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Effect of Lipid Peroxidation on the Fluidity of Erythrocyte Ghost and Phospholipid Liposomal Membranes

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Abstract □ The effects of lipid peroxidation on the fluidity of the lipid bilayers of the human erythrocyte ghosts and egg-lecithin phospholipid liposomes have been studied. For the measurements of the peroxidation extent and the fluidity of the membranes, the thiobarbituric acid-reactive substances and the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene labelled into the membrane were employed, respectively. The lipid peroxidation was performed in hypoxanthine/xanthine oxidase/ferrous ion, and hydrogen peroxide/ferrous ion systems. The results of these experiments show that both of the xanthine oxidase and hydrogen peroxide systems effectively induced the lipid peroxidation of the ghosts, and the liposomal membranes, respectively. The lipid peroxidation decreased the fluidity of the membranes, especially at the very early stage of the peroxidation reaction. The decrease in the fluidity of membrane by the lipid peroxidation has been ascribed to the alteration of the polyunsaturated acyl chains of lipids and cross-linkings among the membrane components. However, under drastic condition of lipid peroxidation, the fluidity of the membrane rather increased possibly due to the deterioration of the membrane integrity by the peroxidation. Morphological change of the erythrocyte on peroxidation has also been observed.

Keywords □ Lipid peroxidation, fluidity of membranes, erythrocyte ghosts, liposomes, fluorescence polarization, morphology

Recently, there have been extensive researches on the effects of peroxidation on the structure and function of the biological membranes[1-5]. The peroxidation of biological membranes is a highly deteriorative process through various free-radical-mediated chain reactions. It is believed that membrane lipids are the major target for cellular damage induced by oxygen radicals and the peroxidation of polyunsaturated lipids in biological membranes is thought to constitute a serious threat to the membrane integrity. Lipid peroxidation of the biological membranes has also been attributed to exert a primary effect in the process of a variety of pathological events[6-10].

Lipid peroxidation of biological membranes results in changes in their lipid organization such as a descrease of the lipid fluidity and increase in the permeability of the membranes[11-14]. Moreover, many evidences showing the damage of membrane proteins and DNA by peroxidation have been reported, i.e., protein fragmentation, intermolecular cross-links formation, amino acid modification and conformational changes[15-18]. These alterations of the structure of the biological membranes should induce some modifications of their functions.

Therefore, it is important to study the effects of lipid peroxidation on these dynamic features in the membrane components in order to understand the molecular mechanisms mediating the modification of the membrane functions induced by the lipid peroxidation. Considerable efforts have been made along this line. However, they are still poorly un-

derstood.

In this study, human erythrocyte ghosts were employed for peroxidation, and liposomes of egg-yolk lecithin were also employed for comparison. Red blood cells offer a number of advantages for peroxidation studies; they are the most readily available source of a pure membrane in human body, rich in polyunsaturated fatty acids, and oxygen radicals are produced continually in red cells by hemoglobin autooxidation. For the measurement of the fluidity of the interior of the lipid bilayers of the biological membranes, the fluorescence polarization of the 1,6-diphenyl-1,3,5-hexatriene(DPH) labelled into the interior of the lipid bilayers of the membranes was measured and employed as the parameter indicating the fluidity.

EXPERIMENTAL

Chemicals

Egg lecithin (chloroform solution), hypoxanthine (HX), xanthine oxidase(XO from butter milk), trichloroacetic acid(TCA), 2-thiobarbituric acid(TBA), butylated hydroxytoluene(BHT), and 1,6-diphenyl-1, 3,5-hexatriene(DPH) were purchased from Sigma Chemical(U.S.A.). Hydrogen peroxide was obtained from Junsei Chemical(Japan). All other chemicals used were of reagent grade.

Preparation of human erythrocyte ghost membranes

Whole blood of adult human donors was taken into a heparinized test tube. After removing plasma by centrifuging (2500 rpm for 10 min. at 4℃), erythrocyte was washed and centrifuged three times with 10 volumes of isotonic tris buffered saline(TBS) containing 0.87% NaCl and 10 mM tris, pH 7.4 to remove buffy coats. After the erythrocyte was lysed with tris buffer, pH 7.4, erythrocyte ghosts were collected by centrifuging (2500 rpm for 10 min. at 4℃). After the final washing, the human erythrocyte ghost membranes were suspended in 10 mM tris buffer, pH 7.4, and the solutions were sonicated by an Ultrasonic Ltd sonicator at a setting of 70 μA for 5 min. Lipid contents in ghosts were extracted and determined by the method described in the following section. A ghost suspension was prepared at a concentration of 0.5 mg lipids/m*l* ghosts.

Lipid extraction of erythrocytes and preparation of liposome of erythrocyte lipids

Whole blood from adult human donors was taken and anticoagulated. It was washed with TBS and centrifuged three times. One mililiter of the final stroma was peroxidized in 10 m*l* of TBS containing hydrogen peroxide or hypoxanthine and xanthine oxidase for 30 min with ferrous ion. The erythrocyte or the peroxidized erythrocyte was extracted with 10 m*l* of ethyl ether with vigorous shaking in a separating funnel for 10 min. The ether extract of the peroxidized erythrocyte lipids was reduced to dryness by rotary evaporation, forming a film on the inside of a round-bottom flask. The liposomes were formed by suspending the erythrocyte lipid film in an appropriate quantity of 10 mM tris buffer (pH 7.4) by sonication for 20 min. at room temperature. The concentration of the extracted lipids was approximately weighed and adjusted to 0.5 mg/m*l* in liposomal suspensions.

Preparation of phospholipid liposomes

A chloroform solution of the phospholipids was reduced to dryness by rotary evaporation, forming a film on the inside of a round-bottom flask. The sample was stored overnight under vacuum to remove the residual solvent. The liposomes were formed by suspending the phospholipid film in an appropriate quantity of 10 mM phosphate buffer (pH 7.4) and sonication for 15 min on the ice bath. The final concentration of phospholipid was 0.5 mg/m*l*.

Peroxidation systems

To 15 m*l* of the erythrocyte ghost suspensions or phospholipid liposomes was added various concentration of hypoxanthine/xanthine oxidase system or hydrogen peroxide and/or ferrous chloride. The suspensions were incubated at 37℃. During the time course of incubation, some parts of the sample were taken out at specified intervals, and thiobarbituric acid-reactive substances(TBARS) and the fluorescence polarization of DPH labelled into the lipid bilayers of the membranes were measured. The TBARS were used as an index of lipid peroxidation, and the fluorescence polarization of DPH was the parameter of the fluidity of the lipid bilayers of the membrane.

The TBA test

Erythrocyte ghost suspensions were incubated at 37℃ and TBARS formed in the suspensions during the time course were measured as follows. On adding 0.14% butylated hydroxytoluene to the reaction mixture, the peroxidation reaction was stopped. An adequate amount of the incubated cell suspension was boiled above 80℃ temperature for 15 min. with TCA-HCl-TBA solution (0.375% thiobarbituric acid/15% trichloroacetic acid/0.25 N HCl). After boiling, the sample was cooled to room temperature, centrifuged at 1000×g for 10 min and the absorbance of the supernatant was measured at 532 nm. Unless otherwise indicated, the data are represented as the average of five measurements.

Morphological change of erythrocyte induced by peroxidation

Fresh peripheral blood, drawn into a vacuum test tube containing ethylene-diaminetetraacetic acid disodium salt (EDTA·2Na), was washed three times with 10 volumes of 150 mM NaCl/5 mM Hepes-NaOH (pH 7.4). The final stroma was diluted by adding 150 mM NaCl/5 mM glucose/5 mM Hepes-NaOH (pH 7.4) containing H_2O_2 or HX+XO, and was incubated at 37℃ for 30 min. The final concentration of erythrocyte was 10% hematocrit. After incubation, a small amount of the cell suspension was pipetted and added into small amount of 2% glutaraldehyde dissolved in 1/30M NaH_2PO_4/Na_2HPO_4 (pH 7.4) for the fixation of cells. The shapes of the cells were observed under an optical microscope.

Fluorescence polarization measurement

The labelling of the probe into the membranes was carried out by adding an aliquots of a stock solution of DPH in tetrahydrofuran (2 mM) to the erythrocyte membrane suspensions, the liposomes of erythrocyte lipids, or phospholipid liposomes. DPH was injected, while vortexing into the suspensions to give final lipid-to-probe weight ratio of about 500. The fluorescence measurements were carried out with a Perkin-Elmer Luminescence spectrometer, Model LS 5, provided with a polarimeter and thermostated cell holder. The excitation wavelength was 358 nm and emission wavelength was 430 nm. The fluorescence polarization was calculated from the intensity measurements by:

$$P = \frac{I_{vv} - G(I_{vh})}{I_{vv} + G(I_{vh})}$$

where I_{vv} is the intensity measured when the polarizer and analyzer prism are in the vertical position and I_{vh} is the intensity when the analyzer prism is in the horizontal position. G is the correction factor given by the ratio of the vertical to the horizontal components when the excitation light is polarized in the horizontal direction. Under the experimental conditions, the lipid peroxidation itself did not produce fluorescence enough to significantly interfere with these measurements. Unless otherwise indicated, the data are represented as the average of five measurements.

RESULTS AND DISCUSSION

Lipid peroxidation in HX and XO system

The peroxidation of the human erythrocyte ghost membranes and phospholipid liposomes was initiated by incubating the samples in an aqueous solution containing various concentration of HX/XO or hydrogen peroxide with or without ferrous chloride at 37℃. With time course of the peroxidation, the TBA assay of the formed TBARS and the measurement of the changes in the fluorescence polarization of DPH labelled into the lipid layers of the ghost membranes or the phospholipid liposomes were carried out. The results of these measurements were illustrated in Figs. 1 and 2. The fluorescence polarization of the DPH labelled into the lipid bilayers of the ghost membranes was 0.300 (n=10, S.D=0.015). On the other hand, the fluorescence polarization of DPH labelled into the liposomal membranes of egg lecithin was 0.190 (n=10, S.D =0.018). This was far below than that of the erythrocyte ghosts, which means that the lipid layers of the egg-lecithin liposomes are far more fluidity than that of erythrocyte ghosts. This less fluidity of the erythrocyte ghost membranes should be ascribed to the interaction of proteins with the lipid matrix in the ghosts rather than the difference in the lipid compositions of the two systems. The TBARS were insignificant in the erythrocyte ghosts as well as in the phospholipid liposomes. This reveals that during the sample preparations, the lipid peroxidation did not occur to any significant extent.

Fig. 1 shows that the HX/XO system peroxidized

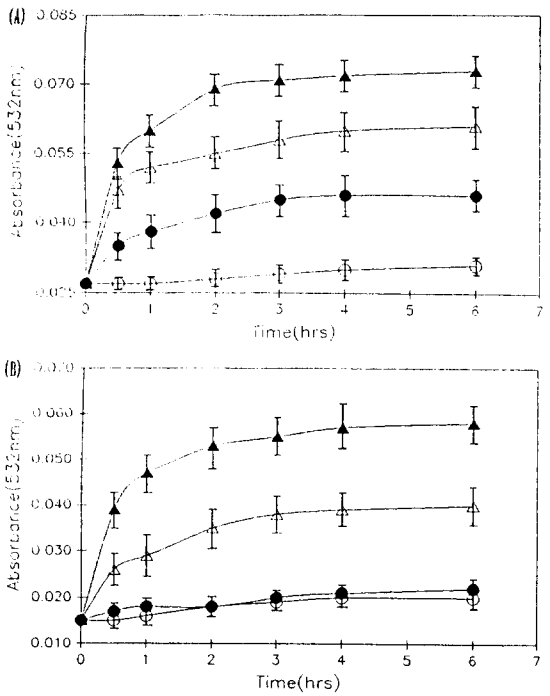

Fig. 1. Time course of the formation of TBARS during peroxidation of the erythrocyte ghosts (A) and the phosphatidyl choline liposomes (B) in HX/XO system.

○; control, ●; 0.3 mM HX+0.015 unit XO, △; 0.3 mM HX+0.015 unit XO+0.1 mM Fe^{2+}, ▲; 0.3 mM HX+0.1 unit XO+0.1 mM Fe^{2+}.

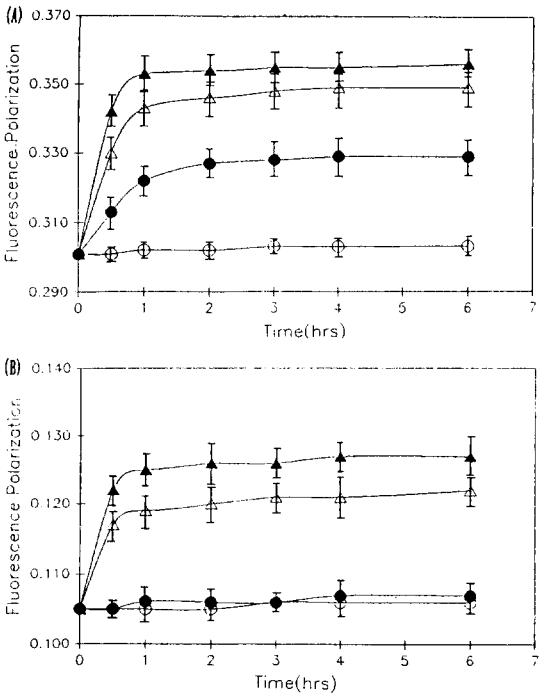

Fig. 2. Time course of the changes of the fluorescence polarization of DPH labelled into lipid bilayers of the erythrocyte ghost membranes (A) and the phosphatidyl choline liposomes (B) were peroxided in HX/XO system.

○; control, ●; 0.3 mM HX+0.015 unit XO, △; 0.3 mM HX+0.015 unit XO+0.1 mM Fe^{2+}, ▲; 0.3 mM HX+0.1 unit XO+0.1 mM Fe^{2+}.

the erythrocyte ghosts, and the higher concentration of xanthine oxidase, the more TBARS was formed, which means that the peroxidation proceeded more effectively with higher concentration of xanthine oxidase. The TBARS increased at early stage of the peroxidation and reached a plateau. On the other hand, the control of the erythrocyte ghost untreated with oxidizing system did not show any significant increase in TBARS. Fig. 2 shows that the fluorescence polarization of DPH labelled into the control of the human erythrocyte ghost membranes maintained relatively uniform values during the experiment time. However, the fluorescence polarization of DPH labelled into the human erythrocyte ghost membranes also increased rapidly at the initial stage of the peroxidation and reached a plateau value. The higher the concentration of XO, the higher values of fluorescence polarization were also observed. These results suggest that the peroxidation of human erythrocyte ghost membrane occurred with HX/XO system and the lipid peroxidation significantly decreased the fluidity of the ghost membranes. The increase in the fluorescence polarization of the probe labelled into the ghost membranes occurred more abruptly at the initial stage of the peroxidation than the increase in TBARS did. This means that the fluidity-decreasing reaction occurred at earlier stage of peroxidation prior to the formation of TBARS. It was also noted that in the absence of added ferrous ion, the lipid peroxidation was less effective. The presence of ferrous ion accelerated the lipid peroxidation. This observation agrees with the results of other experiments[19-21]. The HX/XO system generates the superoxide, and the dismutation of the superoxide produces hydrogen peroxide, which undergoes a Fenton-type reaction with the ferrous ion, producing a free radical. This free radi-

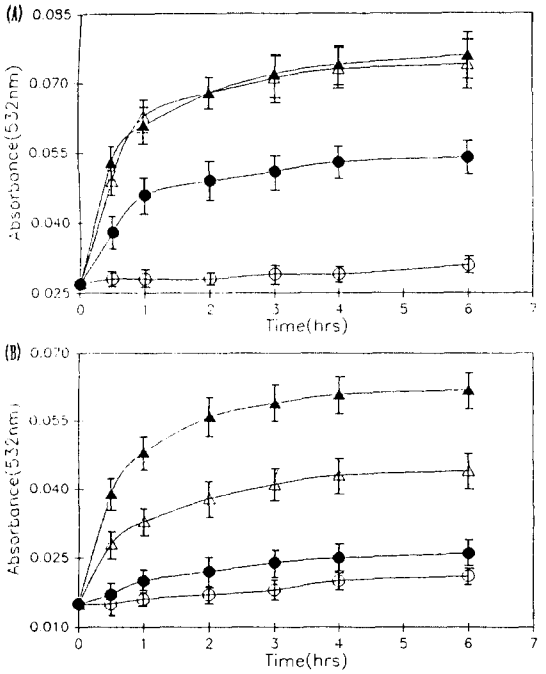

Fig. 3. Time course of the formation of TBARS during peroxidation of the erythrocyte ghosts (A) and the phosphatidyl choline liposomes (B) in hydrogen peroxide system.
○; control, ●; 10 mM H_2O_2, △; 10 mM H_2O_2+0.1 mM Fe^{2+}, ▲; 20 mM H_2O_2+0.1 mM Fe^{2+}.

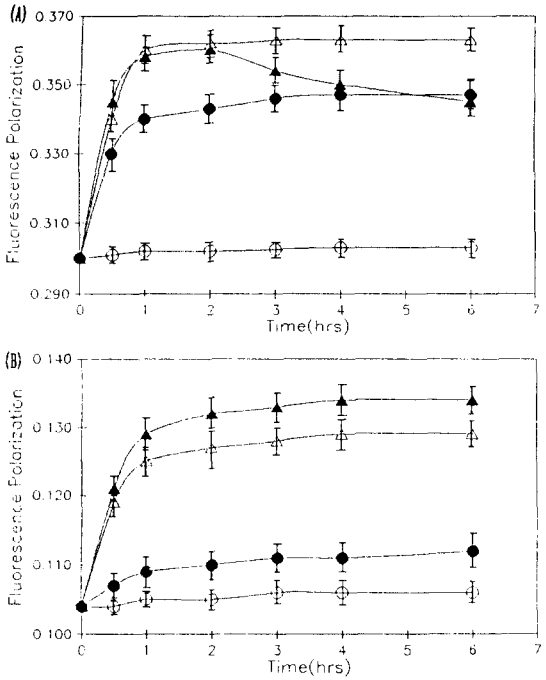

Fig. 4. Time course of the changes of the fluorescence polarization of DPH labelled into lipid bilayers of the erythrocyte ghost membranes (A) and the phosphatidyl choline liposomes (B) were peroxided in hydrogen peroxide system.
○; control, ●; 10 mM H_2O_2, △; 10 mM H_2O_2+0.1 mM Fe^{2+}, ▲; 20 mM H_2O_2+0.1 mM Fe^{2+}.

cal initiates the chain reaction of the peroxidation. This chain reaction possibly includes the alterations of the chemical structures of lipid components, and cross-linking of the lipids with other components of the membranes. This should induce the decrease in the fluidity.

The figures show that the HX/XO/ferrous ion system also induced the lipid peroxidation of the liposomal membranes and the TBARS increased significantly with the reaction going on. The peroxidation was more effective when higher unit of XO was employed. However, without ferrous ion, the peroxidation did not occur to any measurable extent in this experiment. The fluorescence polarization value of DPH labelled into the liposomal membranes increased significantly as the peroxidation reaction proceeded, and the higher unit of XO induced higher values of the fluorescence polarization. This also suggests that the lipid peroxidation of the liposomal membrane reduced the fluidity of the lipid matrix of the liposomal membranes.

The results of the experiments with both of the ghosts and liposomal membranes suggest that the HX/XO system effectively induced the lipid peroxidation in the presence of ferrous ion, and this lipid peroxidation decreased the fluidity of the lipid bilayers of the membranes. It seems that the presence of ferrous ion is critically important in inducing the lipid peroxidation in the liposomal system, while the lipid peroxidation of the erythrocyte ghost membranes virtually occurred without added ferrous ion. This might be ascribed to the residual ferrous ion in the ghosts due to the incomplete removal of the ferrous ion from the system.

Lipid peroxidation in hydrogen peroxide system

The lipid peroxidation experiments were also performed by incubating the erythrocyte ghost suspensions or the phospholipid liposomal membranes in

the presence of various concentration of hydrogen peroxide with ferrous ion at 37℃. The results of the measurements of the changes in TBARS and the fluorescence polarization of DPH labelled into the ghost or liposomal membranes with time were shown in Figs. 3 and 4. Fig. 3 shows that in the case of the erythrocyte ghosts the TBARS increased with the incubation time, and the presence of ferrous ion accelerated the peroxidation. However, concentrations of hydrogen peroxide up to 10 mM was high enough for the maximum formation of TBARS under the experimental condition. This reveals that hydrogen peroxide effectively peroxidized the ghosts in relatively low concentration of hydrogen peroxide in the presence of ferrous ion. As in the case of the peroxidation of the erythrocyte ghosts by the HX/XO system, the TBARS increased abruptly at the initial stage of the peroxidation, and rather reached a plateau as the peroxidation proceeded. The fluorescence polarization value of DPH labelled into the erythrocyte membranes also increased with the incubation time in proportion to the concentration of hydrogen peroxide as shown in Fig. 4. They also increased abruptly at the initial stage of the peroxidation and reached a plateau as in the case of the measurement of the TBARS. However, the initial increase of the fluorescence polarization values were more abrupt than the changes in the TBARS. It was worthwhile to note that, as the concentration of hydrogen peroxide was as high as 20 mM, it's fluorescence polarization -incresing ability was rather reduced. These results suggest that the lipid peroxidation of the erythrocyte ghost membranes occurred effectively at the early stage of the reaction, and this reduced the fluidity of the lipid bilayers of the erythrocyte membranes. This might be ascribed to the alteration of the polyunsaturated fatty acid moieties and possibly cross-linking the lipids with other lipid components or the proteins in the membranes by peroxidation. However, drastic peroxidation by higher concentration of hydrogen peroxide rather decreased the fluidity-decreasing activity. This should be due to the excessive deteriolation of the membrane matrix by excessive peroxidation.

The measurements of the TBARS and the fluoresence polarization of DPH in the phospholipid liposomal membranes show that the trends of the changes of these two parameters with time were generally similar to the data of the experiments in the peroxidation of the erythrocyte ghost membranes except the observation that even higher concentration of hydrogen peroxide up to 20 mM did not reduced its fluorescence polarization-increasing ability. This might be ascribed to the fact that the content of polyunsaturated fatty acids in the egg yolk lecithin is far less than the erythrocyte ghost membranes and this provides the phospholipid membranes with more mechanical strength to keep the integrity of the membranes to peroxidation.

Fluidity of the liposomal membranes of peroxidized erythrocyte lipids

The fluorescence polarization values of the DPH labelled into liposomal membranes which were made of the extracted erythrocyte lipids and the extracted lipids of erythrocyte peroxided with HX /XO/ferrous ion system were measured, respectively. The value for the liposomal membranes of the extracted erythrocyte lipids was 0.13 (n=5, S.D= 0.012), while the value for the liposomal membranes of the peroxidized erythrocyte lipids was 0.17 (n=5, S.D=0.021). Compared with the fluorescence polarization value, 0.300 of DPH labelled in the erythrocyte ghosts, it was clear that the fluidity of the liposomal membranes of the extracted lipids of the erythrocyte was far greater than that of the erythrocyte ghost membranes. The less fluidity of the ghost membranes should be attributed to the less mobility of the lipids due to the restriction through interactions with proteins in ghosts. The increase in the fluorescence polarization in the liposomal membrane of the extracted lipids of peroxidized erythrocyte suggests that the lipid peroxidation reduced the fluidity of the liposomal membranes, and this activity originates from the alteractions in lipid structure and organization in membranes and cross-linking among the lipid components by peroxidation.

Morphological changes of erythrocyte cell by peroxidation

Erythrocytes were incubated at 37℃ for 30 min with 5 mM H_2O_2 or HX/XO system containing 0.3 mM hypoxanthine and 0.1 unit of xanthine oxidase, and changes in the morphology of erythrocyte were observed under an optical microscope after being fixed with glutaraldehyde. It was found that the shape of erythrocytes is very sensitive to the peroxi-

dation. While normal erythrocyte cells were in discoidal shape, the peroxidized erythrocyte cells were crenated (echinocytes) in both cases of peroxidation. This result agrees with the observation of Rice-Evans[22], which reported echinocytes were produced by peroxide stress of erythrocyte. It has been known that environmental changes and drug substances induced morphological changes of the erythrocyte cell[23], and there might be some possibility that the change in the fluidity of the membranes might do some role in the morphological changes of erythrocyte cells. Further studies along this line would be of interest.

CONCLUSION

The HX/XO and H_2O_2 effectively peroxidized the erythrocyte ghosts and phospholipid liposomal membranes in the presence of ferrous ion, respectively. The peroxidation increases the fluorescence polarization of DPH labelled into the lipid bilayers of the membranes, which means that the peroxidation decrease the fluidity of the lipid bilayer of the membranes.

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