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EFFECTS OF MEMBRANE-STABILIZING AGENTS, CHOLESTEROL AND CEPHARANTHIN, ON RADIATION-INDUCED LIPID PEROXIDATION AND PERMEABILITY IN LIPOSOMES

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Effects of two membrane-stabilizing agents, cholesterol and cepharanthin, on radiation-induced lipid peroxidation and membrane permeability were examined. Radiation-induced lipid peroxidation caused an increase in membrane permeability in phosphatidylcholine liposomes. The presence of cholesterol in liposomal membranes caused a decrease in the degree of membrane permeability in spite of an increased lipid peroxidation. On the other hand, cepharanthin suppressed both lipid peroxidation and the changes in permeability induced by radiation. The membrane-stabilizing effect of cholesterol against radiation-induced changes in permeability seemed to depend on the rigidification of membranes, which was estimated by ESR studies. Cepharanthin suppressed the degree of membrane permeability mainly by inhibiting the radiation-induced lipid peroxidation. However, cepharanthin did not exhibit a radical-trapping ability.

Introduction

It is generally regarded that ionizing radiation or ultraviolet light (UV)-radiation causes peroxidation of membrane lipids [1–5]. Extensive studies have suggested that lipid peroxidation might play an important role in radiation-induced damages of membrane functions. Microsomal drug metabolizing activities decreased with increasing radiation-induced lipid peroxidation [6,7]. The degree of hemolysis of erythrocytes was related to lipid peroxidation induced by X- or UV-irradiation [4,8]. Lipid peroxidation was also related to changes in membrane permeability both in biomembranes [9] and in model membranes [4,10]. Although these studies indicate the importance of lipid peroxidation in radiation-induced membrane damages, the exact mechanisms involved in the processes are still unclear.

Model membrane systems are suitable for the investigation of the processes of membrane damage because each membrane component can be analyzed separately. Therefore, examinations on liposomes would be one of the effective ways to elucidate a role of lipid peroxidation in radiation-induced damages to membranes. Previously we reported that glucose permeability of liposomal membranes increased proportionally to radiation-induced lipid peroxidation [10]. The increase in membrane permeability was assumed to be due to perturbation in the lipid bilayer by peroxidation of unsaturated fatty acid side chains [12,13].

In the present study, we have attempted to examine the effects of cholesterol and cepharanthin on the relationship between radiation-induced lipid peroxidation and changes in membrane permeability in liposomes. Cholesterol is often found in high concentrations in a variety of membrane systems

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Abbreviation: DPPH, 1,1-diphenyl-2-picrylhydrazyl.

and can reduce the membrane permeability both in model membranes [14,15] and in biomembranes [16,17]. Extensive studies have indicated that cholesterol tends to rigidify the membrane phospholipids above their phase transition temperature [18–20]. Therefore, it is likely to alter the relationship between lipid peroxidation and membrane permeability after irradiation. Cepharanthin, a biscoclaurin alkaloid, is a clinical drug which has antihemolytic activity. It has been indicated that the alkaloid suppresses the potassium release from red cells or mitochondria induced by snake venom, lysophosphatidylcholine and heavy metal ions [21,22]. However, the molecular mechanism of the action of cepharanthin on the membranes is still unclear. Effects of the agents on membrane fluidity and the radical-trapping ability of the agents were also examined in relation to the oxidative damages produced in liposomes.

Materials and Methods

Commercial soybean phosphatidylcholine was twice washed with acetone as described previously [11]. The acetone-washed phosphatidylcholine was then applied on a silicic acid column (3 × 21 cm) and fractionated with a step-wise gradient of $CHCl_3$-CH_3OH according to the method of Hanahan et al. [23]. The purity of phosphatidylcholine was checked by thin-layer chromatography on a silica gel plate with $CHCl_3/CH_3OH$/8 M NH_4OH/H_2O (24:16:2:1, by vol.) as a solvent system. The fractions which gave a single spot of phosphatidylcholine were collected and stored at −20°C until use. The fatty acid composition of purified phosphatidylcholine was as follows: 14.3% palmitic acid, 4.3% stearic acid, 7.8% oleic acid, 63.9% linoleic acid, 6.9% linolenic acid.

The small amount of phosphatidylcholine (10–20 mg) and appropriate quantities of cholesterol or cepharanthin were mixed in a round-bottomed flask to the desired molar ratio. The interior of the flask was coated with a thin film of lipids by evaporation of their solution in chloroform. After removal of the solvent, a small volume (12.5 μl/mg of phosphatidylcholine) of 0.3 M glucose in 0.15 M NaCl was added. The thin film of dried lipids was then dispersed by agitation with a Vortex mixer under a nitrogen atmosphere. To remove most of the untrapped glucose, the liposomal suspensions were dialyzed overnight against 0.15 M NaCl. The dialyzed suspensions were diluted to phosphatidylcholine concentrations of 0.8 mg/ml with 0.15 M NaCl.

The liposomal suspensions were then irradiated at room temperature using a 1900 Ci ^{60}Co γ-ray beam at various dose rates, usually at 1.6 Gy/min. They were put on ice for 2 h until measurements were performed to accumulate the released glucose.

Radiation-induced lipid peroxidation was determined as malondialdehyde formation by thiobarbituric acid reaction [24] as described previously [11].

Glucose was assayed spectrophotometrically according to the method of Bergmeyer et al. [25] as described previously [11]. The percentage of radiation-induced glucose efflux was expressed as

$$(1 - (G_r/G_c)) \times 100$$

where G_c is the amount of glucose inside the unirradiated liposomes and G_r is the amount inside the irradiated liposomes.

Effects of the agents on membrane fluidity were estimated from order parameter S of 5-doxylstearic acid used as the spin label [26]. Order parameter S represents the degree of molecular motion of fatty acid side chains. According to Hubbel et al. [26], S can be expressed by the following equation:

$$S = (3\cos^2\theta - 1)/2$$

where θ is the mean angle between the axis perpendicular to the membrane surface and the longitudinal axis of the spin label. Therefore, the suppression of molecular motion of fatty acid side chains results in an increase of the S value (a decrease of θ) which manifests a decrease in membrane fluidity. Labeled liposomes were prepared in 0.15 M NaCl with 10 mM sodium phosphate buffer (pH 7.4) from dried film of the mixture of phosphatidylcholine, spin label and various quantities of cholesterol or cepharanthin. Final concentrations of phosphatidylcholine and spin label were 2 mg/ml and 40 μg/ml, respectively. Labeled liposomes were transferred to a capillary tube used as a sample cell. The sample cell was sealed and

centered in a sample holder, which was then inserted in the microwave cavity. All ESR spectra were obtained on a JEOL PE-1X ESR spectrometer at X band with the microwave cavity.

The radical-trapping ability of the agents was estimated using a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Blois [27]. The reaction mixture contained the following reagents: 1 ml of 0.1 mM DPPH in ethanol, 1 ml of sample in ethanol and 1 ml of 0.1 M acetate buffer (pH 5.5). After mixing, the decrease in absorption at 520 nm was measured using a Hitachi 557 double beam spectrophotometer.

Soybean phosphatidylcholine was purchased from Wako Pure Chemical, Co. Cholesterol was obtained from Tokyo Kasei Kogyo Co., Ltd. Silicic acid was obtained from Mallinckrodt Inc. 5-Doxylstearic acid was obtained from Syva Co. Silica gel plates (60 F-254) were obtained from Merk Inc. Cepharanthin was kindly supplied as a gift from Kaken-Drug Co., Ltd. All other chemicals used in the present study were commercial grade.

Results

Effect of radiation doses on lipid peroxidation and membrane permeability

Effect of radiation doses on lipid peroxidation (Fig. 1A) and membrane permeability (Fig. 1B) was examined at a dose rate of 1.6 Gy/min. In purified phosphatidylcholine liposomes (control), both lipid peroxidation and glucose efflux increased linearly with increasing radiation doses and a distinct threshold dose at about 15 Gy was observed in both processes. The relationship between lipid peroxidation and glucose efflux was linear through the origin ($r = 0.958$). These results were similar to those in liposomes prepared from acetone-washed phosphatidylcholine [11].

The degree of radiation-induced glucose efflux was markedly suppressed by cholesterol (20 mol%) or cepharanthin (0.5 mol%). Radiation-induced lipid peroxidation was also suppressed by cepharanthin, while increased lipid peroxidation as compared with control. A threshold dose for the lipid peroxidation was not seen in liposomes containing cholesterol.

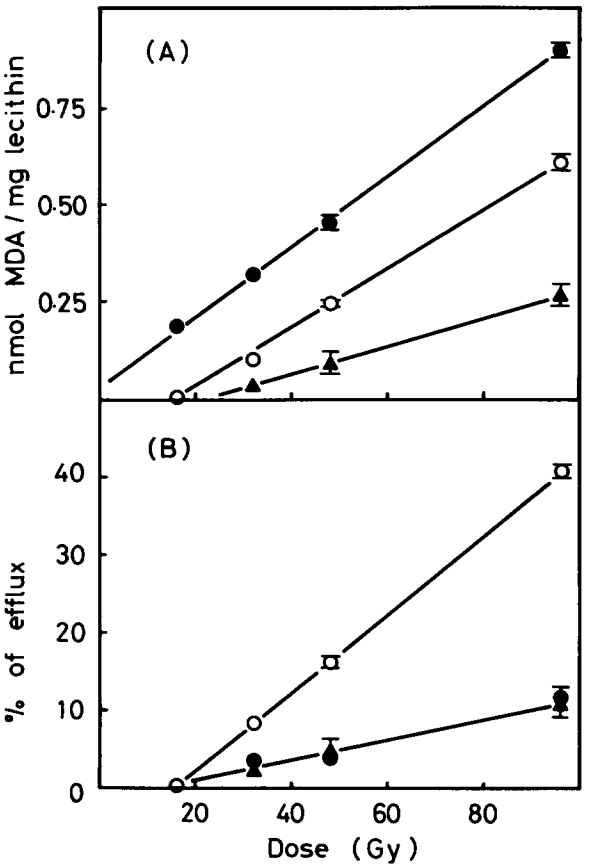

Fig. 1. Effect of radiation-doses on lipid peroxidation (A) and glucose efflux (B) in liposomes. Purified phosphatidylcholine (lecithin) liposomes (○), liposomes containing 20 mol% cholesterol (●) or 0.5 mol% cepharanthin (▲) were irradiated at a dose rate of 1.6 Gy/min.

Effect of different dose rates on lipid peroxidation and membrane permeability

It is characteristic of radiation-induced lipid peroxidation that, at a constant dose, a protracted irradiation is more effective than an acute one [3,7,11]. The inverse dependency of lipid peroxidation on dose rate is shown in Table I (at a total dose of 16 Gy). As shown in Fig. 1, lipid peroxidation could not be detected after irradiation at a total dose of 16 Gy (at a dose rate of 1.6 Gy/min) in phosphatidylcholine liposomes. However, a significant increase in lipid peroxidation was observed after irradiation at a total dose of 16 Gy at lower dose rates. A similar enhancement by the protracted irradiation was seen in liposomes containing cholesterol or cepharanthin. The degree of

TABLE I

DOSE RATE EFFECTS ON LIPID PEROXIDATION AND GLUCOSE EFFLUX IN LIPOSOMES

Purified phosphatidylcholine liposomes and liposomes containing 20 mol% cholesterol and 0.5 mol% cepharanthin were irradiated at a total dose of 16 Gy. Each value represents the average of three experiments with the standard error of mean. MDA, malondialdehyde.

Dose rate (Gy/min)	Lipid peroxidation (nmol MDA/mg lipid)		
	Control	+Cholesterol	+Cepharanthin
0.05	1.42±0.01	2.06±0.02	1.00±0.01
0.1	0.77±0.00	1.19±0.02	0.67±0.01
0.2	0.40±0.01	0.75±0.02	0.26±0.01
0.8	0.09±0.02	0.12±0.00	0.07±0.00
	Glucose efflux (percent of total content)		
	Control	+Cholesterol	+Cepharanthin
0.05	75.2±0.8	19.0±0.8	45.8±3.5
0.1	41.2±2.1	13.0±0.1	24.4±0.4
0.2	19.8±1.2	8.3±0.4	9.0±0.7
0.8	6.2±0.7	4.1±0.3	3.0±0.0

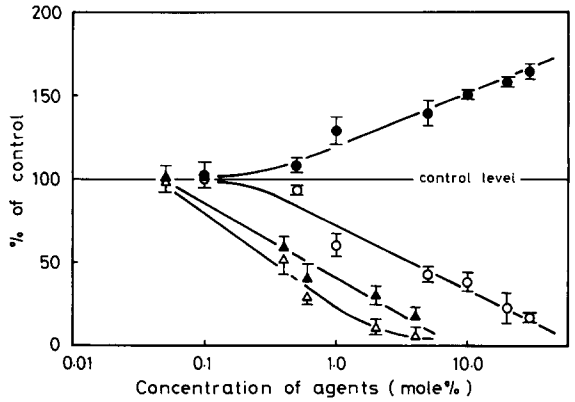

Fig. 2. Concentration effect of cholesterol and cepharanthin on lipid peroxidation and glucose efflux in liposomes. Radiation-induced lipid peroxidation in liposomes containing cholesterol (●) or cepharanthin (▲) and glucose efflux in liposomes containing cholesterol (○) or cepharanthin (△) were examined at a total dose of 96 Gy (dose rate: 1.6 Gy/min). The degrees of radiation-induced lipid peroxidation and glucose efflux in control (purified phosphatidylcholine liposomes) were 0.610±0.005 nmol of malondialdehyde formed/mg of phosphatidylcholine and 40.9±1.0%, respectively.

lipid peroxidation at each dose rate was suppressed by cepharanthin and enhanced by cholesterol as compared with control.

The enhancement of glucose efflux was also seen in lower dose rates (Table I). In liposomes containing cholesterol, however, the degree of the enhancement by the protracted irradiation was less than that in control or in liposomes containing cepharanthin. At each dose rate, the degree of radiation-induced glucose efflux in liposomes containing cholesterol or cepharanthin was less than that in control.

Concentration effect of cholesterol and cepharanthin on lipid peroxidation and membrane permeability after irradiation

Fig. 2 shows the effect of different concentrations of cholesterol and cepharanthin on lipid peroxidation and glucose efflux in liposomes after irradiation at a total dose of 96 Gy at a dose rate of 1.6 Gy/min. Up to the concentration at 0.5 mol%, cholesterol had no effect on the degree of lipid peroxidation and glucose efflux. A significant enhancement of lipid peroxidation and a suppression of glucose efflux were seen at 1 mol% of cholesterol, and larger effects were observed at higher concentrations. On the other hand, an increase of cepharanthin in liposomal membranes caused a decrease in the degree of radiation-induced lipid peroxidation and glucose efflux (at more than 0.05 mol%). The median effective concentrations of cepharanthin to suppress lipid peroxidation and glucose efflux were at about 0.5 mol% and 0.3 mol%, respectively.

Effect of cholesterol and cepharanthin on membrane fluidity of liposomes

It is generally regarded that cholesterol causes a decrease in membrane fluidity above phase transition temperatures of membrane lipids [18–20] and that the rigidification of membranes by cholesterol is the main cause of the decreased permeability. On the other hand, little is known about the effect of cepharanthin on membrane fluidity.

Fig. 3 shows the concentration effect of cholesterol and cepharanthin on order parameter S of 5-doxylstearic acid in liposomal membranes.

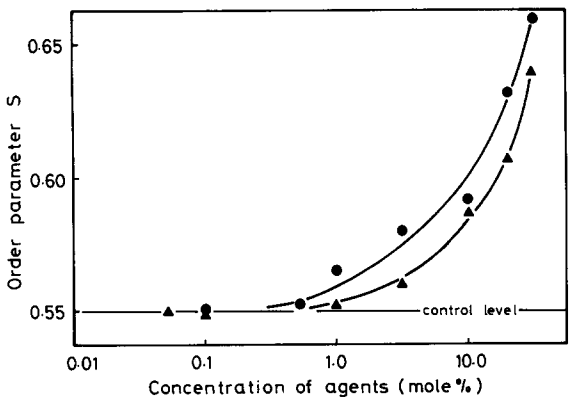

Fig. 3. Effect of different concentrations of cholesterol and cepharanthin on order parameter S of 5-doxylstearic acid in liposomal membranes. Labeled liposomes containing cholesterol (●) or cepharanthin (▲) were prepared by the method described in Materials and Methods. Order parameter S was calculated using the following equation:

$$S = \frac{T_{\parallel} - T_{\perp}}{T_z - T_x} \cdot \frac{T_z + 2T_x}{T_{\parallel} + 2T_{\perp}}$$

where $T_{\parallel}$ and $T_{\perp}$ are hyperfine interactions estimated from the observed ESR spectrum, T_z (=32.9 G) and T_x (=5.9 G) are the elements of hyperfine tensors of the spin label.

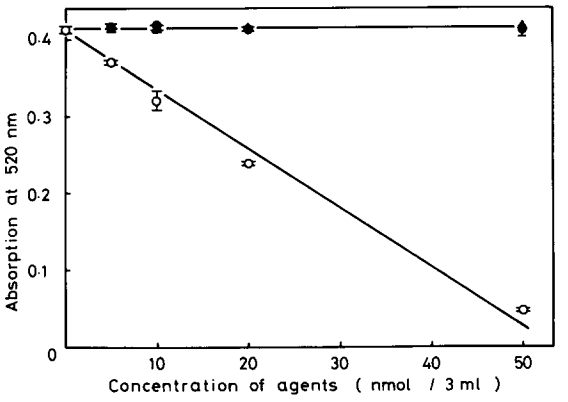

Fig. 4. Radical-trapping ability of agents. The reaction mixture contained 100 nmol DPPH and various quantities of α-tocopherol (○), cholesterol (●) or cepharanthin (▲) in 3 ml as described in Materials and Methods.

Up to the concentration at about 0.5 mol%, both agents had no effect on the S value. At higher concentrations, the S value increased with increasing cholesterol or cepharanthin.

These data indicate that cepharanthin also causes a decrease in membrane fluidity, however it was less effective than cholesterol. The minimum concentration of cholesterol that affected membrane fluidity was almost the same that affected membrane permeability, while cepharanthin caused a significant suppression of the changes in permeability under the concentrations that affected membrane fluidity (Figs. 2 and 3).

Radical-trapping ability of agents

Since radiation-induced lipid peroxidation is due to the interaction between free radicals formed by radiation and unsaturated fatty acids in membrane lipids, the presence of radical scavengers such as superoxide dismutase, tinoridine or α-tocopherol causes decrease in lipid peroxidation [3,7,11]. It is important to investigate the radical-trapping ability of cepharanthin because it suppresses radiation-induced lipid peroxidation effectively as described above.

In order to estimate the radical-trapping ability of agents, we used DPPH solution which exhibited deep violet color (absorption maximum was at 520 nm in 67% ethanol). The color is due to the vibration of unpaired electron so that the DPPH solution is decolorized when radical scavengers are added [26]. As shown in Fig. 4, α-tocopherol trapped DPPH in the ratio of 1 : 2, while cepharanthin and cholesterol did not decolorize DPPH at all. The results suggest that neither cepharanthin nor cholesterol has radical-trapping ability.

Discussion

Recent studies in model membranes have suggested that lipid peroxidation causes an increase in membrane permeability [4,10–13]. Although the exact process of peroxidation-induced changes in permeability has not been well understood, it is supposed that perturbation of lipid bilayer such as formation of conjugated double bonds or breaks in fatty acid side chains may be responsible for the changes in barrier function of membranes. The responsibility of lipid peroxidation for the radiation-induced changes in membrane permeability was indicated by our previous studies with radical scavengers, i.e. there was one-to-one relationship

between the inhibition of radiation-induced lipid peroxidation with radical scavengers and the suppression of changes in permeability in liposomes [11]. The efficacy of radical scavengers against oxidative damages to membranes has important pharmacological implications. On the other hand, mechanisms involved in the membrane-stabilizing effect of cholesterol and cepharanthin seemed to be different from those of radical scavengers.

Presence of cholesterol in liposomal membranes decreased the degree of radiation-induced changes in permeability, in spite of an increased lipid peroxidation. These results suggest that the suppression of changes in membrane permeability is due to the rigidification of membranes by cholesterol because the suppression is seen in the concentration range where cholesterol affects membrane fluidity (Figs. 2 and 3). Therefore the membrane-stabilizing effect of cholesterol against radiation-induced changes in permeability appears to be due to its action on membrane fluidity, and the direct relationship between lipid peroxidation and membrane permeability in liposomes is lost by the presence of cholesterol.

There are at least two possible mechanisms of the increased lipid peroxidation by the presence of cholesterol. First, cholesterol peroxides might cause peroxidation of membrane lipids, or cholesterol peroxides themselves might give a positive value in the thiobarbituric acid reaction. However, it has been indicated that cholesterol peroxides and unsaturated phospholipids easily form hydrogen bonded complexes and such complexes do not give rise to formation of malondialdehyde [28]. Actually, addition of cholesterol irradiated in chloroform into liposmal membranes did not cause formation of malondialdehyde as compared with non-added control (data not shown). Second, the efficiency of the radical reaction in the lipid bilayer might be altered by the presence of cholesterol. It is supposed that membrane lipid molecules are packed by the presence of cholesterol [18–20]. Such molecular packing is likely to alter the efficiency of the radiation-induced chain reaction of radicals.

Cepharanthin suppressed both radiation-induced lipid peroxidation and changes in membrane permeability in liposomes. The membrane-stabilizing effect of cepharanthin against radiation-induced changes in permeability seemed to depend mainly on the inhibition of lipid peroxidation and additionally on the rigidification of membranes, because the degree of suppression of glucose efflux was higher than that of lipid peroxidation in the concentration range where cepharanthin affected membrane fluidity (Figs. 2 and 3). Whether cepharanthin can trap the free radicals produced during water radiolysis is not clear. Scavenging of free radicals, however, may not be involved in the decrease in lipid peroxidation because cepharanthin does not trap DPPH. It has been indicated that cepharanthin suppresses the changes in membrane permeability induced by snake venom, lysophosphatidylcholine or heavy metal ions [21,22]. The presence of cepharanthin in lipid bilayer might interfere through the interactions between membrane lipids and effectors, such as free radicals, phospholipase and so on. Although accurate mechanism involved in the membrane-stabilizing effect of cepharanthin is still unclear, it gives rise to a pharmacological interest as an effective protector against membrane damages caused by alterations in the lipid bilayer.

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