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Fluorescent and Cross-Linked Proteins of Human Erythrocyte Ghosts Formed by Reaction with Hydroperoxylinoleic Acid, Malonaldehyde and Monofunctional Aldehydes

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Human erythrocyte ghosts modified with 13-hydroperoxylinoleic acid (LOOH) and the secondary degradation products of peroxidized lipids, *i.e.* malonaldehyde (MDA) and monofunctional aldehydes, were analyzed for fluorescence formation and protein cross-linking. Reaction of ghosts with LOOH produced fluorescent ghost proteins with an excitation maximum at 357 nm and an emission maximum at 438 nm. The MDA-modified ghost proteins exhibited fluorescence spectra with a longer maximum wavelength, an excitation maximum at 398 nm and an emission maximum at 467 nm, whereas the fluorescence spectra of the ghost proteins modified with 1-heptanal and 2,4-decadienal were indistinguishable from those observed for LOOH-modified ghost proteins. Similar results were obtained for lipid extracts of the modified ghosts. Like LOOH and MDA, the monofunctional aldehydes such as 1-hexanal, 1-heptanal and 2,4-decadienal were capable of cross-linking the ghost proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The importance of MDA in the formation of fluorescence and cross-links may have been exaggerated in earlier reports.

Keywords——lipid peroxidation; 13-hydroperoxylinoleic acid; malonaldehyde; monofunctional aldehyde; fluorescence; cross-linking; erythrocyte membrane

Lipid peroxidation in biological membranes is believed to be a toxic and destructive sequence of reactions leading to cell damage.[1] This phenomenon has also been implicated in aging of cells and tissues, particularly in fluorescent lipofuscin formation.[2] In these reactions, polyunsaturated fatty acids undergo oxidative deterioration to produce lipid hydroperoxides and their secondary degradation products including various aldehydes and ketones.[3] The reactive species thus generated react with the adjacent proteins and phospholipids, yielding modified proteins and lipids.[4] Among these reactive species, malonaldehyde (MDA) has been regarded as a major causative molecule of fluorescence formation and cross-linking in proteins and phospholipids. Tappel and his associate[1,5] reported that MDA produced highly fluorescent conjugated Schiff bases between inter- and intramolecular ε-amino groups in proteins. In contrast, our recent studies demonstrated that fluorescence of MDA-modified hemoglobin and polylysine was due to the highly fluorescent 1,4-dihydropyridine-3,5-dicarbaldehyde residues, and cross-links to the conjugated Schiff bases.[6,7]

Monofunctional aldehydes such as 1-hexanal and 2,4-decadienal can be derived from 13-hydroperoxylinoleic acid and 9-hydroperoxylinoleic acid, respectively.[3,8] These monofunctional aldehydes may play an important role in fluorescence formation and cross-linking of biological membranes, since it was found that reaction of polylysine with these monofunctional aldehydes produced fluorescent and cross-linked polylysines.[7]

In this paper we demonstrate that the *in vitro* reaction of erythrocyte membranes with 13-hydroperoxylinoleic acid (LOOH), MDA and several monofuctional aldehydes resulted in the

formation of fluorescence and cross-links, and that the fluorescence spectra of the ghost proteins and lipids treated with LOOH were different from the spectra of those treated with MDA but indistinguishable from the spectra of those treated with the monofuctional aldehydes.

Materials and Methods

Chemicals——LOOH was prepared according to the method of Gardner[9] by the use of linoleic acid (Sigma Chemical Company, St. Louis, MO, U.S.A.) and soybean lipoxygenase (Lipoxidase Type I, Sigma Chemical Company). The purity of the hydroperoxide was 95% as estimated based on a molecular extinction coefficient of ε (233 nm): 24500.[10] MDA was obtained as the sodium salt (MDA · Na) according to the method of Marnett and Tuttle[11] with slight modifications as previously described.[12] This preparation contained about 3 mol of water as determined by nuclear magnetic resonance (NMR) analysis. *trans, trans*-2,4-Decadienal was obtained from Aldrich Chemical Company (Milwaukee, WIS, U.S.A.). All other chemicals were reagent grade products of Wako Pure Chemical Industries, Ltd., Tokyo.

Erythrocyte Ghost Preparations——Venous blood (200 ml) from a healthy donor was collected in a blood transfusion bag containing 28 ml of citrate–phosphate–dextrose solution. The erythrocytes were spun down, stored at 4°C and used within 3 d. Erythrocyte ghosts were isolated according to the method of Steck,[13] and stored at −20 °C in aliquots until use. Hemoglobin content in the ghost preparations, determined by the method of Dodge,[14] was usually 1.0—3.0% of the total proteins. Protein concentrations were determined by the method of Lowry.[15]

Reaction of Ghosts with LOOH and the Aldehydes——Reactions were carried out by mixing the ghost suspensions (5 mg protein/ml) in 0.1 M phosphate buffer, pH 7.0, with an equal volume of LOOH or aldehyde solution in the same buffer. The mixture was incubated under aerobic conditions at 37°C for 1 h, then the modified ghosts were recovered by centrifugation (15000 *g*, 20 min), and washed 4 times in 5 mM phosphate buffer, pH 7.0, at 4 °C. The washed ghosts were resuspended in water to make the protein concentration 2.5 mg/ml and analyzed as soon as possible.

Protein and Lipid Fractions from the Modified Ghosts——Total lipid of the ghosts was extracted by the method of Bligh and Dyer.[16] A mixture of 1 ml of chloroform and 2 ml of methanol was added to each 0.8 ml (2 mg protein) of the ghost suspension, and mixed well. A clear supernatant and a protein precipitate were obtained by centrifugation at 3000 rpm for 20 min. Next, 1 ml of chloroform and 1 ml of water were added to the clear supernatant. The resulting biphasic mixture was centrifuged, and the chloroform layer was taken and clarified by addition of 0.1 ml of methanol (lipid fraction). The residual protein precipitate was washed twice with chloroform–methanol–water (1 : 2 : 0.8; v/v), then solubilized in 0.4 ml of 10% sodium dodecyl sulfate (SDS). Complete solubilization was achieved by sonication at 20 °C for 20 min. An untreated ghost protein fraction exhibited the regular electrophoretic pattern on an SDS-polyacrylamide gel,[17] and the protein recovery was usually 80% of the original ghost protein.

Measurement of Fluorescence——For fluorescence measurement, 0.5 ml of lipid fraction was diluted with 1.5 ml of chloroform, and 0.2 ml of the SDS-solubilized protein fraction was diluted with 1.8 ml of 10 mM sodium phosphate-buffered saline, pH 7.2. Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer; the solvent background was subtracted automatically. Fluorescence intensities were determined against 0.1 μM quinine sulfate in 0.1 N sulfuric acid measured at an excitation maximum of 353 nm and an emission maximum of 453 nm.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)——SDS-PAGE was done in the discontinuous buffer system of Laemmli[18] with a 10% separating gel and a 4% stacking gel. Samples were prepared by solubilizing the washed ghosts in the sample buffer containing 5% 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor, and incubated at 37 °C for 1 h. Gel bands were visualized by Coomassie Brilliant Blue R-250 staining.

Thiobarbituric Acid (TBA) Test——The washed protein residue was solubilized in 1.0 ml of 50% acetic acid, then mixed with 1.0 ml of 20 mM TBA solution in 50% acetic acid. The mixture was heated at 100 °C for 20 min,[19] then allowed to cool, and the absorbance at 532 nm was measured. Standard solutions of MDA · Na (1—20 μM) were similarly treated.

Results

Incubation of human erythrocyte ghosts with LOOH at 37 °C for 1 h resulted in fluorescence formation of both protein and lipid fractions (Figs. 1 A and B). The protein fractions exhibited fluorescence spectra with excitation maxima at 357 nm and emission

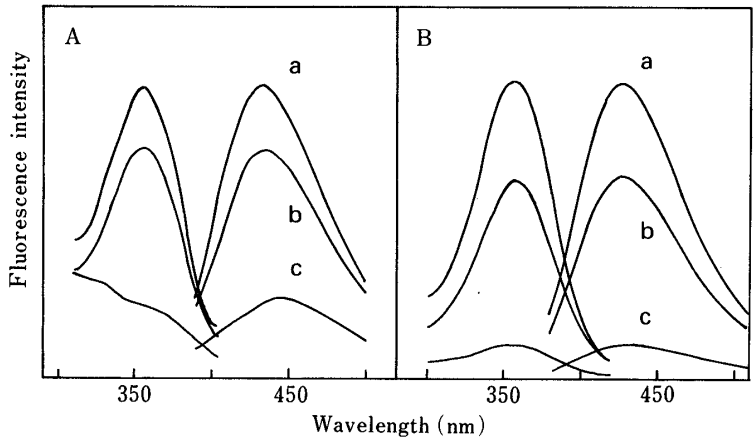

Fig. 1. Fluorescence Spectra of Proteins (A) and Lipids (B) from Ghosts Treated with LOOH

Ghosts were treated with 2 mM (a), 0.5 mM (b) LOOH, or without LOOH (c) at 37 °C for 1 h. The modified ghosts were fractionated into proteins and lipids as described in Materials and Methods.

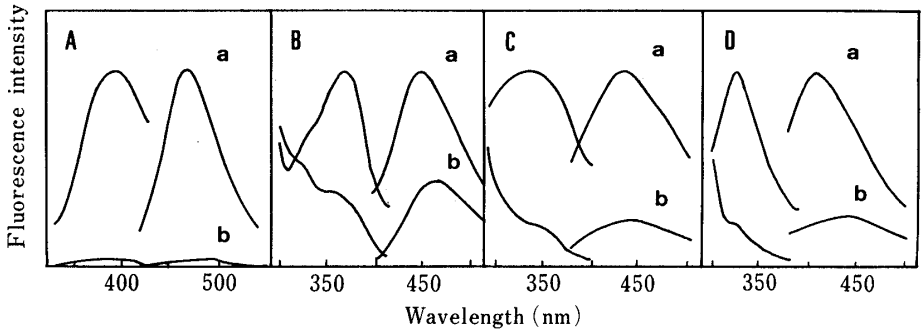

Fig. 2. Fluorescence Spectra of Proteins from Ghosts Treated with Various Aldehydes

A, MDA (25 mM); B, 1-heptanal (50 mM); C, 2,4-decadienal (0.5 mM); D, glutaraldehyde (0.5 mM). Ghosts were incubated (a) with or (b) without each of the aldehydes at 37 °C for 1 h, followed by washing and fractionation as described in Materials and Methods.

maxima at 438 nm, which are similar to those of the lipid fractions with excitation maxima at 357 nm and emission maxima at 428 nm. The fluorescence intensities increased in a dose-dependent manner for both fractions. Time-dependent fluorescence increase was observed for both fractions (data not shown). Slight variations in the maximum emission wavelength for protein fluorescence (between 430 and 445 nm) were observed depending upon the ghost preparations.

The exogenously added lipid hydroperoxides can act as initiators of peroxidation of membrane lipids if metal ions such as ferrous and ferric ions, or hemoproteins are present.[20] Since the ghost preparations used in the present experiments contained hemoglobin corresponding to 1—3% of the total proteins, the fluorescence observed was presumably due to the reaction of membrane constituents with the secondary breakdown products of the added LOOH and with the lipid hydroperoxides endogenously generated during the incubation with LOOH. Therefore, we examined the capacity for fluorescence formation and protein cross-linking of various aldehydes which may have been produced by the reaction of LOOH with the ghosts. These aldehydes include 1-hexanal and 1-heptanal as monofunctional saturated aldehydes, 2,4-decadienal as a monofunctional unsaturated aldehyde, and MDA as a dialdehyde.[3] For comparison, several other aldehydes such as formaldehyde, acetaldehyde

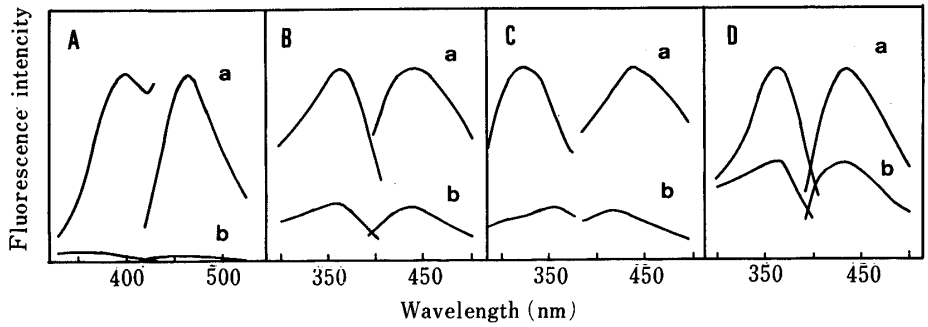

Fig. 3. Fluorescence Spectra of Lipids from the Ghosts Treated with Various Aldehydes

A, MDA (25 mM); B, 1-heptanal (50 mM); C, 2,4-decadienal (0.5 mM); D, glutaraldehyde (0.5 mM). Ghosts were incubated (a) with or (b) without each of the aldehydes at 37 °C for 1 h, followed by washing and fractionation as described in Materials and Methods.

TABLE I. Fluorescence of Proteins and Lipids from Ghosts Treated with LOOH and Various Aldehydes

Ghosts treated with		Fluorescence of proteins			Fluorescence of lipids		
	(mM)	Excitation maximum (nm)	Emission maximum (nm)	Relative fluorescence intensity[a)]	Excitation maximum (nm)	Emission maximum (nm)	Relative fluorescence intensity[a)]
LOOH	0.5	357	438	4.1	357	428	4.3
	2			5.7			7.0
MDA	1	398	467	1.2	398	467	0.6
	5			10.0			3.9
	25			24.8			18.1
1-Heptanal	25	366	442	3.0	366	442	0.0
	50			3.2			5.3
2,4-Decadienal	0.5	330	430	11.4	325	430	4.8
Glutaraldehyde	0.2	324	410	4.6	363	432	1.4
	0.5			10.2			2.3

a) Relative fluorescence intensity against 0.1 μM quinine sulfate for the ghost concentration of 10 mg protein per milliliter. Values are shown after subtracting the control values without treatment.

and glutaraldehyde were also examined.

Fluorescence of the ghost proteins and lipids arising from the reaction with these aldehydes was measured. MDA, 1-heptanal, 2,4-decadienal and glutaraldehyde produced significant fluorescence in the protein and lipid fractions (Figs. 2 and 3), but formaldehyde, acetaldehyde and 1-hexanal produced little fluorescence even at the highest concentrations tested (50 mM). The maximum wavelengths for excitation and emission of the fluorescence spectra and the relative fluorescence intensities of these modified protein and lipid fractions are summarized in Table I. MDA-modified ghost proteins fluoresced at 398 (excitation maximum) and 467 nm (emission maximum) (Fig. 2A and Table I). The excitation and emission maxima of the spectrum were much longer than those of the proteins from LOOH-modified ghosts (Fig. 1A and Table I). On the other hand, the fluorescence spectra of the membrane proteins modified with 1-heptanal, 2,4-decadienal and glutaraldehyde, each exhibiting excitation maxima at 320—370 nm and emission maxima at 410—450 nm, were similar to the spectrum of those modified with LOOH (Figs. 2B-D and Table I).

For all the modifications of the protein fractions, dose-dependent increases in fluorescence intensities were observed (Table I). Fluorescence intensities of the protein fractions of

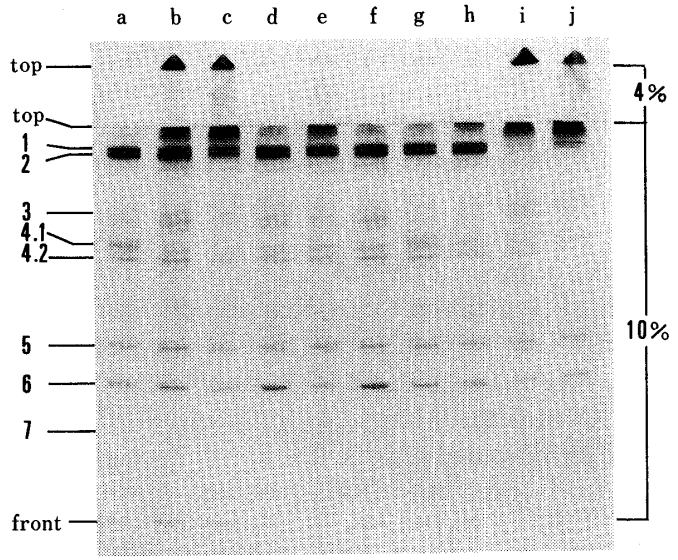

Fig. 4. SDS-PAGE of the Ghosts Treated with LOOH and Various Aldehydes

a, control; b, 0.5 mM LOOH; c, 2 mM LOOH; d, 5 mM MDA; e, 25 mM MDA; f, 50 mM acetaldehyde; g, 50 mM 1-hexanal; h, 25 mM 1-heptanal; i, 0.5 mM 2,4-decadienal; j, 0.5 mM glutaraldehyde. A 20 μg sample of the ghost proteins was loaded per lane. Major bands are indicated according to the numbering of Steck.[17]

TABLE II. TBA-Reaction of Ghost Proteins Treated with LOOH and MDA

Ghosts incubated with	(mM)	TBA-reactive substances associated with proteins ($A_{532\,nm}$[a]/mg protein)
None		0.004
MDA	1	0.040
	5	0.146
	25	0.456
LOOH	0.5	0.008
	2	0.016

a) Standard MDA·Na (10 nmol) gave an absorbance of 0.351.

the ghosts modified with 2,4-decadienal, LOOH and MDA were higher in that order, when compared at concentrations of the reagents lower than 1 mM. Similar results were obtained for the lipid fractions (Figs. 3B-D and Table I). The pronounced dissimilarity of the fluorescence spectra between LOOH-modified and MDA-modified ghost proteins/lipids means that the involvement of MDA in fluorescence formation of the peroxidized ghosts is questionable.

We compared the effects of LOOH, MDA and the monofunctional aldehydes on the membrane protein cross-linking. Figure 4 shows SDS-PAGE patterns of the ghosts treated with these reagents. When the ghosts were treated with LOOH at 0.5 and 2 mM concentrations, the membrane proteins were extensively cross-linked, yielding high-molecular-weight aggregates at the top of the separating and the stacking gels (Fig. 4 lanes b and c). These aggregates must be cross-linked products of band 1 and 2 proteins (spectrin) and band 4.1 protein, since these proteins in the ghosts modified with 2 mM LOOH (lane c) were significantly more weakly stained than those of the unmodified ghosts (lane a). In contrast, MDA gave fewer aggregates at the top of the separating gel even at a higher concentration of the reagent (25 mM) (lanes d and e), and in this case the aggregates might be derived from spectrin since only the spectrin bands were less intensely stained. Several monofunctional aldehydes were also capable of cross-linking the ghost proteins. Acetaldehyde and 1-hexanal were effective in cross-linking the ghost proteins at the highest concentration tested (50 mM) (lanes f and g). Treatment of the ghosts with 1-heptanal at 25 mM gave a high-molecular-weight band at the top of the separating gel (lane h). When the ghosts were treated with 2,4-

decadienal at 0.5 mM, the membrane proteins were extensively cross-linked, yielding high-molecular-weight aggregates at the top of the separating and the stacking gels (lane i). In this modification, the spectrin bands disappeared. The cross-linking ability of 2,4-decadienal was comparable to that of LOOH. Glutaraldehyde, a known bifunctional cross-linking agent, showed similar cross-linking ability (lane j). Spectrin seemed to be the most susceptible protein to cross-linking by LOOH, MDA, 2,4-decadienal and glutaraldehyde.

The higher fluorescence formation and protein cross-linking by LOOH than by MDA cannot be ascribed to the possibility that effective numbers of MDA molecules were produced in LOOH-modified ghosts. This possibility was also ruled out by comparison of the TBA-test results of MDA- and LOOH-modified ghost proteins. Table II shows that TBA coloration of LOOH-modified ghost proteins was much weaker than that of MDA-modified ones at the concentrations examined. The numbers of MDA molecules bound to the proteins of LOOH-modified ghosts were much smaller than in MDA-modified ghosts. These results suggest that most of the fluorescence and the cross-links produced in LOOH-modified ghosts is not attributable to MDA.

Discussion

In a series of studies on MDA-derived fluorescent compounds, we showed that the fluorophores produced in the reaction of MDA with amino groups were 1,4-dihydropyridine-3,5-dicarbaldehyde residues with excitation maxima at 390—405 nm and emission maxima at 450—465 nm.[6,7,12,21] It was also suggested that the conjugated Schiff bases produced by these reactions, which were originally reported to be highly fluorescent,[5,22] were less fluorescent and were likely to provide cross-links between amino groups.[6,7,12] Furthermore, our previous work showed that, as well as LOOH and MDA, monofuctional aldehydes produced fluorescent and cross-linked polylysines,[7] and that the fluorescence spectra of polylysines modified with LOOH were different from those obtained with MDA but similar to those obtained with monofunctional aldehydes.[7]

Since their function is to carry oxygen, erythrocytes are susceptible to lipid peroxidation, and thereby provide a good model system for the study of membrane lipid peroxidation and of cell aging. Elucidation of the mechanisms of the fluorescence formation and protein cross-linking using erythrocyte membranes would be an important step forward in clarifying these events. In the present study, human erythrocyte ghosts were treated with LOOH and the secondary oxidation products of peroxidized lipids (MDA and the monofunctional aldehydes), and the products were analyzed in terms of fluorescence formation and protein cross-linking.

Fluorescence spectra of MDA-modified ghost proteins and lipids gave the same maximum wavelengths for excitation (398 nm) and emission (467 nm) as those of MDA-modified polylysines,[7] suggesting that the fluorophores in MDA-modified ghost proteins and lipids have 1,4-dihydropyridine type structures as discussed above. Jain and Hochstein[23] have reported that the fluorescent chromolipids of MDA-treated erythrocytes showed similar spectra with excitation maxima at 390—400 nm and emission maxima at 460 nm.

The fluorescence spectra of the protein and lipid fractions from LOOH-modified ghosts were consistent with those reported for peroxidized erythrocyte membrane lipids[24-26] and LOOH-modified serum albumin,[27] both exhibiting excitation maxima at 360 nm and emission maxima at 430—440 nm. Since MDA is claimed to be a degradation product of 5-membered cyclic peroxides of fatty acids with triene structures, such as linolenic and arachidonic acids,[28-30] LOOH itself may not produce MDA. MDA and the monofunctional aldehydes can be generated upon addition of LOOH as a result of the initiation of ghost lipid peroxidation in the presence of hemoglobin.[20] The present results demonstrate that

fluorescence formation and protein cross-linking of LOOH-modified ghosts are not ascribable to MDA. The TBA-test results of the modified ghost proteins also indicated that the involvement of MDA was not likely.

While the mechanisms of fluorophore formation in the reaction of amino groups with the monofunctional aldehydes are not known, the abilities of these aldehydes to cross-link the membrane proteins might be explained by mechanisms including self-condensation of these molecules, in which bifunctional aldehydes are formed, as proposed previously.[7] Reactivity of these aldehydes with the membranes may be due to the hydrophobicities of the molecules, since the aldehydes with double bonds or longer chains were potent in the formation of fluorescence and cross-links. 2,4-Decadienal showed potent reactivity in the formation of fluorescence and cross-links in erythrocyte membranes. 1-Heptanal was more effective in both fluorescence formation and cross-linking than 1-hexanal. It is conceivable that these monofunctional aldehydes play an important role in the formation of fluorescence and cross-links of the peroxidized membranes. If this is the case, the higher reactivity of LOOH than of 1-hexanal may be explained as follows. LOOH readily associates with membranes, where it is degraded to produce 1-hexanal which is much more effective than in solution. Alternatively, the effect of LOOH may be mainly due to other more potent monofunctional aldehydes produced by the induced autoxidation of membrane lipids.

Koster and his colleagues[25,26] induced lipid peroxidation in erythrocyte ghosts using organic hydroperoxides or LOOH, and they suggested that the cross-linking of the membrane proteins might be independent of the process of MDA production. Fujimoto *et al.*[31] reported that deoxyribonucleic acid forms fluorescent products with secondary oxidation products other than MDA.

Our present results suggest that the secondary oxidation products of lipid peroxidation other than MDA play an important role in the formation of fluorescence and cross-links of the peroxidized membranes. Monofunctional aldehydes appeared to be candidate molecules. Further investigations are needed to characterize and identify the molecules involved in these events in peroxidized membranes. In any case, the importance of MDA in the formation of fluorescence and cross-links may have been exaggerated in earlier reports.

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