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Original Contribution

MODULATION OF CARDIAC MITOCHONDRIAL MEMBRANE FLUIDITY BY AGE AND CALORIE INTAKE

JAEWON LEE, BYUNG P. YU, and JEREMIAH T. HERLIHY

Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

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Abstract—The aim of the present study was to determine the effects of dietary restriction (DR) on the age-related changes in membrane fluidity, fatty acid composition and free radical damage of mitochondrial membranes obtained from the rat left ventricle. Mitochondrial membrane preparations were obtained from the left ventricles of 6- and 24-month-old, male, Fischer 344 rats that were allowed to eat throughout their life either ad lib (Group A) or only 60% of the amount consumed by the ad lib fed group (Group B). Our results show that the membrane fluidity of the 24 month Group A hearts was less than that of the 6 month group A hearts. No differences in membrane fluidity were observed between the 6 and 24 month DR groups. The fatty acid composition of the mitochondrial membranes of the two ad lib fed groups differed: the long-chain polyunsaturated 22:4 fatty acid was higher in the older group, although linoleic acid (18:2) was lower. DR eliminated the differences. No statistically significant difference in the overall polyunsaturated fatty acid content was noted. However, the peroxidizability index was higher in the membranes of the 24 month Group A hearts but not in the 24 month Group B hearts. Finally, the degree of lipid damage, as assessed in vitro by the induced production of reactive oxygen species, was elevated in the 24 month Group A hearts. No difference was observed between the young and old DR groups. Considered together, these results suggest that DR maintains the integrity of the cardiac mitochondrial membrane fluidity by minimizing membrane damage through modulation of membrane fatty acid profile. © 1998 Elsevier Science Inc.

Keywords—Rat, Reactive oxidant species, Lipid peroxidation, Fatty acid composition, Dietary restriction, Heart, Free radicals, Membrane fluidity

INTRODUCTION

The oxidative stress theory of aging holds that the progressive deterioration and time associated alterations arising during aging are the cumulative result of incessant free radical generation occurring in the course of normal cellular metabolism [1,2]. Although all components of the cell are subjected to reactive oxidant attack, biological membranes, because they are composed mainly of lipid, are especially susceptible to oxidative damage. The injury appears mainly as an increase in membrane lipid peroxidation that compromises membrane integrity. Because most reactive oxidants are produced during oxidative processes occurring in the mitochondria, the membranes of these organelles run an increased risk of incurring oxidative damage. This is especially true in highly oxidative tissues such as the left ventricle of the heart. Dietary restriction (DR) exerts a strong anti-aging action. It increases longevity, decreases the occurrence and severity of age-related diseases and retards the physiological decline associated with aging [3,4]. One mechanism by which DR may retard the aging processes is its remarkable ability to reduce oxidative damage [5-8]. With regard to the heart, previous work from our laboratory [9] has shown that DR decreases the malondialdehyde content of cardiac mitochondria, indicating a decrease in lipid peroxidation damage. DR also enhances the functional characteristics of cardiac synaptosomes [10] and β-adrenergic stimulated inotropic responses [11]. It also retards the age-related decline in β-adrenergic stimulation of the adenylyl cyclase activity and its inhibition by adenosine receptor activation [12]. The present study was designed to expand upon our previous findings by determining the effects of age and

Address correspondence to: Dr. Jeremiah T. Herlihy, Department of Physiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284, USA; Tel: (210) 567-4333; Fax: (210) 567-4410.

DR on the cardiac mitochondrial membrane fluidity and lipid profile as well as the degree of oxidative damage.

MATERIALS AND METHODS

Animals

The rats utilized in these experiments were supplied by the core facility in San Antonio that maintains a population of aging rats treated as follows. Male, Specific Pathogen Free (SPF), Fischer 344 rats were obtained from Charles River Laboratories (Portage, MI) at 1.0 ± 0.07 months of age and maintained in a barrier facility as described previously [13]. At 1.5 months of age the rats were divided into 2 groups: Group A consisting of rats allowed free access to food and Group B, consisting of rats allowed to eat 60% of the amount consumed by Group A. An extensive description of the implementation of the DR paradigm and its effects on longevity and pathology has been published [14]. At 24 months of age, approximately 70% of the Group A rats survive and greater than 90% of the Group B rats are alive. The hearts from 24 month old Group A rats exhibit some morphological alterations at the microscopic level, although no gross pathology is evident. Hearts from Group B animals at this age are virtually free of these changes.

Preparation of cardiac mitochondrial membranes

Rats were sacrificed by decapitation at 6 and 24 months of age. The hearts were quickly excised and placed in cold, isotonic saline. The atria and large vessels were removed and the left ventricle separated from the free wall of the right ventricle. The left ventricle was snap frozen between tongs cooled to the temperature of liquid nitrogen and stored at 70°C. For each mitochondrial sample analyzed, two hearts were pooled and a mitochondrial fraction was prepared by differential centrifugation by methods used in our laboratory [9]. Ten hearts from each age and dietary group (total of 40 hearts) were utilized to obtain 5 samples to be analyzed from each group. Whenever mitochondrial material was sufficient, determinations of membrane fluidity, lipid profiles and reactive oxygen species (see below) were all performed on the same sample. Protein was measured according to the method of Bradford [15].

Determination of membrane fluidity

Membrane fluidity was assessed by measuring fluorescence polarization of 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5 hexatriene (TMA-DPH) [16]. Mitochondria (0.2 mg of protein) were added to 46 mM potassium phosphate buffer (pH 7.2) to give a final volume of 3 ml. After the mixture was vortexed for 60 s, 0.167 nmol of TMA-DPH was added to the reaction medium to achieve a 1:600 mol ratio of probe/phospholipid. TMA-DPH was allowed to incorporate into the lipid moiety by incubating the mixture at 37°C for 30 min with gentle shaking. Fluorescence was measured on a Perkin-Elmer LS-50 luminescence spectrophotometer with the excitation and emission wavelengths set at 360 nm and 430 nm, respectively. An emission filter (390 nm) minimized stray light. The mixture was stirred constantly and the temperature maintained at 22 ± 0.2°C. Because fluidity is inversely related to polarization, changes in membrane fluidity are expressed as the reciprocal of polarization (1/P), as described previously [16, 17].

Lipid analysis

Fatty acid analysis was carried out by the method of Laganiere and Yu [18]. Briefly, mitochondrial lipids were extracted according to the method of Folch et al. [19] using a 2:1 (v/v) mixture of chloroform/methanol. Fatty acid methyl esters were derived in sealed tubes at 90°C for 2 h using methanolic HCl (Supelco, Bellefonte, PA). The prepared esters were extracted with hexane and analyzed on a Perkin-Elmer Model 8420 gas-liquid chromatograph equipped with a fused silica capillary column (DB-225, J & W Scientific, Folsom, CA). Injection port, oven and detector temperatures were set at 250°C, 210°C and 250°C, respectively, and the helium carrier gas flow maintained at 0.9 ml/min. Peak identification was obtained by comparison of retention times with mixtures of fatty acid standards. The determination of the peroxidizability index (PI) was performed as described previously [18] and modified by Pamplona et al. [20]. Briefly, the PI = (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 3) + (% pentaenoic acid × 4) + (% hexaenoic acid × 5).

Quantification of reactive oxidant species (ROS)

The various ROS, including superoxide, hydrogen peroxide, hydroxyl radicals and lipid hydroperoxides, were quantified by previously published methods [21-23], using the probe, 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). Mitochondria (0.1 mg protein) were added to ice-cold potassium phosphate buffer (50 mM, pH 7.4) containing 5 uM DCFH-DA. The final volume of the mixture was 3.0 ml. DCFH-DA incorporation into the membranes was allowed to proceed by pre-incubation at 37°C for 15 min. To remove the unloaded DCFH-DA, the mixture was centrifuged for 8 min at 12,500 ×

g at 4°C. The pellet was resuspended in 3.0 ml of 50 mM potassium phosphate buffer and incubated with 0.5 mM *tert*-butyl hydroperoxide at 37°C for 30 min to induce lipid peroxidation. The changes in fluorescence intensity was measured at 10, 15, 20, 25 and 30 min on a Perkin-Elmer LS-50 luminescence spectrophotometer with excitation and emission wavelengths set at 488 nm and 525 nm, respectively.

Chemicals

TMA-DPH and DCFH-DA were obtained from Molecular Probes (Eugene, OR). 2′,7′-Dichlorofluorescein and *tert*-butyl hydroperoxide were obtained from Sigma; the methanolic-HCl kit was purchased from Supelco (Bellefonte, PA). All other compounds were of reagent grade.

Statistical analysis

The statistical significance of the differences between the treatments (age and diet) was determined by two-factor ANOVA. Differences between the means of the individual groups were assessed by one-factor ANOVA in conjunction with Duncan's New Multiple-range test. Values are expressed as the mean ± SEM for 4 or 5 samples in each group. A value of $p < .05$ was set as the level of significance.

RESULTS

Membrane fluidity

Both age and diet affected membrane structure as indirectly shown by changes in membrane fluidity (Fig. 1). Mitochondrial membranes from 24 month old Group A rats were less fluid compared to those of 6 month old Group A rats. The age-related decline in membrane fluidity was prevented by DR. The membranes from 24 month old Group B rats possessed the same fluidity as the 6 month old rats. Except for the 24 month old Group A rats, no differences were observed between the means of the other groups.

Analysis of fatty acid composition

The fatty acid composition of the total lipids of mitochondria from the left ventricle was also affected by age and diet (Table 1). Aging was associated with a decrease in the less saturated 18:2 fatty acid (linoleic acid) and an increase in the highly unsaturated 22:4 fatty acid (Table 1, Groups 6A and 24A). Membranes obtained from DR hearts at both 6 and 24 months exhibited higher 18:2 fatty acid and lower 22:4 fatty acid contents than their respective age-matched, ad lib fed controls. DR modulated the effect of age on the contents of the 18:2 and 22:4 fatty acids because no differences were observed between the content of the fatty acids in Group 6B and Group 24B.

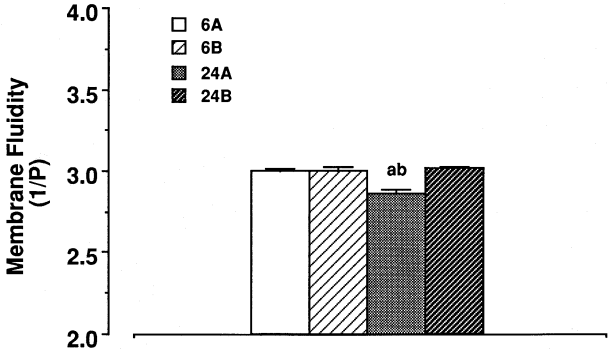

Fig. 1. Effects of age and calorie restriction on membrane fluidity of cardiac ventricular mitochondria. Fluidity is expressed on the ordinate as the inverse of membrane fluorescence polarization detected with TMA-DPH. Columns and bars represent the means ± SEM for 4 samples (2 hearts/sample, total of 8 hearts utilized) in each group. [ab]Represents a significant difference between the mean of Group 24A and that of Group 6A and Group 24B. Both age and diet treatments significantly affected membrane fluidity ($p < .01$ and $p < .005$, respectively).

Although no noticeable differences were observed in the total percentage of polyunsaturated fatty acids (Table 1), an examination of the peroxidizability index (PI) reveals that the membranes from Group 24A were significantly more peroxidizable than membranes from Group 6A, indicating an age-dependent shift in the fatty acid pattern. DR attenuated the age effect; the PI of the Group 24B membranes was the same as that for Group 6B (Table 1).

Induction of in vitro lipid peroxidation

To assess the degree of lipid peroxidation in each group, the ROS formation of the mitochondrial membranes was determined after ROS induction by t-butyl hydroperoxide. ROS formation increased with age in both dietary groups (Fig. 2). However, the ROS production of the Group B membranes was significantly lower at both 6 and 24 months than that observed in Group A. In fact, the older Group 24B had a lower ROS formation than the younger Group 6A.

DISCUSSION

Aging is associated with alterations in many membrane-related properties and activities, including a decrease in membrane fluidity [8]. The results of the present study (Fig. 1) confirm the age-related loss in

Table 1. Effects of Age and Calorie Restriction on the Fatty Acid Composition* of Left Ventricular Mitochondrial Membranes

Group	6A	6B	24A	24B
C16:0	11.84 ± 0.39	11.62 ± 0.65	12.22 ± 0.26	11.77 ± 0.52
C18:0	21.84 ± 0.31	21.59 ± 0.21	21.00 ± 0.51	21.57 ± 0.16
C18:1	7.55 ± 0.34	8.08 ± 0.28	8.53 ± 0.34†,‡	7.47 ± 0.16
C18:2	19.09 ± 0.68‡	22.58 ± 1.07	12.96 ± 0.28†,‡	22.93 ± 0.52
C20:0	1.10 ± 0.71	0.51 ± 0.12	0.59 ± 0.24	0.41 ± 0.10
C20:4	22.58 ± 0.30	22.38 ± 0.27	23.61 ± 0.70	23.37 ± 0.46
C22:0	1.13 ± 0.43	0.65 ± 0.09	0.70 ± 0.21	0.41 ± 0.07
C22:3	2.84 ± 0.11‡	2.23 ± 0.21	3.22 ± 0.23‡	1.92 ± 0.10
C22:4	5.25 ± 0.20‡	4.10 ± 0.50	8.72 ± 0.19†,‡	3.38 ± 0.30
C22:5	1.76 ± 0.17	1.46 ± 0.02	2.11 ± 0.48	1.86 ± 0.06
C22:6	4.84 ± 0.12	4.60 ± 0.14	6.13 ± 0.97	4.58 ± 0.19
PUFA	56.35 ± 0.77	57.36 ± 0.58	56.76 ± 0.96	58.05 ± 0.58
PI	139.5 ± 1.2	135.4 ± 1.66	155.5 ± 5.3†,‡	137.4 ± 2.3

* Values are expressed as mol % of total lipids and represent the means ± SEM for 5 samples in each group, except Group 24B which had 4 samples.
† Different from the group on the same diet ($p < .05$).
‡ Different from the group of the same age ($p < .05$).

membrane fluidity and are in agreement with the results of previous studies from this laboratory on the aging brain [24] and liver [16,25,26]. A major, new finding of the present work is that DR, which is known to retard the aging processes [3,4], significantly attenuated the loss in cardiac mitochondrial membrane fluidity (Fig. 1). These results extend to cardiac mitochondrial membranes the anti-aging effects of DR on membrane fluidity previously observed in subcellular membranes [16,24–26].

Zs.-Nagy [27] was among the first to suggest that physicochemical changes in membrane structure form the basis for the increase in membrane rigidity observed in the aging cell. Since then, several mechanisms have been proposed [28] to explain the effects of age and diet on membrane fluidity. The fatty acid composition of the membrane lipids and lipoperoxidation are major determinants of age-related changes in membrane fluidity as documented by earlier work from this laboratory [25]. It was demonstrated convincingly that aging was associated with a profound change in the lipid profiles of organelle membranes [18,29-32]. In these organs, the membranes from older rats possessed elevated levels of the highly unsaturated, long-chain fatty acids with lower levels of the less saturated fatty acids. A similar shift in the fatty acid profile was observed here in the heart mitochondria (Table 1). Mitochondrial membranes from 24 month Group A hearts had greater amounts of the 22:4, highly unsaturated fatty acids, exhibited a trend toward the relatively higher unsaturated 22:5 and 22:6 fatty acids and possessed lower levels of the less saturated 18:2 fatty acids (Table 1). Because polyunsaturated fatty acids posses lower melting points as compared to saturated fatty acids, a relative increase in the content of polyunsaturated fatty acids would be expected to render the membranes more fluid. Based solely on the fatty acid content shown in Table 1, the membranes from the older Group 24A should exhibit greater fluidity than those from Group 6A. Paradoxically, just the opposite occurred; membrane fluidity decreased with age (Fig. 1).

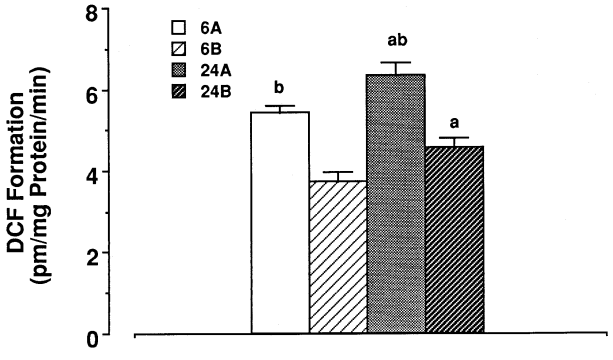

Fig. 2. Effects of age and calorie restriction on the production of reactive oxygen species (ROS) induced by *tert*-butyl hydroperoxide addition. DCF formation (ordinate) is used as an index of ROS production. Columns and bars represent the means ± SEM for 5 samples (2 hearts/sample, total of 10 hearts utilized) in each group, except Group 6B that had 4 samples. [a]Represents a significant difference between the means of the groups on the same diet (age effect); [b]represents a significant difference between the means of the groups of the same age (diet effect). Both age and diet treatments significantly affected membrane fluidity ($p < .003$ and $p < .0001$, respectively).

The paradox may be resolved by a careful consideration of the age-related changes in the oxidative status of the organ. Aging is associated with a decline in the anti-oxidant protective mechanisms [8] and cellular components experience increased oxidative stress with the passage of time. Because the highly unsaturated fatty acids are more vulnerable to free radical attack [25], they experience greater lipid oxidative damage. Lipid peroxi-

dation has been shown to contribute significantly to membrane rigidity [16,25,33,34].

In the present study, the ROS production of mitochondrial membranes from older hearts was higher than that of the younger hearts (Fig. 2), confirming the notion that the peroxidation level of the membrane lipids was higher in the older group. Because lipid peroxidation and peroxidized lipids favor an increase in membrane rigidity, it is not surprising that aging is associated with a decrease in membrane fluidity (Fig. 1). Thus, although the highly unsaturated fatty acid content of cardiac mitochondrial membranes increases with age (tending to increase membrane fluidity), the degree of peroxidation of these membranes also increases (which favors a decrease in membrane fluidity). The net effect of these two age-related processes overall is a decrease in membrane fluidity, as observed in Fig. 1.

The validity of this explanation is supported by our results with DR. DR retarded the age-related changes in mitochondrial membrane fluidity (Fig. 1) and eliminated the alterations in the lipid profile (Table 1). Mitochondrial membranes from rats subjected to DR possessed a higher 18:2 fatty acid content and a lower 22:4 content than ad libitum fed rats (Table 1). Again, based solely on the lipid profile, one would expect membranes from the DR group to exhibit a decrease in membrane fluidity. The decrease is not observed. Instead, membrane fluidity is maintained unchanged throughout most of the life span (Fig. 1). This apparent contradiction can be resolved by employing the same reasoning presented above for the aging effect. Although the change in the lipid profile induced by DR favors a decrease in membrane fluidity, DR is also associated with a decrease in ROS production (Fig. 2) and lipid peroxidation, leading to an increase in membrane fluidity. The overall net effect of DR is an increase in membrane fluidity (Fig. 1).

A general schematic for the various processes is shown in Fig. 3. The fatty acid composition of the membrane affects the degree of peroxidizability for the membrane lipids; the more highly unsaturated the fatty acid, the greater is its peroxidizability. The presence of ROS leads to an increase in lipid peroxidation especially in the more peroxidizable fatty acids that, in turn, increases membrane rigidity. The cell attempts to correct the increase in rigidity by increasing the content of highly unsaturated fatty acids in the membrane. Although these fatty acids tend to decrease membrane rigidity, they are also more vulnerable to free radical attack and the consequent lipid peroxidation. Thus, the cycle is complete and a positive feedback loop is established. DR exerts its anti-aging action on membrane rigidity by decreasing the level of free radical damage and membrane peroxidation and, thus, retarding the development of the positive feedback loop.

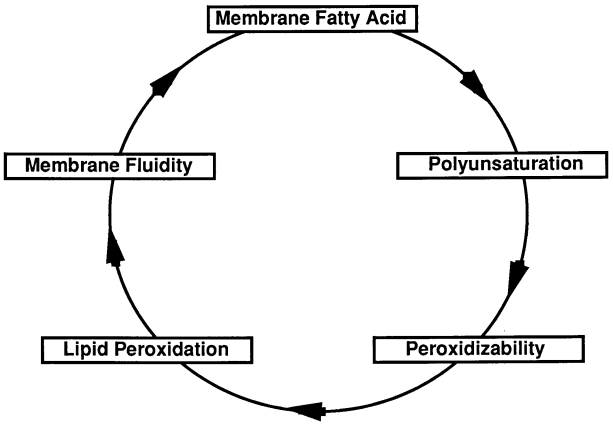

Fig. 3. Schematic representation of the lipid peroxidation cycle. See text for details.

Many studies have shown that dietary fat composition influences the fatty acid patterns of the organism. The significance of our study is the demonstration that restriction of calorie intake alone can also elicit such changes and the findings reported here for changes in the cardiac lipid profile are similar to those reported by us in hepatic organelles [18].

In many tissues, aging is associated with changes in membrane receptor function and DR has been reported to retard some of those alterations. DR attenuates the age-related increase in hepatic beta receptor number [35,36] and retards the loss in alpha-1 adrenergic responsiveness in aorta [37] and parotid gland [38]. In the lung, the decline in the isoproterenol-stimulated increase in adenylyl cyclase activity was also retarded [39]. With regard to the heart, DR was shown to increase the β-adrenergic responsiveness of left atria from young [40] and older [11] rats. Recently, Gao et al. [12] demonstrated that the age-related loss in the β-adrenergic-stimulated adenylyl cyclase activity as well as the decline in the inhibition due to adenosine A1 receptor activation were prevented by DR and suggested that DR acted by maintaining the coupling between the receptor and G-proteins. The mechanisms responsible for these anti-aging actions of DR are not known, although prevention of changes in membrane fluidity [41] or lipid peroxidation [42] has been proposed. The maintenance of membrane fluidity and prevention of lipid peroxidation as seen in the present study are in agreement with these suggestions.

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ABBREVIATIONS

DCFH-DA—2′,7′-dichlorofluorescein-diacetate
DR—dietary restriction
ROS—reactive oxidant species
TMA-DPH1—1-(4-Trimethylammonium phenyl)-6-phenyl-1,3,5 hexatriene