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The effect of ionic strength on the lipid peroxidation of porcine intestinal brush-border membrane vesicles

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The effects of salt concentration gradient (inside to outside) on the lipid peroxidation of porcine intestinal brush-border membrane vesicles have been studied and several interesting features of the peroxidation have been elucidated. The addition of dithiothreitol and Fe^{2+} is far more effective in induction of the lipid peroxidation than any of the other metal ion species tested (Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Cr^{3+}). The peroxidation rate of the membrane vesicles induced by dithiothreitol plus Fe^{2+} was sensitive for the incubation temperature and was increased with increase of the temperature. Imposition of an inward salt concentration gradient on the membrane vesicles preloaded with 300 mM mannitol by addition of 100 mM chloride of K^+, Na^+, Li^+, Rb^+, NH_4^+ or choline to medium produces a very large reduction of the lipid peroxidation induced by dithiothreitol plus Fe^{2+}. The membrane peroxidation is depressed more with the mannitol (300 mM)-preloaded vesicles than with the K_2SO_4 (100 mM)-preloaded vesicles when they are incubated in medium containing 20–100 mM of K_2SO_4. Addition of membrane-permeant anions such as SCN^- and I^-, but not addition of NO_3^-, to incubation medium has been found to decrease markedly the lipid peroxidation of the mannitol-preloaded vesicles. From these results it is suggested that the lipid peroxidation of the brush-border membranes by addition of dithiothreitol plus Fe^{2+} is sensitively changed with change in ionic strength.

Introduction

The problem of membrane lipid peroxidation is of interest in context with aging of living cells as well as tissue injury [1–3].

Recently, it has been reported by several investigators that peroxidation of phospholipids of artificial and biological membrane systems induces notable perturbation of the lipid organization of these systems [4–7] and in consequence modifications of the activities of membrane-bound enzymes, e.g., activation of glucuronyl transferase in liver membranes [8], thiamine diphosphatase in rat brain [9] and adenylate cyclase in rat cerebral cortex [10,11] and inhibition of $(Na^+ + K^+)$-ATPase in monkey cerebral cortex [12].

Although it is generally accepted that peroxidation of the phospholipids of various cellular membranes leads to loss or disruption of the double bonds of unsaturated fatty acids combined to the lipids, the molecular mechanism and causal affiliation of the lipid peroxidation have not been well understood. Therefore, it is important to study what kinds of factors are concerned with the lipid peroxidation reaction in biological membrane systems at the molecular level.

Mowri et al. [13] have recently reported that the degree of lipid peroxidation in liposomes com-

Abbreviation: ANS, 1-anilino-8-naphthalenesulfonate.

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posed of 1-palmitoyl-2-arachidonylphosphatidylcholine induced by ascorbic acid plus Fe^{2+} is related to the physical state of lipid layers in the liposomes.

Previously, we have demonstrated [14,15] that the membrane fluidity of rabbit intestinal brush-border membrane vesicles increases by shielding of the negatively charged groups of the membrane components with increasing ionic strength of medium. It is therefore of interest to study whether change in the membrane surface charge density by addition of salts to medium influences the lipid peroxidation reaction of the membrane vesicles.

In the present study, we have explored the effects of salts on the lipid peroxidation of porcine intestinal brush-border membrane vesicles induced by dithiothreitol plus Fe^{2+} and found an interesting evidence on the correlation between the membrane surface charge and the lipid peroxidation of the membrane vesicles.

Materials and Methods

Preparation of membrane vesicles. Brush-border membrane vesicles were prepared from porcine small intestine by the calcium-precipitation method as described in our previous paper [16]. The membrane vesicles loaded with mannitol (mannitol vesicles) or K_2SO_4 (K_2SO_4 vesicles), as specified in the results, were prepared by incubating the membrane vesicles (5 mg/ml) with 10 mM Tris-HCl buffer (pH 7.4) containing 300 mM mannitol or 100 mM K_2SO_4 at 0°C for 18 h. Protein concentration was assayed by the method of Lowry et al. [17] using bovine serum albumin as standard.

Chemicals. Dithiothreitol and 2-thiobarbituric acid were purchased from Wako Pure Chemical Co. 1-Anilino-8-naphthalenesulfonate (magnesium salt, ANS) was obtained from Nakarai Chemical Co. All other reagents used were of the highest purity obtainable.

Determination of lipid peroxidation of the membranes. Lipid peroxidation of the membranes was induced by the incubation of the membrane vesicles with 10 μM $FeSO_4$ and 5 mM dithiothreitol in 30 mM Tris-HCl buffer (pH 7.4) at 37°C for 30 min, unless otherwise mentioned. The amount of thiobarbituric acid-reactive substances (as malondialdehyde) formed by the reaction was determined fluorometrically as described by Hiraiwa et al. [18] using 1,1,3,3-tetraethoxypropane as a standard, except for the use of 10% trichloroacetic acid in place of the phosphotungstic acid and H_2SO_4 mixture. The heating step in the thiobarbituric acid reaction was carried out at 90°C for 60 min.

Fluorescence measurements. Fluorescence measurements were carried out using a Hitachi MPF-4 fluorescence spectrophotometer equipped with a rhodamine B quantum counter. The sample compartment was maintained at 25°C by circulating water through the cell holder. The fluorescence intensity of the malondialdehyde-thiobarbituric acid complex was measured by recording the intensity at 553 nm with excitation of 515 nm. On the other hand, the excitation and emission wavelengths used for ANS fluorescence measurements were 350 and 490 nm, respectively. The emission light was passed through a 350 nm cut-off filter to exclude light scattering in the case of ANS fluorescence measurement. The apparent dissociation constants of the ANS-membrane complex were estimated according to the fluorescence titration procedure proposed by Wang and Edelman [19] as described in our previous paper [14].

Results and Discussion

Time-course of lipid peroxidation of the membranes

When the porcine intestinal brush-border membrane vesicles were incubated with 5 mM dithiothreitol plus 10 μM Fe^{2+} at 37°C, the amount of thiobarbituric acid-reactive substances gradually increased depending on the incubation time (Fig. 1). On the other hand, the membrane vesicles in the absence of dithiothreitol and Fe^{2+} showed no appreciable formation of thiobarbituric acid-reactive substances within 180 min.

Effect of temperature on lipid peroxidation of the membranes

Fig. 2 shows the temperature-dependence profiles of the formation of thiobarbituric acid-reactive substances of the membrane vesicles in the presence and absence of dithiothreitol plus Fe^{2+}.

As can be seen in this figure, the amount of thiobarbituric acid-reactive substances sigmoidally increases depending on the incubation temperature after the addition of dithiothreitol plus Fe^{2+}

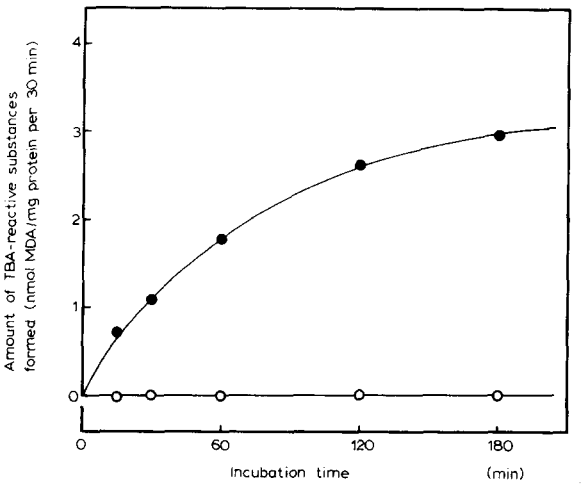

Fig. 1. Time-course of lipid peroxidation of the membranes in the presence (●) and absence (○) of 5 mM dithiothreitol and 10 μM Fe^{2+}. Membrane protein concentration was 0.465 mg/ml. TBA, thiobarbituric acid; MDA, malondialdehyde.

TABLE I

THE EFFECTS OF SPECIES OF METAL ION ON THE LIPID PEROXIDATION OF THE MEMBRANES IN THE PRESENCE AND ABSENCE OF 5 mM DITHIOTHREITOL (DTT) AT 37°C

Membrane protein concentration was 0.390 mg/ml. The concentration of metal ions was 10 μM in each system. Results are expressed as means ± S.D. of triplicate determinations. TBA, thiobarbituric acid; MDA, malondialdehyde.

Metal ions	Amount of TBA-reactive substances formed (nmol MDA/mg protein per 30 min)	
	− DTT	+ DTT
$FeSO_4$	1.07 ± 0.05	1.83 ± 0.07
$FeCl_3$	0.24 ± 0.02	0.40 ± 0.03
$CuSO_4$	0.22 ± 0.04	0.30 ± 0.02
$NiSO_4$	0.11 ± 0.02	0.14 ± 0.01
$ZnSO_4$	0.04 ± 0.01	0.06 ± 0.01
$CrCl_3$	0.04 ± 0.01	0.03 ± 0.01

to the system. From the midpoint of the plot, it is suggested that the temperature, at which the half-maximal amount of thiobarbituric acid-reactive substances is formed, is about 22°C. On the other hand, in the system without dithiothreitol plus Fe^{2+}, the formation of thiobarbituric acid substances was not observed throughout the temperature range tested.

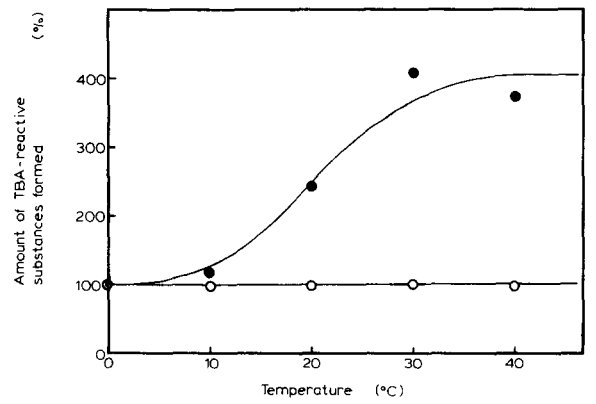

Fig. 2. Temperature dependence of lipid peroxidation of the membranes induced by dithiothreitol plus Fe^{2+}. Membrane protein concentration was 0.465 mg/ml. Temperature was varied from 0 to 50°C. The amount of thiobarbituric acid-reactive substances formed was expressed as the relative to that formed at 0°C in each system. ○, in the absence of dithiothreitol plus Fe^{2+}; ●, in the presence of 5 mM dithiothreitol plus 10 μM Fe^{2+}. TBA, thiobarbituric acid.

Metal ion specificity for membrane lipid peroxidation

Table I shows the amount of thiobarbituric acid-reactive substances formed after incubation (37°C, 30 min) of the membrane vesicles with various metal ions (10 μM each) in the presence and absence of 5 mM dithiothreitol.

Among various species of metal ion tested, Fe^{2+} was the most effective in induction of formation of thiobarbituric acid-reactive substances of the membranes in both the presence and absence of dithiothreitol, the induction being far more effective in the presence of dithiothreitol. On the other hand, 4 mM dithiothreitol alone showed no appreciable formation of thiobarbituric acid-reactive substances.

This result, which implies that a combination of Fe^{2+} and dithiothreitol is a very powerful exogenous catalytic system for lipid peroxidation of the membranes, is compatible with observations by other investigators [9–11].

Effect of 3-tert-butyl-4-hydroxyanisole on membrane lipid peroxidation

Since 3-*tert*-butyl-4-hydroxyanisole has been

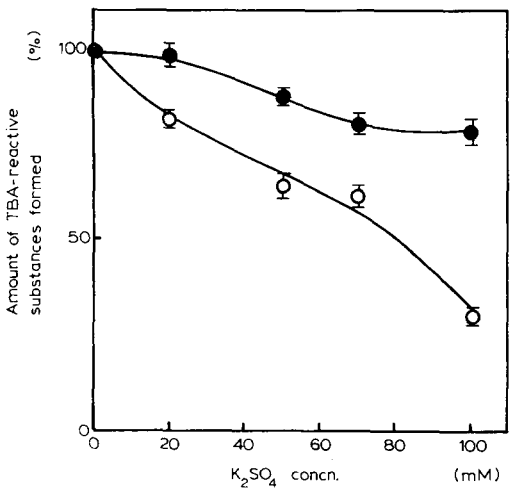

Fig. 3. K_2SO_4 concentration dependence on the membrane lipid peroxidation induced by dithiothreitol plus Fe^{2+} at 37°C. Membrane protein concentrations were 0.405 and 0.385 mg/ml for the mannitol vesicles (○) and the K_2SO_4 vesicles (●), respectively. K_2SO_4 concentration in medium was varied within a range from 20 to 100 mM. The total osmolarity of the incubation medium was maintained at 300 mM as mannitol concentration by the addition of adequate amounts of 1 M mannitol. The amount of thiobarbituric acid-reactive substances formed was expressed as the relative to that formed in the absence of K_2SO_4 in each system. The dithiothreitol and Fe^{2+} concentrations were 5 mM and 10 μM, respectively. Values are expressed as mean ± S.D. of triplicate experiments. TBA, thiobarbituric acid.

TABLE II

THE EFFECTS OF EXTRAVESICULAR SALTS ON THE LIPID PEROXIDATION OF THE MANNITOL VESICLES INDUCED BY DITHIOTHREITOL PLUS Fe^{2+}

Membrane protein concentration was 0.440 mg/ml. The final concentration of salts was 100 mM. Other experimental conditions were the same as described in the legend to Fig. 3. Results are expressed as mean ± S.D. of three membrane preparations. Abbreviations, see Table I.

Salts	Amount of TBA-reactive substances formed (nmol MDA/mg protein per 30 min)
No addition	2.24 ± 0.34
KCl	0.67 ± 0.07
NaCl	0.79 ± 0.07
NH_4Cl	0.60 ± 0.10
LiCl	0.96 ± 0.10
RbCl	0.79 ± 0.08
Choline chloride	0.97 ± 0.15

shown as a potent antioxidant [20], we tested its effect on the lipid peroxidation of the membrane vesicles induced by dithiothreitol plus Fe^{2+}. The addition of 3-*tert*-butyl-4-hydroxyanisole suppressed the formation of thiobarbituric acid-reactive substances depending on its concentration. The amount of thiobarbituric acid-reactive substances formed by mixing with 5 mM dithiothreitol plus 10 μM Fe^{2+} at 37°C (1.74 nmol/mg protein per 30 min) decreased to 0.22, 0.12 and 0.02 nmol/mg protein per 30 min by addition of 0.1, 1 and 5 mM 3-*tert*-butyl-4-hydroxyanisole, respectively.

From these results, it is clear that the formation of thiobarbituric acid-reactive substances induced by addition of dithiothreitol plus Fe^{2+} to the membrane vesicles is due to the lipid peroxidation reaction of the membrane vesicles, although the amount of thiobarbituric acid-reactive substances formed at 30 min after the addition of dithiothreitol plus Fe^{2+} was extremely low as compared to those of other membrane systems such as rat cerebral cortex [9–11] and rabbit liver microsomes [21].

The effect of salt concentration gradient on the lipid peroxidation

Fig. 3 shows the effect of K_2SO_4 concentration gradient on the induction of lipid peroxidation of the membrane vesicles by dithiothreitol plus Fe^{2+}.

In the case of the mannitol vesicles, the amount of thiobarbituric acid-reactive substances formed by dithiothreitol plus Fe^{2+} was sharply decreased with increasing K_2SO_4 concentration in the medium, reduced by about 70% in 100 mM K_2SO_4. The profile of the change in the formation of thiobarbituric acid-reactive substances against varying extravesicular K_2SO_4 concentrations proved very different in the K_2SO_4 vesicles from that in the mannitol vesicles. Formation of thiobarbituric acid-reactive substances was scarcely influenced in the lower range of extravesicular K_2SO_4 concentrations (up to 20 mM) and its reduction at 100 mM K_2SO_4 was only about 20% in the K_2SO_4 vesicles.

In this experiment, the amounts of thiobarbituric acid-reactive substances formed by addition of dithiothreitol plus Fe^{2+} were almost the same in the systems with and without 300 mM mannitol

TABLE III

THE EFFECTS OF DIFFERENT ANIONS ON THE LIPID PEROXIDATION OF THE MANNITOL VESICLES INDUCED BY DITHIOTHREITOL PLUS Fe^{2+}

Membrane protein concentration was 0.441 mg/ml. The final concentration of salts was 100 mM. Other experimental conditions were the same as described in the legend to Fig. 3. Results are expressed as mean ± S.D. of three membrane preparations. Abbreviations, see Table I.

Salts	Amount of TBA-reactive substances formed (nmol MDA/mg protein per 30 min)
No addition	1.92 ± 0.19
KCl	0.58 ± 0.07
KBr	0.75 ± 0.11
KF	1.32 ± 0.22
KNO_3	1.46 ± 0.13
KSCN	0.14 ± 0.08
KI	0.24 ± 0.02

in the vesicles (1.20 ± 0.07 and 1.32 ± 0.06 nmol/mg protein per 30 min for the systems with and without 300 mM mannitol, respectively). This indicates that mannitol, which was used to load the vesicles, did not affect the membrane lipid peroxidation by dithiothreitol plus Fe^{2+}. Furthermore, the addition of 300 mM mannitol to the extravesicular medium also did almost not influence the formulation of thiobarbituric acid-reactive substances (1.10 ± 0.08 nmol/mg protein per 30 min).

Next, we explored the effect of several monovalent cations in chlorides on the formation of thiobarbituric acid-reactive substances of the mannitol vesicles induced by dithiothreitol plus Fe^{2+}. As can be seen in Table II, the amount of thiobarbituric acid-reactive substances formed was gradually reduced to an almost comparable level by these salts. In this experiment, all of these salts used did not affect the fluorescence measurement of the malondialdehyde-thiobarbituric acid complex.

From these results, it is suggested that the membrane lipid peroxidation is sensitive to the change in charge density on the membrane surface. Namely, modification of the electrostatic property of the membranes such as neutralization of the surface negative charge by a high concentration of salt [22] may favor the suppression of the formation of thiobarbituric acid-reactive substances.

The effect of anions on the lipid peroxidation

As shown in Table III, 100 mM of I^- or SCN^- in incubation medium reduced the lipid peroxidation of the mannitol vesicles far more greatly than other anions such as Cl^-, Br^-, F^- and NO_3^-. This concentration of I^- and SCN^- in the medium also proved not to have any influence on the subsequent fluorescence measurement of the malondialdehyde-thiobarbituric acid complex. This result may imply that lipid peroxidation is extremely suppressed as the interior of the vesicles becomes negative with respect to the outside, because I^- and SCN^- are anions that readily penetrate into biological membrane vesicles [23]. The anion, NO_3^-, also is considered to be membrane-permeant [23,24]. But this anion was far less effective in reduction of the formation of thiobarbituric acid-reactive substances than the other two permeant anions.

Then, the details of concentration dependence of the inhibitory effect of these anions, NO_3^-, SCN^- and Cl^-, on the formation of thiobarbituric acid-reactive substances were investigated (Fig. 4). It is noted from the graphs that NO_3^- is the anion essentially distinct in the mode of de-

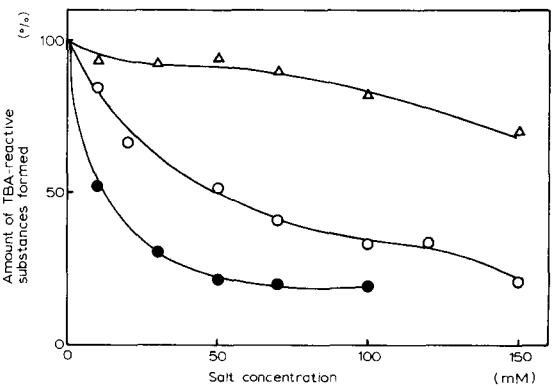

Fig. 4. Dependence of membrane lipid peroxidation on anions and their concentration. Membrane protein concentration was 0.425 mg/ml. The concentration was varied up to 150 mM. The values of thiobarbituric acid-reactive substances formed are expressed as the relative to that obtained in the absence of salts in each system. ○, KCl; ●, KSCN; △, KNO_3. Other experimental conditions were the same as described in the legend to Fig. 3. TBA, thiobarbituric acid.

TABLE IV

THE EFFECTS OF DIFFERENT ANIONS ON THE LIPID PEROXIDATION OF THE MANNITOL VESICLES IN THE SYSTEM OF ASCORBIC ACID PLUS Fe^{2+}

Membrane protein concentration was 0.404 mg/ml. The final concentration of salts was 100 mM. Concentration of ascorbic acid and Fe^{2+} were 100 and 10 μM, respectively. Other experimental conditions were the same as the case of dithiothreitol plus Fe^{2+}. Results are expressed as mean ± S.D. of three membrane preparations. Abbreviations, see Table I.

Salts	Amount of TBA-reactive substances formed (nmol MDA/mg protein per 30 min)
No addition	2.36 ± 0.01
KCl	1.76 ± 0.05
KNO_3	1.83 ± 0.01
KSCN	0.69 ± 0.02
KI	0.34 ± 0.01

TABLE V

THE EFFECTS OF SALTS ON THE ANS-BINDING AFFINITY FOR THE MANNITOL VESICLES AT 25°C

The apparent dissociation constants (K_d) of the ANS-membrane complex were determined from the slope in the double reciprocal plots of fluorescence intensities versus ANS concentrations. Membrane protein concentration was 0.142 mg/ml. ANS concentration was varied from 3.32 to 62.5 μM. Salt concentration was 100 mM. Change in the osmolarity of medium by addition of salts was corrected with addition of mannitol. Results are expressed as mean ± S.D. of three membrane preparations.

Salts	K_d (μM)
No addition	71.1 ± 1.20
KCl	51.1 ± 1.01
KBr	51.1 ± 1.12
KF	51.1 ± 1.15
KSCN	63.3 ± 1.05
KNO_3	57.8 ± 1.07
KI	55.6 ± 1.05

pression of lipid peroxidation from the other two over 150 mM of their concentrations. The reason for the difference is not known at present. NO_2^- was checked as a possible contaminant in the reagent, KNO_3, on its effect on the inhibitory action of permeant anion on the formation of thiobarbituric acid-reactive substances determined with the systems containing dithiothreitol and Fe^{2+}. But NO_2^- was conclusively without effect on the inhibitory action of SCN^- (100 mM) either at 50 or 500 μM (data not shown).

The uniqueness of NO_3^- among the permeant anions in the effect on the peroxidation of membrane lipid has also been demonstrated in another system containing ascorbate in place of dithiothreitol (Table IV). NO_3^- (100 mM) had no effect on the formation of thiobarbituric acid-reactive substances of the membranes in incomplete systems consisting either one of Fe^{2+} and dithiothreitol or ascorbate (data not shown). From this experimental result, it is also confirmed that the action of NO_3^- on membrane lipid peroxidation is certainly different from other permeant anions such as I^- and SCN^-.

ANS fluorescence measurement

Finally, we have checked using a fluorescence probe, ANS, whether the addition of these salts to the membrane vesicles induces modification of the membrane surface charge density and/or membrane potential, because it has been well recognized that the ANS fluorescence in biological membrane systems conveniently reflects the change in the nature of the membrane surface [14,15,25].

As can be seen in Table V, the binding affinity of ANS molecules for the membranes was markedly increased by addition of salts, but there was a distinct difference in the effectiveness on ANS-binding affinity between membrane-permeant (I^-, SCN^- and NO_3^-) and less permeant (Cl^-, Br^- and F^-) anions. These results suggest that a decrease in surface charge density as a result of binding of ions to the membrane surface will lead to a reduction in the electrorepulsion force between ANS and the membranes, resulting in an increase in the binding affinity of the dye molecules for the membranes. On the other hand, the difference in effect of permeant and less permeant anions on the ANS-binding affinity is probably explained as due to a difference in the degree of anion-generated membrane potential change, as reported with the membrane of *Physarum polycephalum* [26].

On the basis of all these results, it seems likely that the nature of membrane surface charge is one of the important elements controlling lipid peroxidation reaction of porcine intestinal

brush-border membranes, although it is difficult at present to know whether the membrane charge density is related directly or indirectly to the mechanism of membrane lipid peroxidation.

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