the lipid peroxidation and slightly increased the zeta potential (△ (2)). On the other hand, poly-Glu (□) and poly-Lys (△ (1)) inhibited Fe^{3+}-NTA-dependent lipid peroxidation without changing membrane charges in DCP-EYPC and SA-EYPC liposomes, respectively, which have the same type of charges as these polypeptides have.

As mentioned above, both BSAs and polypeptides inhibited the lipid peroxidation with and without changing the membrane charge. In liposomes whose zeta potential was neutralized by them, their inhibitory effect was caused by their direct interaction with membranes, whereas in liposomes whose zeta potentials were not affected by them, their inhibitory effect was caused by their binding to Fe^{3+}-chelate and keeping it away from membranes, but not by binding to membranes. In order to prove the binding of the additives to Fe^{3+}-NTA, therefore, we chose systems in which zeta potentials of liposomes were not affected by BSAs and polypeptides, and the binding of BSAs or polypeotides to Fe^{3+}-NTA was estimated by the equilibrium dialysis experiment (see Materials and methods). The percentages binding of Fe^{3+}-NTA to the BSAs were

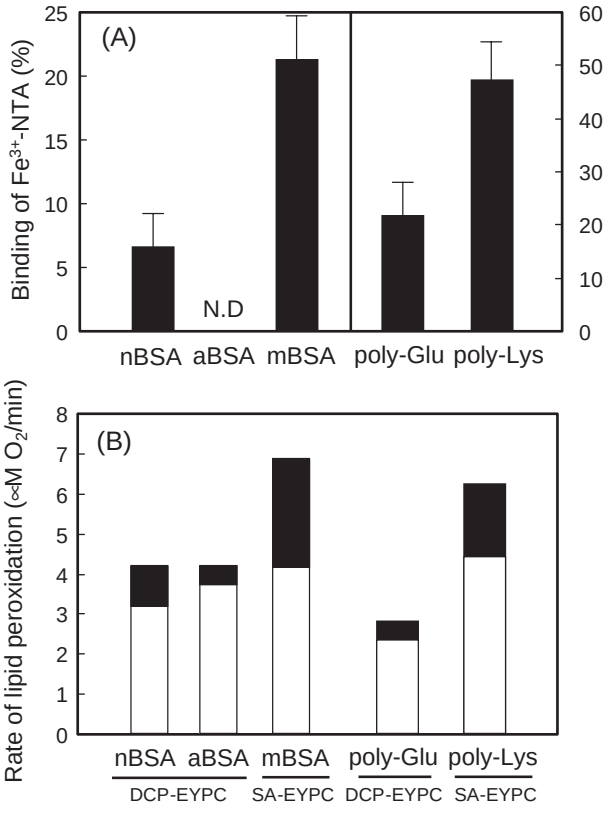

Fig. 9. Correlations between the abilities of BSAs and polypeptides to bind to Fe^{3+}-NTA and to inhibit Fe^{3+}-NTA/X-XO-induced lipid peroxidation without change of the membrane charge. Panel A: concentrations of reagents were: 3 mg BSA/ml and 90 μM Fe^{3+}-NTA for the binding of BSAs to Fe^{3+}-NTA, and poly-Lys (645 μg/ml) or poly-Glu (645 μg/ml) and 60 μM Fe^{3+}-NTA for the binding of polypeptides to Fe^{3+}-NTA. Binding percents are means±S.D. for three experiments. Panel B: (□) BSAs or polypeptides were present; (■+□) BSAs or polypeptides were absent. Values are means for two experiments. Concentrations of BSAs and polypeptides were 1.0 mg/ml and 645 μg/ml, respectively. Concentrations of EYPC, DCP, and SA were 1 mM, 0.2 mM, and 0.2 mM, respectively, in the experiments with BSAs. Concentrations of EYPC, DCP, and SA were 1 mM, 0.1 mM, and 0.1 mM, respectively, in the experiments with polypeptides. Other experimental conditions were as shown in Fig. 3.

6.6±2.4% for nBSA, 0% for aBSA, and 21.2±3.5% for mBSA, and those to poly-Glu and poly-Lys were 21.8±5.9% and 47.0±7.3%, respectively. The results are compared with their inhibiting abilities on Fe^{3+}-NTA-induced lipid peroxidation (Fig. 9). As was expected, there was a good correlation between their relative abilities to bind to Fe^{3+}-NTA (in Fig. 9(A)) and to inhibit lipid peroxidation (in Fig. 9(B)).

4. Discussion

Recently, we proposed a mechanism of peroxidation of membrane lipids exposed to $O_2^{\cdot-}$: an initiation reaction that first requires the binding of iron-chelate to the membrane and is followed by the Haber-Weiss-like reaction of iron reduction by $O_2^{\cdot-}$ with the hydroperoxyl group of preformed lipid peroxides at the membrane surface to form alkoxyl radicals, which penetrate into the hydrophobic inner region and trigger the initiation reaction [15,16].

This hypothesis is supported by the results in Fig. 1 showing that the rate of $O_2^{\cdot-}$ driven lipid peroxidation induced by Fe^{3+}-NTA, which, charged either positively and negatively, can interact with membranes of either charge, was increased with the increase of positive and negative zeta potentials of liposomes, possibly by an increase in coulombic attraction between the charged membrane and Fe^{3+}-NTA. The rates of $O_2^{\cdot-}$-driven lipid peroxidation induced by Fe^{3+}-EGTA and Fe^{3+}-EDTA, which interact with negatively and positively charged membranes, respectively, also increased with an increase of negative and positive zeta potentials, respectively. These results further support the proposal that the binding of iron chelate to the membrane surface triggered the initiation of the lipid peroxidation. In this study, we used this most-likely initiation system for membrane lipid peroxidation in biological circulation because it is most suitable to investigate the antioxidant effect of BSA from the point of view of preventing the occurrence of the Fenton-like reaction at the membrane surface.

The orders of the inhibitions by BSAs of Fe^{3+}-NTA-dependent lipid peroxidation were: (−) charged aBSA>weakly (−) charged nBSA>(+) charged mBSA in positively charged SA-EYPC liposomes, and mBSA>nBSA->aBSA in negatively charged DCP-EYPC liposomes (Fig. 1). These results indicate that BSAs inhibit lipid peroxidation more strongly in oppositely charged membranes than in those with the same charge, suggesting that the inhibition effect of BSA is due to a decrease in the availability of Fe^{3+}-NTA by lowering the membrane charge, followed by a decrease in the amount of Fe^{3+}-NTA bound to membranes. This suggestion was supported by the findings shown in Fig. 4 that BSA-concentration dependencies of the neutralization of the membrane charges, which are opposite to charges of BSAs, were well correlated to the dependencies of the decrease of the rates of Fe^{3+}-NTA-induced lipid peroxidation. The similarly good correlations observed in Fe^{3+}-EDTA- and Fe^{3+}-EGTA-induced lipid peroxidation systems (Figs. 6(A) and 7(A)) further support this suggestion.

To further investigate the inhibiting effect of BSA on lipid peroxidation from its changing effect on the zeta potentials, we compared the effect of BSAs on the rates of lipid peroxidation in two types of liposomes with the same zeta potentials: one was liposomes whose zeta potential was neutralized by the addition of opposite charged BSA, and the other is the liposomes, zeta potential of which was neutralized by the subtraction of charged substances from the membrane. At the same zeta potential, the rates of lipid peroxidation induced by Fe^{3+}-EGTA and Fe^{3+}-EDTA were always higher in the system to which BSAs were added than in the system in which membranous charge substances were reduced (Figs. 6(B) and 7(B)). Similar results were observed when polypeptides such as poly-Glu and poly-Lys were used instead of BSAs; Fig. 8 (A) shows that the rates of lipid peroxidation were always higher in the presence of poly-Glu in SA-EYPC liposomes and poly-Lys in DCP-EYPC liposomes than in their absence in liposomes with the same zeta potential. These results would be explained as follows: that Fe^{3+}-chelate is more available in systems to which BSAs and polypeptides were added than in systems in which membranous charge substances were reduced, possibly because the number of membranous charge molecules available for binding with Fe^{3+}-chelate was larger in the former system, even though the zeta potential of both systems was the same. In cases that the membrane charge was reversed by the addition of BSA, inhibition of lipid peroxidation by BSA as shown in Fig. 6(B) (○ (3) and ○ (4)), as examples, can be explained as follows. The decreased rates of Fe^{3+}-EGTA-dependent lipid peroxidation due to the addition of mBSA at a high concentration to the system of DCP-EYPC liposomes, charges of which became net positive by binding a large amount of positively charged mBSA, might be due to decreased availability of Fe^{3+}-EGTA, because only a small amount of residual positively charged Fe^{3+}-EGTA interacted with the negatively charged membranous DCP left from interaction with mBSA. A similar mechanism would also be applicable to the Fe^{3+}-EDTA-dependent lipid peroxidation decreased by the addition of aBSA in SA-EYPC liposomes (□ (3), □ (4) in Fig. 7(B)). Based on this proposal, the results shown in Fig. 5(C) (○ (2) and ○ (3)) that the rates of Fe^{3+}-NTA-dependent lipid peroxidation by the addition of mBSA at high concentrations to the system of DCP-EYPC liposomes, the apparent charge of which becomes positive, were lower than the rates in the positively charged SA-EYPC liposome system without mBSA (dotted line in Fig. 5(C)) at same zeta potential can be explained as follows. In the mBSA-added system, a large amount of negatively charged DCP is occupied by positively charged mBSA, resulting in a large increase of the amount of silent Fe^{3+}-NTA, which is unable to bind with DCP, for the initiation of lipid peroxidation. Accord-

ingly, the amount of DCP bindable with positively charged Fe^{3+}-NTA remains small, and thus only a small amount of residual Fe^{3+}-NTA is available to initiate lipid peroxidation. Under conditions with the same zeta potential, the lower rate of lipid peroxidation in the DCP-liposome system to which mBSA was added than in the SA-liposome system to which no BSA was added, would be due to the lower availability of Fe^{3+}-NTA depending on the amount bound to charge molecules. Similarly, the higher availability of Fe^{3+}-NTA would cause the higher rate of lipid peroxidation in the SA-liposome system to which aBSA was added than in the DCP-liposome system to which no BSA was added under conditions with the same zeta potential (□ (2), □ (3) in Fig. 5(B)).

BSAs and polypeptides inhibited lipid peroxidation without affecting the zeta potentials of the liposomes, which have the same type of charges as BSAs and polypeptides; as shown in Fig. 9(B), nBSA, aBSA, and poly-Glu did not affect the zeta potential but weakly inhibited lipid peroxidation in DCP-EYPC liposomes, while mBSA and poly-Lys also lowered lipid peroxidation in SA-EYPC liposomes without affecting their zeta potential. This indicates that they inhibit lipid peroxidation without direct interaction with membranes. We concluded that their inhibiting effect on lipid peroxidation was due to their binding with Fe^{3+}-NTA and excluding it from membranes. A good correlation between their abilities to bind to Fe^{3+}-NTA and to inhibit lipid peroxidation (Fig. 9(A) and (B)) supports this conclusion.

Surprisingly, an amphiphilic polypeptide, poly (Lys-Phe), increased the rate of Fe^{3+}-NTA-dependent lipid peroxidation and the zeta potential of EYPC liposomes, but a hydrophilic poly-Lys decreased the rate of Fe^{3+}-NTA-dependent lipid peroxidation and increased their zeta potential (○ (2), ⊙ (2), and △ (2) in Fig. 8(B)). In EYPC liposomes, poly (Lys-Phe) binds to the membranes by hydrophobic interaction and increases their membrane charge by acting as a membrane charge molecule like SA, resulting in an increase in the amount of Fe^{3+}-NTA bound to membranous poly (Lys-Phe), and an increase in lipid peroxidation follows. Similar behaviors by poly (Lys-Phe) were observed in negatively charged DCP-EYPC liposomes (○ (1), ⊙ (1) in Fig. 8(B)). In DCP-EYPC liposomes, some parts of membranes charged positively with poly-(Lys-Phe) and other of parts charged negatively with DCP results in an increase of the total number of membrane-charged molecules, that is, an increase of the amount of membranous sites bound with Fe^{3+}-NTA, which can interact with molecules of either charge. Accordingly, the initiation reaction induced by Fe^{3+}-NTA occurs charge-site dependently, and the initiation ability of Fe^{3+}-NTA depends on the total number of sites charged positively and negatively in a membrane rather than the net charge of the membrane.

Recently, Anraku et al. [7] reported that additional net negative charge was increased in oxidized human serum albumin. This finding suggests that the oxidized BSA functions just like an aBSA. It is very interesting to investigate the antioxidant effect of the oxidized BSA from this point.

Finally, we will discuss the antioxidant effect of the native type of BSA based on the above-mentioned considerations. The rate of lipid peroxidation depending on the Fe^{3+}-NTA concentration in SA-EYPC liposomes increased with an increase of SA concentration (Figs. 2 and 3). At concentrations of Fe^{3+}-NTA higher than 20 μM, the decrease of the Fe^{3+}-NTA concentration-dependent lipid peroxidation due to the addition of nBSA (1 mg/ml) to SA-EYPC liposomes (0.2 mM/1.0 mM) with the zeta potential of +41 mV was similar to that of the lipid peroxidation rates decreased by a lower SA concentration in liposomes (Fig. 2), which had about +20 mV of zeta potential as approximated from the rate of lipid peroxidation in SA-EYPC liposomes shown in Fig. 3. These results suggest that net negatively charged nBSA binds to positively charged SA and decreases the amount of membranous SA available for Fe^{3+}-NTA binding, resulting in a decrease in the rate of lipid peroxidation. The zeta potential measured when nBSA (1 mg/ml) was added to SA-EYPC (0.2 mM/1.0 mM) liposomes was about −5 mV, which corresponds to the zeta potential of EYPC liposomes containing no charged substance (Fig. 5(A)). The rate of lipid peroxidation depending on the Fe^{3+}-NTA concentration in the nBSA-added system of SA-EYPC liposomes with −5 mV (● in Fig. 2) was higher than that in nBSA-free system of the EYPC liposome with a charge of −5 mV (□ in Fig. 2), indicating that the membranes of SA-EYPC liposomes that interact with nBSA would be more likely to bind with Fe^{3+}-NTA than with the membranes of EYPC liposomes, even if their membrane zeta potentials were almost the same.

On the contrary, at a concentration of Fe^{3+}-NTA lower than 10 μM, the rate of lipid peroxidation was slower in the system in which nBSA (1 mg/ml) was added to SA-EYPC (0.2 mM/1 mM EYPC) liposomes (● in Fig. 2) than in the nBSA-free system of EYPC liposomes containing no SA (□ in Fig. 2), although the zeta potentials of liposomes in both systems were almost the same (−5 mV). These results suggest that nBSA binds with most of the Fe^{3+}-NTA and prevents it from interacting with membranes by keeping it away from the membrane, resulting in little induction of lipid peroxidation.

In conclusion, the binding of Fe-chelate to membrane surfaces triggered initiation of the Fe-chelate/$O_2^{\cdot-}$-dependent lipid peroxidation of the membrane. BSA inhibits this lipid peroxidation by decreasing the availability of Fe-chelate in two manners, which are dependent on Fe-chelate concentration: ① at high concentrations of Fe-chelate, BSA directly interacts with the membrane and prevents the interaction of Fe-chelate with the membrane surface, where lipid peroxidation is initiated; and ② at low concentrations of Fe-chelate, BSA interacts with Fe-chelate and excludes it from the membrane.

Acknowledgement

We thank Dr. M. Anraku (Kumamoto University) for technical assistance for measurement of the net charge of the alubumins by determining their migration times in capillary electrophoresis.

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