Damage to Microsomal Membrane by Lipid Peroxidation

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ABSTRACT

Alterations of microsomal membrane integrity were examined during lipid peroxidation. Loss of membrane-bound NADPH cytochrome *c* reductase activity and protein release into the aqueous phase were related to disruption of the lipophilic region of the membrane. Formation of fluorescent products in the lipid phase of the membrane occurred only in the presence of peroxidation products. Changes in the membrane lipid phase during peroxidation included a decrease in the reactive amino groups of the phospholipids and a decrease in detectable phosphatidyl ethanolamine. In an ultrafiltration continuous flow chamber, peroxidized microsomal membranes were hydrolyzed to a lesser degree by solubilized lysosomal cathepsins and to a greater degree by lysosomal nucleases than were nonperoxidized membranes.

INTRODUCTION

Biological membranes, especially those of subcellular organelles, are labile to lipid peroxidation because of their high content of polyunsaturated lipids (1). Among the products of oxidative deterioration of these unsaturated lipids are free radical intermediates, semistable peroxides and reactive carbonyls (2). The mechanisms by which cellular systems are damaged include free radical polymerization (3,4), aldehyde crosslinking polymerization (5,6) and disruption of membrane lipid-protein integrity (7). Alterations in phospholipid structure also occur during peroxidation of purified phospholipids (8,9) and of complex phospholipids of membranes (10,11).

Several models for membrane structure, including the interaction between lipid-protein complexes, have been discussed by Branton and Deamer (12). The importance of examining changes in structural integrity caused by lipid peroxidation is apparent. An understanding of these changes would enable a better understanding of the pathological conditions induced by lipid peroxidation in vivo. Several pathological disorders may involve lipid peroxidation damage, including some phases of atherosclerosis (13,14), aging (15,16), neuronal ceroid-lipofuscinosis or Batten's syndrome (17) and liver injury caused by ethanol (18) or chlorinated hydrocarbons (19). The microsomal membrane was chosen as a model system to evaluate alterations in membrane integrity induced by lipid peroxidation.

MATERIALS AND METHODS

Source of Materials

The materials obtained from Sigma Chemical Co., St. Louis, Mo., included 2-thiobarbituric acid (TBA), D glucose-6-phosphate, cytochrome *c* (horse heart), NADPH and dithioerythritol. 1,1,3,3-Tetraethoxypropane bis (diethyl acetal) was purchased from J.T. Baker Chemical Co., Phillipsburg, N.J. Butylated hydroxytoluene (BHT) was obtained from Eastman Chemical Co., Kingsport, Tenn. Precoated Silica Gel G thin layer chromatography (TLC) plates were purchased from Quantum Industries, Fairfield, N.J. The phospholipid standards, phosphatidyl ethanolamine and phosphatidyl choline, were purchased from Supelco, Inc., Bellefonte, Pa.; and phosphatidyl serine, phosphatidyl inositol, lysolecithin and sphingomyelin from Schwarz/Mann, Orangeburg, N.Y. The Diaflo ultrafiltration apparatus and ultrafilters (UM-2) were obtained from Amicon Corporation, Lexington, Mass.

Preparation of Subcellular Organelles

Rat liver was fractionated into nuclear, mitochondrial, light mitochondrial-lysosomal and microsomal fractions by the method of de Duve et al. (20) after homogenization in 250 mM sucrose and 1 mM EDTA.

Microsomal Peroxidation System

Aliquots of 25 mg microsomal membrane protein were suspended in 10 ml 10 mM Tris-HCl buffer, pH 7.4, in 50 ml flasks. Peroxidation was initiated by addition of 0.5 ml each of 10 mM ascorbate and 10 mM $FeCl_3$. The sealed flasks were shaken at 225 rpm in oxygen at 37 C. Flasks were removed for analyses of contents at 0, 2, 4, 6, 8, 10, 12, 18 and 24 hr. Controls contained in addition 1% BHT.

Analytical Methods

Lipid peroxidation was followed by the measurement of TBA reactive substances (TBRS). Aliquots of 0.5 ml of the microsomal suspension, 0.5 ml distilled water and 0.5 ml

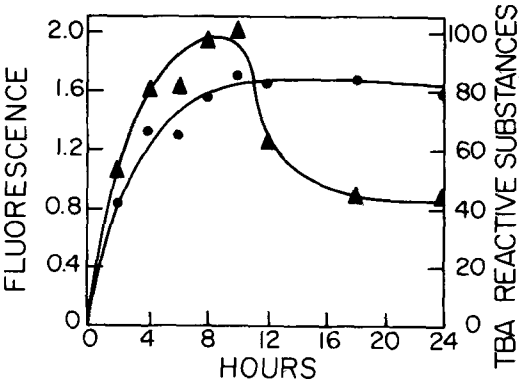

FIG. 1. Formation of fluorescent pigments (•) and production of TBA reactive substances (▲) during peroxidation of microsomal membranes. TBA reactive substances are expressed as nmoles of MA per milligram membrane protein. Fluorescence is expressed as the fluorescence times meter multiplier setting per milligram membrane protein.

30% trichloroacetic acid were mixed and centrifuged at ca. 3000 rpm for 3 min. The supernatant was added to an equal volume of aqueous 0.67% TBA, heated in a boiling water bath for 15 min, cooled to room temperature, and the absorbance measured at 532 nm was expressed as nanomoles of malonaldehyde (MA). The MA standard was prepared from 1,1,3,3-tetraethoxypropane bis (diethyl acetal) (21).

Extraction for measurement of fluorescence was done as follows. Aliquots of 2.5 ml microsomal suspensions were homogenized with 5.0 ml chloroform-methanol 2:1 for 1 min at room temperature. After addition of 2.5 ml distilled water the slurry was mixed for 15 sec and briefly centrifuged. Fluorescence spectra of the chloroform layers were recorded with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.). The slit arrangement was 3, 1 and 3 mm for slits 3, 4 and 6, respectively; the sensitivity was set at 50 and the meter multiplier was set in accordance with the fluorescence intensity of each sample. The quinine sulfate standard (1 μg/ml 0.1 N H_2SO_4) had a fluorescence intensity of 70 at a meter multiplier setting of 0.3.

Phospholipids were quantitated (22) and expressed as micromoles of inorganic phosphate (Pi). A 1.0 ml aliquot of the chloroform extract was concentrated and applied to a Silica Gel G plate. The phospholipids were separated by chloroform-methanol-water 80:25:3 and detected by iodine vapor. The fractions were removed from the TLC plates, hydrolyzed (22) and Pi determined.

Free amino groups of the phospholipids were determined by ninhydrin analysis on 0.5 ml aliquots of chloroform extracts after drying under nitrogen. The absorbance was measured at 570 nm and expressed as nanomoles of ethanolamine.

The protein released from the membrane during peroxidation was determined by the method of Miller (23) after centrifugation of 2.5 ml of the microsomal suspension at 100,000 x g for 1 hr.

NADPH cytochrome *c* reductase was assayed by addition of a 0.1 ml aliquot of the microsomal suspension to a spectrophotometer cuvette that contained 0.2 ml NADPH (1 mg/ml), 0.1 ml oxidized cytochrome *c* (10 mg/ml) and 1.6 ml 10 mM Tris-HCl buffer, pH 7.4. Absorbance was recorded at 550 nm for 3 min against a cytochrome *c* blank (24). Glucose-6-phosphatase was assayed by the method of Dallner (25), and the released Pi was measured by the method of Fiske and Subba-Row (26).

Preparation of Soluble Lysosomal Enzyme Fraction

The light mitochondrial-lysosomal fraction, resuspended in a small volume of 10 mM sodium acetate buffer, pH 5.4, was frozen and thawed 15 times to release the lysosomal enzymes. The membrane was removed by centrifugation at 100,000 x g for 60 min. The supernatant, containing the solubilized lysosomal enzymes, was dialyzed against 10 mM acetate buffer for 20 hr at 2 C with three changes of buffer. The transpeptidase activity of cathepsin C (27) in this preparation, which was used in the hydrolysis experiments, was 1525 nmoles of tyrosyl hydroxamate per minute per milligram of protein.

Hydrolysis of Peroxidized Microsomal Membrane

A 2.0 ml aliquot of the microsomal suspension (5.0 mg protein) and 1.0 ml of the lysosomal enzyme preparation (2.95 mg protein) were added to 7.0 ml 0.1 M sodium acetate buffer, pH 5.4, that contained 10 mM dithioerythritol. The mixture was placed in a 10 ml Diaflo-ultrafiltration apparatus with a UM-2 ultrafilter, which allowed molecules of less than 1000 mol wt to pass through. The reaction system was placed under nitrogen pressure to ensure a constant flow of buffer into and of hydrolysate out of the reaction chamber. A reaction temperature of 37 C and a flow rate of 2.0 ml/hr were maintained. The hydrolysate (1.65 ml fractions) was examined for products released by the cathepsin system (28) and acid nucleases (29). The products of proteolysis were determined with ninhydrin and the acid nuclease products were measured with orcinol (30).

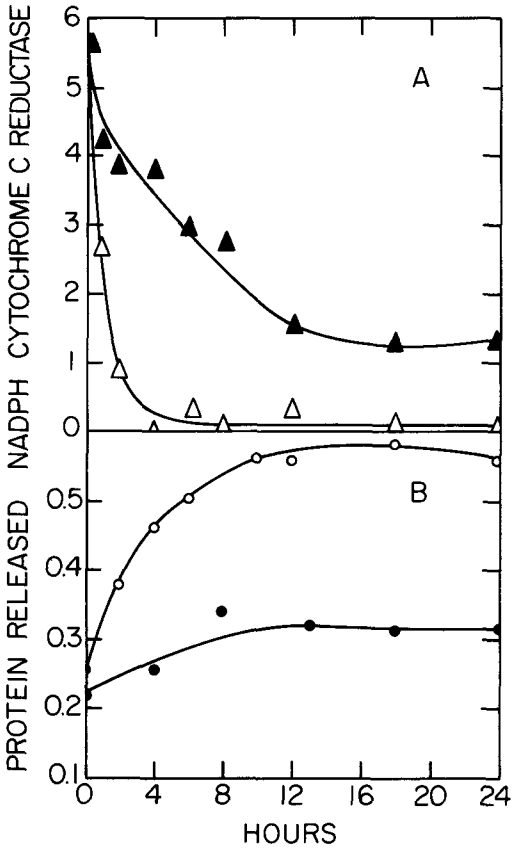

FIG. 2. (A) Loss of NADPH cytochrome *c* reductase activity in the absence (Δ) and presence (▲) of antioxidant during peroxidation of microsomal membrane. Activity is expressed as nmoles of cytochrome *c* reduced per minute per milligram protein. (B) Release of protein from microsomal membrane in the absence (○) and presence (●) of antioxidant during lipid peroxidation. Protein released is expressed as milligram solubilized protein per milligram remaining membrane protein.

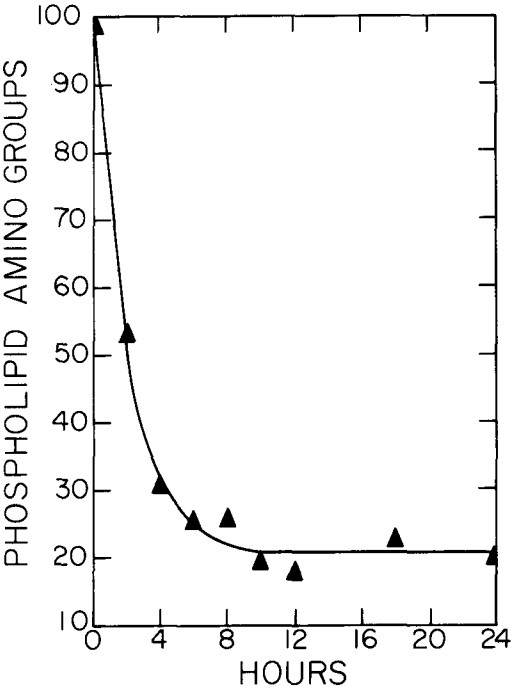

FIG. 3. Decrease in free amino groups of phospholipids during microsomal membrane peroxidation. The free phospholipid amino groups are expressed as percentage of the initial value in nmoles ethanolamine per μmole P_i.

Membrane remaining in the reaction chamber after hydrolysis by lysosomal enzymes was recovered by centrifugation at 100,000 x g for 60 min. Protein was determined on an aliquot; the remainder was extracted with chloroform-methanol and examined for fluorescence.

RESULTS

Microsomal membrane was chosen as a model to evaluate alterations in membrane integrity resulting from lipid peroxidation. The microsomes contain a complete complement of phospholipids and enzyme activities which can be examined. Addition of antioxidant, 1% BHT, to the microsomal suspension protected the membrane from peroxidation. These samples served as controls and allowed differentiation of changes in membrane integrity due to temperature and those changes caused by lipid peroxidation.

Figure 1 shows amounts of TBRS and fluorescent products as a function of time. TBRS increased for 8-10 hr and then decreased. Fluorescent products developed similarly until TBRS decreased, whereupon fluorescence development plateaued. All fluorescent products had maximum emission at 430 nm when excited at its maximum of 360 nm. In the presence of antioxidant the microsomes showed neither the formation of TBRS nor the development of significant fluorescence.

To evaluate the effect of lipid peroxidation on the activity of membrane-bound enzymes, NADPH cytochrome *c* reductase and glucose-6-phosphatase were measured. Peroxidizing membrane lost 50% of its initial NADPH cytochrome *c* reductase activity within the 1st hr (Fig. 2A). The antioxidant protected system lost activity also, but at a much slower rate. However loss of glucose-6-phosphatase activity was much more rapid and was not protected by antioxidant. Within 1 hr each sample contained less than 10% of its initial activity. The increased protein content of the microsomal supernatant (Fig. 2B) indicates that disruption

TABLE I

Phospholipid Composition of Peroxidized Microsomal Membrane

Peroxidation time, hr	Percentage of total phospholipid		
	Phosphatidyl ethanolamine	Phosphatidyl choline	Other[a]
0	22.0	66.6	11.4
2	12.6	60.6	26.8
4	8.2	60.2	31.7
6	12.4	60.6	27.8
8	11.8	57.7	30.8
10	6.5	60.8	36.0
12	6.8	57.5	35.8
18	3.1	59.8	38.7
24	2.6	60.6	37.0
24 (Control)	16.7	67.3	17.0
Literature[b]	24.0	64.0	12.0

[a]Phospholipids with an R_f less than 0.20, including phosphatidyl serine, phosphatidyl inositol, lysolecithin and sphingomyelin.

[b]See Reference 11.

of the membrane occurred. Added antioxidant partially stabilized the membrane and decreased the release of protein from the membrane. The results of these studies on peroxidizing membrane indicated that the lipophilic portion of the membrane was disrupted, which decreased the interaction between the enzyme and the membrane lipids.

Alterations in the lipid phase were examined by analysis of the phospholipid extract. During peroxidation the amount of extractable phospholipid decreased, with a larger decrease in amino phospholipids (Fig. 3). In the presence of antioxidant these alterations did not occur.

The percentage of each phospholipid fraction is shown in Table I as a function of peroxidation time. The lower R_f phospholipids were combined, since they were not as clearly separated from each other as were phosphatidyl ethanolamine and phosphatidyl choline. The trend observed was that the phosphatidyl ethanolamine fraction decreased with increased peroxidation, while the percentage of the lower R_f phospholipids increased.

Using the Diaflo ultrafiltration apparatus as a reaction chamber, microsomal membrane damaged by peroxidation for various intervals of time was hydrolyzed by lysosomal enzymes. Rates of hydrolysis appeared to be first order. The results of these experiments are best shown by the total products of hydrolysis given in Table II. Two indicators of lysosomal hydrolysis were measured: catheptic activity, which releases amino acids and peptides, and acid nuclease activity, which releases nucleotide fragments from the membranes. Results are expressed in terms of standards as micromoles of leucine and micromoles of adenosine. A decrease in hydrolyzability of the protein part of the peroxidized membrane is indicated by the accumulation of products released by cathepsin hydrolysis, while the products released by acid nuclease hydrolysis suggest increased hydrolyzability of the nucleic acids. Although both of these trends seem apparent, they are not statistically significant. Examination of hydrolysate fractions for aqueous fluorescent damage products, which could have been released during hydrolysis of the peroxidized membrane, was negative. Only the natural fluorescence of the aromatic amino acids was observed.

The chloroform-methanol extract of the residual membrane after 12.8 hr of hydrolysis was examined for accumulated fluorescent products. The fluorescence spectra were similar to those of the fluorescent products measured as a function of peroxidation time (Fig. 1) and to that of lipofuscin pigments (2). Maximum fluorescence was at 430 nm when excited at 360 nm.

The concentration of fluorescent products in the microsomal membrane was examined before and after lysosomal hydrolysis. The nonperoxidized membrane (0 hr) had an increase in fluorescence from less than 0.1 to 1.3 fluorescence units per milligram membrane protein during hydrolysis. It apparently underwent peroxidation during the hydrolysis reaction. The peroxidized membrane samples of 4-24 hr had higher initial fluorescence but increased from 0.6 to 0.9-1.1 fluorescence units per milligram membrane protein. In both cases the fluorescent pigment concentration was greater after hydrolysis.

DISCUSSION

The microsomal membrane has been used in

TABLE II

Products of Peroxidized Microsomal Membranes Hydrolyzed by Lysosomal Enzymes

Time of peroxidation, hr	Amino acids, mmol[a]	Nucleotides, μmol[a]
0	0.30	9
4	0.23	13
8	0.21	12
12	0.16	12
18	0.15	15
24	0.21	12

[a]Total moles of product released from 5 mg microsomal membrane during 12.8 hr hydrolysis with 2.95 mg lysosomal enzymes in 10 ml 100 mM sodium acetate buffer, pH 5.4.

many laboratories as a model in studies of lipid peroxidation (7,10,11,31-33), and antioxidants have been used to protect membrane systems from peroxidative damage (34-37). The lack of formation of TBRS in the presence of added BHT indicates inhibition of lipid peroxidation. In microsomal membranes the formation of fluorescent products with a fluorescence maximum of 430 nm and an excitation maximum at 360 nm occurred only when the membranes underwent lipid peroxidation. This was noted earlier for peroxidation of amino phospholipids (34) and for the organelle membranes examined for dietary antioxidant protection (33).

The accumulation of TBRS in the peroxidizing membrane results from the formation of aldehydes and ketones. The eventual decline of TBRS is due to a decrease in the available unsaturated lipid content; to the reaction of TBRS, especially MA, with amino groups of amino acids, proteins or amino phospholipids; to the further oxidative decomposition of TBRS; and to condensation reactions in which TBRS form unreactable polymers.

The loss of membrane-associated enzyme activity is most likely the result of a combination of damaging processes, including free radical polymerization (3), aldehyde polymerization (6) and disruption of lipid-protein interaction (7). At the membrane surface, disruption of lipophilic sites by peroxidation simulates a detergent effect in the inactivation of enzyme systems (7). The observation of increased protein release into the microsomal supernatant during peroxidation strongly supports this theory. The fact that glucose-6-phosphatase activity was lost much more rapidly than the NADPH cytochrome *c* reductase activity suggests that the latter enzyme system is more tightly bound to the membrane. These experiments show that the integrity of the membrane is dependent upon the retention of lipid-protein interaction.

A decrease in the phosphatidyl ethanolamine content and the loss of reactive amino groups during peroxidation indicates the formation of aldehyde-amine reaction products, some of which are fluorescent. During peroxidation of phosphatidyl ethanolamine, a decrease in free amino groups was shown (8) and Schiff base products were formed between aliphatic aldehydes and the amino group of the phospholipid (9).

Decrease of phosphatidyl ethanolamine, with an equal and concomitant increase in the lower R_f phospholipid fractions, is in accord with preliminary studies with a dipalmityl phosphatidyl ethanolamine-malonaldehyde model system wherein the reaction products migrated at a lower R_f than the initial phosphatidyl ethanolamine (34). Although this evidence does not prove the formation of any particular chromophoric group, it does suggest the formation of modified phosphatidyl ethanolamine with a reduced R_f.

How do alterations in membrane structure like these affect their degradation and turnover by the lysosomal enzyme system? The theory for the formation of lipofuscin pigments involves ingestion of membrane components which cannot be completely hydrolyzed by the lysosome. The lysosome does not release these products and they accumulate as lipofuscin pigment (38). The fluorescence of lipofuscin pigments has been tentatively identified as characteristic of a conjugated Schiff base formed by the reaction of malonaldehyde with two amino compounds and having a 1-amino-3-iminopropene structure (39). A model system was used to mimic the lysosome and to evaluate the effect of peroxidative damage on the hydrolysis of microsomal membrane. Since the protein released into the soluble phase increased with peroxidation damage, a greater rate of hydrolysis by lysosomal enzymes might be expected. A possible explanation for the apparent decrease in hydrolysis is that the

released protein formed aggregates, by interaction of their lipophilic regions, which could not be hydrolyzed easily by the lysosomal enzymes. Aggregation of microsomal suspensions during peroxidation has been described (31). Increased release of nucleotides probably resulted from hydrolysis of released ribosomal subunits and *m*-RNA. Arstila et al. (40) showed by electron microscopy that increased peroxidation caused detachment of ribosomes from membrane followed by dissociation of the ribosomal particles. The structure of the microsome then changed from round to less regular vesicles and finally to a dense, amorphous precipitate that contained membrane debris.

After hydrolysis of the peroxidized microsomes, the apparent increase in fluorescent pigment concentration was partially caused by a decrease in the protein concentration during hydrolysis by lysosomal enzymes and ultrafiltration. The nonperoxidized membrane (0 hr) had a greater increase in fluorescence than the peroxidized membranes due to the continued peroxidation of the microsomal membrane during the lysosomal hydrolysis. In vivo, the same result might be expected. Once initiated, membrane peroxidation could continue even during the degradation process within the lysosome.

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