MICROSOMAL LIPID PEROXIDATION CAUSES AN INCREASE IN THE ORDER OF THE MEMBRANE LIPID DOMAIN

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1. Introduction

The microsomal fraction isolated from rat liver can undergo an enzymatically catalyzed lipid peroxidation when incubated in the presence of NADPH, concomitant with an enhanced oxygen uptake and oxidation of NADPH [1]. The mechanism of microsomal lipid peroxidation has been extensively studied [2–8]. The first step is the abstraction of a hydrogen atom from an allylic methylen group. This leads to the formation of a lipid radical. Subsequent chain reactions result in a breakdown of poly-unsaturated fatty acids and the production of stable endproducts including ethane, propane, pentane, and also to the production of malondialdehyde.

Several biochemical changes concomitant with, or due to, lipid peroxidation have been investigated [9,10]. However, little is known about the biophysical consequences of lipid peroxidation in biomembranes. Spectral studies of fluorescent probes [11] indicated that lipid peroxidation decreases the fluidity of microsomal membranes, whereas a spin label study showed that γ radiation-induced lipid peroxidation decreases the degree of order in membrane lipids [12].

Using steady state fluorescence anisotropy of diphenylhexatriene we now show that lipid peroxidation in rat liver microsomes increases the order of the microsomal phospholipid acyl chains. This increase in order might be due to the formation of covalent bonds between adjacent lipid radicals. This is the first quantitative study of the change in membrane structure induced by lipid peroxidation.

2. Materials and methods

Chemicals were obtained from the following sources: Boehringer (Mannheim), isocitrate dehydrogenase (pig heart, grade III), nicotinamide adenine dinucleotide phosphate (reduced form, tetrasodium salt). Fluka (Buchs), 2-thiobarbituric acid, 1,6-diphenyl-1,3,5-hexatriene. All other chemicals were obtained from Merck (Darmstadt).

Male Sprague-Dawley rats (150–200 g body wt) were i.p. injected with phenobarbital (80 mg/kg body wt) daily for 4 consecutive days, starved for 24 h, then killed. Isolation and peroxidation of microsomes and the determination of malondialdehyde was as in [13].

For labeling peroxidized microsomes with diphenylhexatriene 1 ml aliquots of the peroxidizing membrane suspension were quickly cooled to 0°C at the time indicated and centrifuged for 30 min at 105 000 × *g*. The pellet was then resuspended in buffer (50 mM potassium phosphate (pH 7.2)) to 80 μg protein/ml and incubated for $\geq$15 min at room temperature with 0.25 μM diphenylhexatriene (2 mM stock solution in tetrahydrofuran).

Steady state fluorescence anisotropy was measured with an Aminco SPF-500 spectrofluorometer. The sample was excited by vertically polarized light at 357 nm, and the sample fluorescence at 450 nm through optical filters and the monochromator was analyzed into vertically and horizontally polarized components, I_V and I_H. The steady state anisotropy, r^s, is obtained by:

$$r^s = (I_V - I_H)/(I_V + 2I_H)$$

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For each measurement of r^S, I_V and I_H were corrected for contribution of intrinsic fluorescence and light scattering.

For the determination of the total fatty acid content of microsomes, lipids were extracted according to [14] as modified [15]. Transesterification was done as in [16]. Margarinic acid was added as an internal standard. The resulting fatty acid methyl esters were analyzed by gas chromatography on a diethyleneglycol succinate column (Packard Instruments Co.). Malondialdehyde was prepared by acid hydrolysis from tetraethoxypropane as in [17]. Phosphorus was determined according to [18], and protein as in [19].

3. Results

Fig.1 shows the time course of malondialdehyde production during peroxidation of rat liver microsomes, and the corresponding increase in the steady state fluorescence anisotropy r^S of diphenylhexatriene incorporated into the membranes. A good correlation between the malondialdehyde production and the increase in r^S is observed. This correlation was highly reproducible in different membrane preparations. The time course, however, varied among different preparations and depended on the age of the membranes. Thus, some freshly prepared microsomes show a lag phase in the malondialdehyde production up to 25 min which upon storage for 48 h at 0°C is almost completely abolished. However, some preparations show no lag phase at all. The observed variation in the time course of malondialdehyde production could be due to the presence of some endogenous antioxidants. Both the production of malondialdehyde and the increase in r^S were found to be dependent on the presence of NADPH in the peroxidation medium.

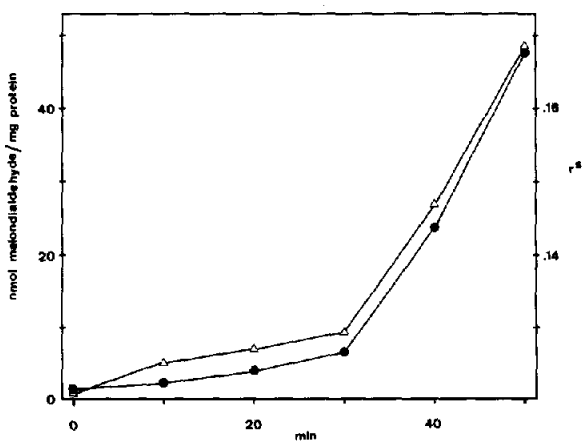

Fig.1. NADPH-dependent lipid peroxidation and steady state fluorescence anisotropy of diphenylhexatriene in rat liver microsomes. The formation of malondialdehyde (●——●) was used as an indicator for lipid peroxidation. Steady state fluorescence anisotropy r^S (△——△) was determined at room temperature.

Control experiments showed that the increase in r^S is most probably not due to the presence of malondialdehyde, a potential cross-linker, per se since addition of up to 100 nmol malondialdehyde/mg protein to non-peroxidized microsomes did not result in an increased value of r^S. After incubation for 30 min at 37°C little binding of malondialdehyde to the membranes (<5 nmol malondialdehyde/mg protein) was detected in these controls. We also determined the amount of added malondialdehyde that could be recovered from microsomes as thiobarbituric acid-reactive material. The recovery was complete indicating no further metabolism of malondialdehyde by microsomes.

The temperature dependence of r^S is shown in fig.2. A continuous decrease in r^S over 5–37°C is

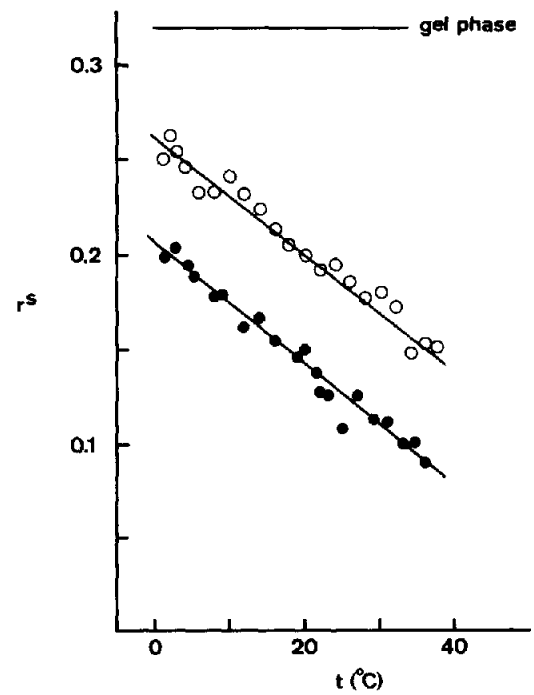

Fig.2. Temperature dependence of steady state fluorescence anisotropy of diphenylhexatriene in rat liver microsomes: (●——●) control microsomes; (○——○) peroxidized microsomes (40 nmol malondialdehyde/mg protein); expected r^S value for the gel phase of lipids.

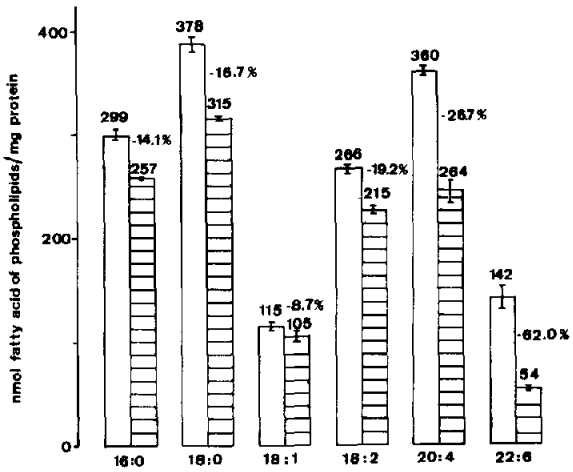

Fig.3. Fatty acid composition of microsomal phospholipids. Phospholipids were isolated from control microsomes (open columns) and peroxidized microsomes (20 nmol malondialdehyde/mg protein, hatched columns), and fatty acids were determined after transesterification. The decrease in the content of fatty acids in peroxidized microsomes is indicated in percent. The bars give the standard error in 4 expt.

observed for both control and peroxidized microsomes, indicating that both types of microsomal membranes are in the liquid–crystalline state over this temperature range.

Fig.3 shows the fatty acid composition of total phospholipids extracted from control and peroxidized (20 nmol malondialdehyde/mg protein) microsomes. There is a decrease in the detectable fatty acid residues from ~1560 nmol/mg protein in control to ~1210 nmol/mg protein in peroxidized microsomes. No lipid phosphorus (18–20 μg phosphorus/mg protein in either control or peroxidized microsomes) nor protein was lost from microsomes by peroxidation. From fig.3 it is evident that predominantly the polyunsaturated acyl chains are decreased by peroxidation. Analysis of the no. double bonds/mol lipid phosphorus (assuming 1 g atom lipid phosphorus = 1 mol phospholipid) shows a decrease of ~38% in the double bond content of the lipids in peroxidized microsomes (3.77 in controls, 2.35 in peroxidized microsomes).

During peroxidation only a minor liberation of fatty acids (<10 nmol/mg protein), possibly from the phospholipid ester bonds, could be observed using 2 independent methods [20,21].

4. Discussion

Diphenylhexatriene has been used extensively in liposomes and natural membranes to probe for what was called 'microviscosity' [22]. However, it has become clear that the steady-state anisotropy r^s of diphenylhexatriene does not give information about 'microviscosity' but about the orientational order of the fatty acid chains of phospholipids [23].

This report shows that there is a good correlation between the production of malondialdehyde and r^s in peroxidized rat liver microsomes. The increase in r^s of diphenylhexatriene in peroxidized membranes is indicative of an increased order of the microsomal lipid acyl chains. The determination of r^s alone, however, is not sufficient to show unequivocally the increase in order, since a decrease in the lifetime of diphenylhexatriene in peroxidized membranes could also lead to an increase in r^s.

A direct measurement of the increase in the orientational order of lipid acyl chains has been obtained with time-resolved fluorescence polarization. The time-dependent anisotropy $r(t)$ of diphenylhexatriene embedded in both control and peroxidized microsomes decayed with a few nanosecond time constant to a time-independent residual anisotropy $r(\infty)$ within the time range of several tens of nanoseconds (S. K., P. Wahl, unpublished). A significant increase in $r(\infty)$ from 0.09 (control) to 0.13 by ~50% was observed by microsomal lipid peroxidation to 60 nmol malondialdehyde/mg protein. These values correspond to the conventional order parameter of $S = 0.48$ (control) and 0.58 (60 nmol malondialdehyde/mg protein), respectively [24,25].

An interesting picture emerges from the quantitative analysis of the peroxidized microsomal membrane lipids and the increase in r^s. There is a large discrepancy between the amount of malondialdehyde produced and the amount of acyl chains lost upon peroxidation as detected by gas chromatography after transesterification. Little binding to and no metabolism of malondialdehyde by microsomes was observed. Only a negligible liberation of fatty acids from microsomal phospholipids could be detected in agreement with the finding [4] that there is little change in the microsomal fatty acid ester content of microsomal phospholipids due to peroxidation. Besides malondialdehyde, ethane, propane, and pentane are well established breakdown products of peroxidizing membrane lipids. The amount in which they are produced relative

to the amount of acyl chains oxidized has not been carefully assessed. However, it can be estimated that the amount of hydrocarbon gas produced in peroxidizing microsomes is at least an order of magnitude smaller than the amount of malondialdehyde [26,27]. It therefore appears that a large part of the acyl side chains that does not appear after transesterification and gas chromatography cannot be accounted for by the production of ethane, propane, and pentane. One probable interpretation for the observed decrease in unsaturated bonds is that during peroxidation neighbouring acyl side chain radicals form covalent bonds between each other and thus decrease the motional freedom of diphenylhexatriene.

The malondialdehyde produced during lipid peroxidation could conceivably react as a bifunctional reagent and cross-link membrane components [28]. Our findings that:

(i) r^s of diphenylhexatriene in peroxidized microsomes increases parallel with the malondialdehyde production;

(ii) Added malondialdehyde does not change r^s indicate that malondialdehyde does not cross-link in the lipid domain.

Acknowledgements

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