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ANTI-LIPOPEROXIDATION ACTION OF FOOD RESTRICTION

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SUMMARY: Chronic food restriction inhibited the age-related increase of malondialdehyde production and lipid hydroperoxides in liver mitochondrial and microsomal membranes of ad libitum fed Fischer 344 rats. The anti-lipoperoxidation action of food restriction could not be attributable to the changes in membrane lipid content nor vitamin E status. Restricting calories modified membrane fatty acid composition by increasing linoleic acid and decreasing docosapentaenoic acid content in both membranes. The significance of the fatty acid modification was discussed in terms of anti-lipoperoxidation and membrane fluidity.

Chronic food restriction is now recognized as the most effective manipuation that extends the lifespan of laboratory rodents (1). Studies from our laboratory and others showed that food restriction also prevents age-related loss of physiological functions (2) and retards the occurrence of many age-related diseases (3).

In 1956, Harman (4) proposed a hypothesis that a major cause of the aging process is deleterious free radical reactions occurring in normal aerobic metabolism. Since these free radicals are known to preferentially attack lipids and lipid molecules in cell membrane structures (5), we addressed the question of lipid oxidation in aging with the proposal that food restriction exerts its anti-aging action by attenuating membrane lipid peroxidation (6). We tested this concept with liver mitochondrial and microsomal membranes and report herein supportive evidence.

MATERIALS & METHODS

Animals and diet.

Specific-pathogen free Fischer 344 male rats (Charles River Lab) were maintained in a barrier facility and fed a semi-synthetic diet (Ralston-Purina) as previously described (7). Food restriction was started at 6 weeks of age and consisted of 60% of the daily caloric allowance of the ad libitum fed controls (7).

Membrane preparations.

Rats were anesthetized with ether and exanguinated. The liver tissue was homogenized (10% w/v in 10 mM HEPES pH 7.4 containing 220 mM mannitol, 60 mM sucrose and 10 mM KCl) and was centrifuged at 500 X g for 5 min. The resulting supernatant was centrifuged at 2400 X g for 10 min. and the pellet was used as mitochondrial fraction. The remaining supernatant was mixed with 8 mM $CaCl_2$ for 5 min. and spun down at 25,400 X g for 15 min. to yield the micro-

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somal fraction. Each fraction was washed once and resuspended in 37.5 mM Tris (pH 7.4) and 112 mM KCl buffer. Purity of the membrane preparations was assessed with the following marker enzymes: Citrate synthase (8), NADPH cytochrome c (P-450) reductase (9) and B-galactosaminidase (10). Protein was determined according to Lowry (11).

Biochemical measurements and lipid analysis.

A. In vitro lipid peroxidation

Non-enzymatic peroxidation was carried out in 1.5 ml total volume of incubation system containing 37.5 mM Tris (pH 7.4) and 112 mM KCl, 10 μM $FeSO_4$ and 0.5 mM ascorbic acid at 30°C with shaking. Enzyme-dependent lipid peroxidation was carried out in presence of 1 mM NADPH, 0.2 mM $FeSO_4$ complexed with 5 mM ADP as described (12). The extent of lipid peroxidation was measured by a modification of the TBA assay reported by Ohkawa et. al. (13) using malonaldehyde (MDA) bisdiethyl acetal as standard.

B. Lipid hydroperoxide and phosphorus.

Total membrane lipids were extracted with 2:1 chloroform/methanol (14). Hydroperoxides were determined with the potassium iodide method of Buege and Aust (15) using cumene hydroperoxide as standard. Lipid phosphorus was assayed as described by Ames (16).

C. Fatty acid analysis and vitamin E

Fatty acid esters were derivatized with methanolic-HCl and analyzed by gas-liquid chromatograph (Model 8420 Perkin-Elmer) using silica capillary column (DB225, J&W Scientific, Ca) at 210°C. Mixtures of fatty acid standards (Nu-check-Prep) were run for peak identification, and further verification of each fatty acid was obtained by mass spectrometry. Membrane vitamin E content was determined by the fluorometric method of Taylor et. al. (17).

RESULTS

The first task in testing our hypothesis was to assess the extent of membrane lipid peroxidation. Fig. 1 illustrates the time course of lipid peroxidation of membranes from ad libitum (AL) and restricted rats (FR) at 6 mo. and 24 mo. of age. In the AL rats, there were marked age-related increases in membrane peroxidation induced by both enzyme-dependent (Fig. 1) and non enzymatic (data not shown) incubation conditions. The age-related increase was 35-40% over the 6 mo. values and this increment was approximately the same in mitochondria as in microsomes. The time course study revealed that peroxidation of the membranes from AL rats was characterized by a rapid burst of MDA production during the first 10 min. of incubation which resulted in extensive peroxidation after 60 min., and in 24 month old rats lipid oxidation was further enhanced. In contrast, membranes from FR animals strongly resisted the induction of in vitro peroxidation. As shown in Fig. 1, MDA production during 60 min. incubation was effectively inhibited in 6 and 24 mo. old FR rat membranes, and the generation of MDA by FR rats at 60 min. was much less than that of the AL fed rats at 5 min. of incubation.

To further ascertain the effects of age and dietary restriction on membrane lipid peroxidation, endogeneous lipid hydroperoxides were measured in mitochondria and microsome from both dietary groups of rats at 6, 12 and 24 mo. of ages. Fig. 2a and Fig. 2b show that mitochondrial and microsomal hydroperoxide contents increased with age in both groups. However, the age-related hydroperoxides were significantly lowered ($p < 0.005$) by food restriction to levels that never exceeded the lowest point (found at 6 mo. of age) in AL rats.

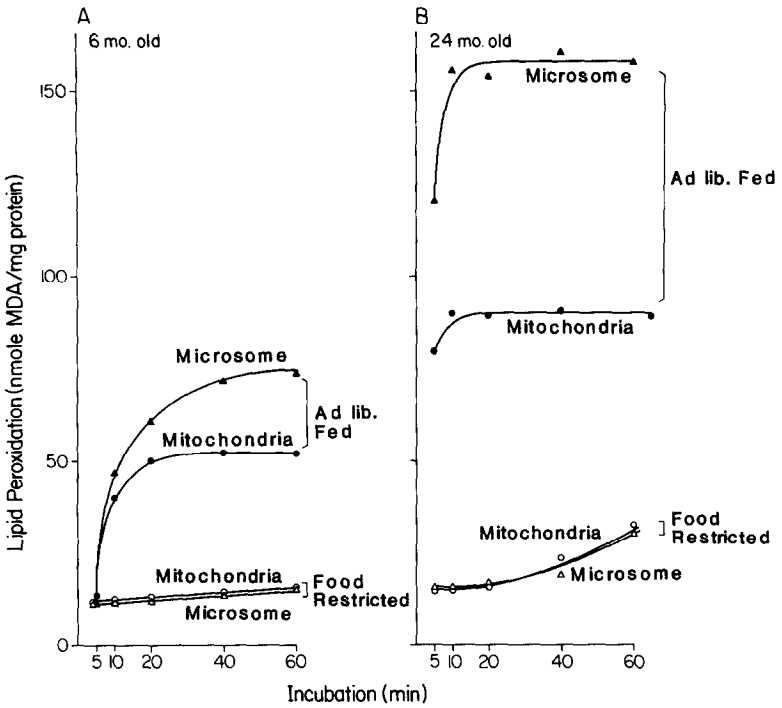

Fig. 1. In vitro enzyme-dependent lipid peroxidation. (A) 6-month-old rats (B) 24-month-old rats. Peroxidation was performed as described in Materials and Methods in the presence of 0.2 mM $FeSO_4$, 5 mM ADP and 1 mM NADPH.

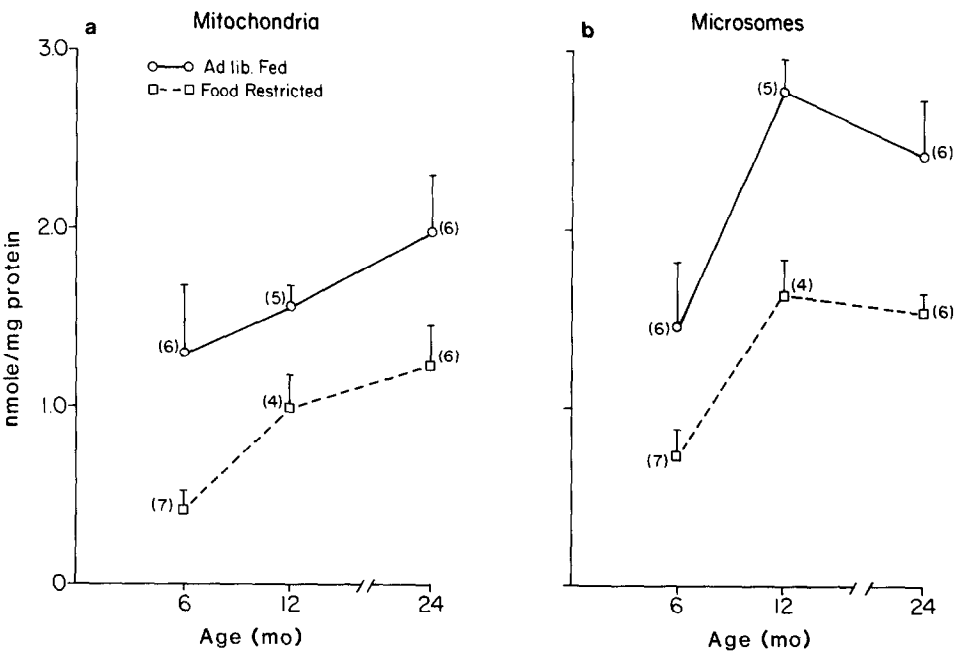

Fig. 2. Endogeneous lipid hydroperoxides of (a) mitochondria and (b) microsomes. Membrane lipid extracts were assayed by the potassium iodide method of Buege and Aust (15). Statistical differences between ad libitum fed rats and restricted rats are $0.01<p<0.05$.

TABLE 1. Phospholipid content of membranes

MEMBRANE FRACTION	LIPID PHOSPHORUS ($\mu g P_i$/mg protein)					
	6 mo.		12 mo.		24 mo.	
	AL	FR	AL	FR	AL	FR
Mitochondria	8.33 ±.45	7.47 ±.53	9.66 ±.44	10.07 ±.44	9.46[a] ±.63	7.34[a] ±.38
Microsomes	11.10 ±1.10	11.46 ±.68	12.13 ±.33	13.27 ±.95	12.50 ±1.13	10.71 ±.66

Mean ± S.E.M. of n = 5 - 9 rats per group.

[a] $p < 0.01$

It is worthy pointing out that microsomes consistently showed higher peroxidative activity than mitochondria. This may be related to the higher lipid content of the microsomal membrane as indicated by the data in Table 1. Interestingly, the amount of membrane phospholipid was not significantly altered by diet nor age, except at 24 mo. of age, when FR rat mitochondria content was slightly lower than in the AL rats.

To gain some insight on membrane structural alteration and protection against lipoperoxidation, vitamin E content of membrane preparations from both dietary groups was determined. As shown in Table 2, vitamin E content was less in FR rat membranes, particularly in microsomes ($p < 0.05$). The interesting finding is that membrane vitamin E content does not show any correlation to the amount of lipid peroxidation and hydroperoxide content of membranes. Regarding the membrane integrity, the membrane composition was assessed by analysis of the microsomal and mitochondrial fatty acid compositions of 12 mo. old rats from the two dietary groups. Table 3 shows that the unsaturation/saturation ratios were significantly higher ($p < 0.01$) in FR rats than in AL

TABLE 2. Membrane Vitamin E

DIETARY GROUPS (12 Months of Age)	VITAMIN E ($\mu g/g$ protein)	
	Mitochondria	Microsomes
Ad Libitum-Fed	200.4 ± 31	388.5 ± 38[a]
Food Restricted	156.4 ± 17	247.5 ± 50[a]

Mean ± S.E.M. of n = 4-5 rats per group.

[a] $p < 0.05$

TABLE 3. Fatty acid composition of membranes (12 months of age)

MAJOR FATTY ACIDS	MITOCHONDRIA		MICROSOMES	
	AL	FR	AL	FR
16:0	19.71 ± 1.1	18.96 ± 0.2	20.33 ± 0.3	18.96 ± 0.4
18:0	21.12 ± 1.2	19.54 ± 0.4	19.30 ± 0.6	18.88 ± 0.3
18:1	9.28 ± 0.9	8.62 ± 0.2	10.20 ± 0.5	8.68 ± 0.2
18:2	11.54 ± 0.4[a]	16.62 ± 0.5[a]	12.03 ± 0.7[a]	16.05 ± 0.6[a]
18:3	0.44 ± 0.7	0.42 ± 0.02	0.43 ± 0.03	0.49 ± 0.04
20:4	23.29 ± 1.1	23.78 ± 0.2	23.12 ± 0.9	23.61 ± 0.3
22:5	3.60 ± 0.3[a]	1.97 ± 0.04[a]	3.30 ± 0.2[a]	1.88 ± 0.03[a]
22:6	3.45 ± 0.1	3.02 ± 0.2	2.97 ± 0.2	2.64 ± 0.1
Unsat/Sat Index	1.28 ± 0.01[a]	1.41 ± 0.02[a]	1.31 ± 0.01[b]	1.43 ± 0.04[b]

Mean ± S.E.M. of n= 4-5 rats per group.

[a] $p < 0.001$ [b] $p < 0.01$

Index is the sum of the percentage of the unsaturated fatty acids divided by the sum of the percentage of the saturated fatty acids.

rats. Food restriction also resulted in higher levels of linoleic acid (18:2) but lower docosapentaenoic acid (22:5) in both mitochondria and microsomes. These trends, higher unsaturation/saturation ratios and inverse changes between 18:2 and 22:5, prevailed until 24 months of age (data not shown). No change in arachidonic acid content was seen in either dietary group.

DISCUSSION

The present studies have demonstrated that age-related increases in lipid peroxidation of subcellular membranes can be effectively inhibited by chronic food restriction. The increased peroxidation of mitochondrial and microsomal membranes from AL fed rats was clearly suppressed in FR rats as indicated by the rate and amount of MDA generated during 60 min. incubation. We substantiated the in vitro lipoperoxidation with a direct measurement of endogeneous peroxides of membrane lipid, because MDA quantitation by TBA test often includes other substances besides MDA (18). The hydroperoxides of both membranes increased almost two-fold from 6 to 24 mo. but this increase was much inhibited in FR rats at every age tested. It should be noted that, although the age-related increase in lipid peroxidation was previously reported (19), to our knowledge, our report is the first of such findings on the modulatory effect of food restriction on the age-related peroxidation of biological membrane. The only related studies in this regard are those by Chipalkatti et. al. (1983) (20) who showed decreased TBA-reactive peroxides in liver homogenates from food restricted mice.

Although the mechanisms for the anti-lipoperoxidation action of food restriction are not definable at present, our current study produced evidence

which eliminates possibilities that are not involved in the anti-lipoperoxidation action. The suppressed MDA production and peroxides in FR rats are not due to a lesser amount of lipid existing in FR rat membrane as shown in Table 1. It can be further evidenced that endogeneous vitamin E in membrane seems unlikely to play a major role in protecting membrane lipid alteration against the age-related lipoperoxidation as shown by the higher vitamin E content in AL rat membranes. However, the possibility remains that food restriction can modulate other cytosolic antioxidants or scavenger enzymes (21, 22) as protectors against membrane peroxidation, and currently this possibility is under extensive investigation in our laboratory (23). Another important finding revealed in this study is that food restriction can effectively modulate membrane fatty acid composition (Table 3) and can maintain membrane fluidity in spite of the increased lipid peroxidation with advancing age. It appears that the FR rats sustain a higher unsaturation/saturation index by modulating 18:2 and 22:5 fatty acids, and at the same time are able to minimize the age-related lipid peroxidation by decreasing a more highly peroxidizable 22:5 fatty acid (24, 25).

We therefore conclude that restriction of food intake provides the rats with means to maintain the integrity of membrane structure and fluidity by reducing the age-related lipid peroxidizability. This action of food restriction may play a major role in preventing loss of physiological functions as it is expressed in extending longevity of animals (1).

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