

AGING AND CALORIC RESTRICTION AFFECT MITOCHONDRIAL RESPIRATION AND LIPID MEMBRANE STATUS: AN ELECTRON PARAMAGNETIC RESONANCE INVESTIGATION

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Abstract—Previous studies have indicated that reactive oxygen species (ROS) are likely involved in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (AD). ROS, generated by succinate-stimulated mitochondria, have been reported to be spin trapped and detected by electron paramagnetic resonance (EPR). Our aim in the current study was to study the impact of aging on the effect of increased metabolic stimuli on mitochondrial respiration in terms of oxy-radical generation and possible lipid peroxidative changes in brain neocortical membranes. A mixed population of brain synaptosomes and mitochondria from brown norway male rats of differing ages being fed either ad lib (AL) or a caloric-restricted diet (DR) was prepared and labeled with 5-nitroxyl stearate (5-NS), a membrane lipid-specific spin label. The changes in anisotropic motion of the intercalated 5-NS spin probe also allows one to evaluate the status of the membrane fluidity in the lipid microenvironment via the order parameter. Upon succinate stimulation of mitochondria, the ROS generated resulted in a decrease in the EPR signal amplitude of the 5-NS reporter molecule indicative of the flux of oxy-radicals produced and possible peroxidation-induced changes in the synaptosomal lipid membrane. The line width remained constant, indicating that the overall intensity was reduced. The results showed a significant overall age effect in the ability to generate oxygen-derived radicals following metabolic stimulation ($p < .0001$). Stimulation of state 4 mitochondrial respiration with 20 mM succinate resulted in greater oxy-radical production in 25-month-old animals as compared to younger animals, suggesting increased mitochondrial leakage with age. Free radical stress induced by metabolic stimulation also causes a concomitant increase in membrane fluidity ($p < .0001$). There was also a significant age effect ($p < .0007$) on the order parameter of the mixed population of membranes. Although caloric restriction attenuated the membrane rigidization caused by aging, it was not found to play a role in limiting the oxy-radical production following metabolic stimulation of mitochondria. The overall effect of age on membrane spin-label intensities EPR signal upon succinate stimulation suggests that progressive mitochondrial dysfunction may be a key factor in the aging process and in development of age-associated diseases. © 1997 Elsevier Science Inc.

Keywords—Brain mitochondria, Electron Paramagnetic Resonance (EPR), Aging, Caloric restriction, 5-Nitroxyl stearate, Membrane fluidity

INTRODUCTION

Multiple lines of evidence have shown that an unavoidable consequence of life in an aerobic environment is the production of oxygen-derived free radicals. The

free radical theory of aging proposes that these free radicals result in a cumulative damage to critical cellular components, eventually leading to many age-related disorders.^{1–2} Consistent with this hypothesis, there is evidence in the literature suggesting that the maximum life span in a majority of mammalian species increases as the aerobic metabolic rate at rest decreases and body size increases.^{3–6} For example, the BMR is

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about seven times higher in rats than in humans, consistent with a much shorter life-span.⁴ Increase in the basal metabolic rate could lead to a substantial production of endogenous oxidants like superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}) and other by-products of normal metabolism. Studies have demonstrated that the level of consequent oxidative DNA damage is roughly related to metabolic rate across a range of mammalian species.⁷⁻⁹ In addition to normal brain aging, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease are particular age-related disorders wherein it is proposed that a free radical-mediated deterioration of neuronal or glial membrane components is etiologically involved in the neurodegenerative process.¹⁰⁻¹³

One cellular source of oxidants on which several disease states are thought to be predicated is the mitochondrion, which apart from being central to the bioenergetic process, is also proposed to be involved in the aging process.¹⁴ Much of the attention on this organelle is due to its ability to generate oxy-radicals during respiration, which is tightly coupled with oxidative phosphorylation. Normal mitochondrial respiration utilizes its electron transport chain to accomplish a four-electron reduction of O_2 to water with a simultaneous production of ATP through phosphorylation of ADP. A temporary or sustained loss of mitochondrial function and ATP production has been implicated in etiology of several neurodegenerative disorders.¹⁵

With respect to neuronal cell membranes, previous studies in other laboratories have shown that membrane fatty acid modification is an age-dependent phenomenon that is related to the extent of lipid peroxidation.¹⁶ Thus, increases in lipid peroxidation with age may play a critical role in modifying membrane-related functions.¹⁷⁻²¹ Many mitochondrial functions are also linked directly to the structural integrity of its membranes, which is dependent primarily on the interaction of lipids and proteins within the membranes.²² The components of the electron transport chain can, under certain abnormal conditions, initiate free radical-mediated peroxidation of mitochondrial lipid membrane system.²³

Several studies regarding synaptosomal membrane fluidity demonstrate an age-related change in the biophysical characteristics of the membrane.^{17,24} Rigidization of the membrane with advancing aging has been correlated to the biochemical interaction between the changing phospholipid composition and increasing cholesterol content.^{25,26} However, recent studies using dietary restriction as a modulator of free radical metabolism suggest that changes in lipid

composition alone are not responsible for age-related alterations in membrane fluidity. Decreased membrane fluidity is now speculated to be a secondary event to two age-dependent processes: (1) the lipid peroxidation process, and (2) increase in accumulation of lipids with a higher degree of unsaturation more prone to oxidation.^{17,24,27,28}

Because 80-90% of cellular oxygen is normally consumed by the activity of the mitochondrial respiratory chain, mitochondria represent the main site of cellular oxygen activation.²⁹ Although, the mechanism underlying the age-related increases in mitochondrial production of $O_2^{\cdot-}$ and H_2O_2 is unknown, it has been well established that the mitochondrial macromolecules undergo damage by self-generated ROS.³⁰ Higher levels of oxygen tension, for example, during exercise, or increased respiratory chain substrate concentrations under induced metabolic stress tend to form higher levels of ROS.²⁹⁻³⁰ These ROS can contribute to oxidative damage of mitochondrial lipids, proteins, and DNA.

In the current study, EPR spectrometry was used in conjunction with a site-specific spin label to investigate: (a) whether succinate stimulation of mitochondria results in oxidative modification of membrane lipids; (b) whether there are age-related differences in the effects of succinate stimulation of mitochondrial respiration; and (c) whether caloric restriction could exert its reported antiaging effect by reducing this free radical production following succinate stimulation of mitochondrial respiration. The hypothesis being tested is that caloric restriction protects brain mitochondria and its biomolecular components and, thus, decreases metabolic generation of oxy-radicals, thereby modulating lipid membrane damage.

MATERIALS AND METHODS

Chemicals

5-Nitroxyl stearate, sucrose (ultrapure grade), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (ultrapure grade), Phenylmethane sulfonyl fluoride (PMSF), trypsin inhibitor, and other standard chemicals were obtained from Sigma Chemicals (St. Louis, MO). Protease inhibitors (leupeptin, pepstatin, aprotinin) were obtained from Calbiochem (San Diego, CA).

Animals and diet

Brown Norway (BN) male rats of specified ages were used in this study. The maximum life span of BN rats being fed ad lib is known to be 40 months. The rats maintained on diet restriction have a higher max-

imum life span of 47 months. The rats were maintained at the *National Institute on Aging* National Center for Toxicological Research Project on Caloric Restriction (NIA/NCTR/PCR) Colonies (Jefferson, AR) until 1 month prior to target ages, whereupon they were shipped by air to the Sanders-Brown Aging Center at the University of Kentucky, Lexington, KY. The rats were housed individually in $21 \times 10 \times 8''$ solid-bottom polycarbonate cages with wire tops for placement of food and water. The rats maintained on diet restriction (DR) had a daily access to 60% of the intake of a companion group of rats given ad lib access (AL) to the diet (NIH-31 pellets, Purina Feeds). The dietary restriction was implemented after the animals were 16 weeks of age. The DR were fed a special NIH-31 formulation providing a correction for intake of essential nutrients.³¹ The rats were maintained on a 12-h light, 12-h dark cycle with light portion beginning at 0600 h. Following a 2-to 3-week adaptation period, the rats were used for experiments.

Isolation of synaptosomes and mitochondria

The brain of the animal was rapidly removed after decapitation, the neocortex dissected free, and immediately suspended in ice-cold isolation buffer [0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bis(amino-ethyl ether)N,N,N',N'-tetraacetic acid (EGTA), and the protease inhibitors: 0.5 μ g/ml leupeptin, 0.5 μ g/ml pepstatin, 0.5 μ g/ml aprotinin, 20 μ g trypsin inhibitor, 0.2 mM PMSF]. Samples were homogenized with 12 passes through a Wheaton motorized teflon-coated homogenizer at 100 rpm with 0.25 mm clearance. The crude homogenate was centrifuged at $1500 \times g$ for 10 min at 4°C, after which the supernatant was collected and recentrifuged at $20,000 \times g$ for 10 min. The resulting pellet, termed the P-2 pellet, was dispersed in Krebs buffer (pH 7.4) containing 1 mM desferroxamine, an iron chelator. The P-2 pellet, which mainly contained synaptosomes and mitochondria, was assayed for protein concentration by the Lowry method.³²

Lipid-specific 5-nitroxyl stearate spin probe

To determine if changes in the physical state of cortical synaptosomal bilayer lipids occurred after metabolic stimulation, the lipid-specific spin probe 5-nitroxyl stearate was used. Unlike isotropic motion exhibited by nonoriented spin probes such as the protein sulfhydryl-specific spin label MAL-6, 5-NS exhibits anisotropic motion.^{20,21} This amphipathic spin probe

intercalates into both leaflets of the lipid bilayer with its fatty acyl chain embedded in the hydrophobic bilayer and its polar head group oriented near the polar head groups of the lipid molecules at the hydrophilic surface of the bilayer. It is conceptualized that the polar head group of 5-NS is held firmly in place by the head groups of the bilayer lipids, while, under normal conditions, the hydrophobic tail is free to undergo rapid anisotropic motion in the interior of the bilayer.^{20,21} Because the nitroxide group (the electron paramagnetic resonance-active portion) is covalently bound to the alkyl chain of the probe, the motion of the nitroxide group reflects the intramembrane motion in the adjacent segment of the molecule.

Therefore, 5-NS is a useful probe for monitoring changes in the membrane lipid microenvironment near the bilayer surface. A typical spectrum of 5-NS intercalated in the lipid bilayers of a mixed population of synaptosomes and mitochondrial membranes is shown in Fig. 1. The rapid anisotropic motion occurs about the long axis of the spin probe, which engenders new effective T tensor elements T'_{\parallel} and T'_{\perp} . An order parameter S is calculated from these T -tensor values by the equation:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{\parallel} - T_{\perp}} * \frac{TrT}{TrT'}$$

$$TrT' = T'_{\parallel} + 2T'_{\perp} \quad (1)$$

where the primed values are spectroscopically measured (Fig. 1) and the unprimed values are known constants obtained from single crystal data.³³ A decrease in S is taken to indicate an increase in the membrane fluidity of the lipid microenvironment about the spin probe.^{20,21,33}

Another parameter of interest was the amplitude (B_0) of the $M_1 = 0$ central line of the 5-NS spectrum.

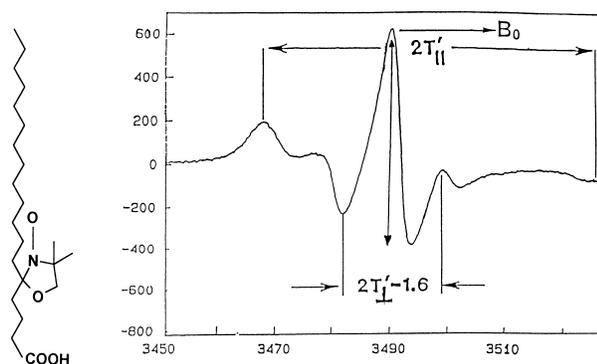


Fig. 1. Structure and representative EPR spectrum of 5-nitroxyl stearate (5-NS). T -tensor measurements from which the order parameter is calculated and the signal amplitude (B_0) are indicated.

This value is a measure of the amplitude of the 5-NS signal and, with a constant linewidth, a decrease in its magnitude is indicative of direct reduction of the nitroxide group by free radical reactive oxygen species like superoxide ($O_2^{\cdot-}$ and hydroxyl radical (OH^{\cdot}) to the corresponding EPR-silent hydroxyl amine.^{11,34–36}

5-NS spin labeling and EPR analysis

The lipid bilayer of membranes of this homogenate were spin labeled by incubation for 30 minutes with gentle shaking at 25°C using 0.6 μ g of the lipid-specific spin label 5-nitroxyl stearate per milligram of protein in Krebs buffer. After spin labeling, the protein concentration was adjusted to 4 mg/ml, aliquoted into Eppendorf tubes, and then treated with differing concentrations of succinate (0, 10, and 20 mM) to stimulate mitochondrial respiration. After a 3-h incubation at 22°C, EPR measurement of the amplitude (B_0) of the $M_I = 0$ central resonance line of the 5-NS signal and the fluidity parameters (T_{\parallel} and T_{\perp}) were performed (Fig. 1). The EPR spectra of labeled samples were acquired on a Bruker 300 EPR spectrometer operating at incident microwave power = 18 mW, modulation amplitude = 0.4 G, time constant = 1.28 ms, conversion time = 10 ms; field sweep width = 80 G.

Statistics

The following table provides a breakdown of the number of animals used in this study. AL represents the animals that were fed ad lib, and DR indicates animals maintained on a calorically restricted diet (Table 1).

To compare mean responses, an analysis of variance (ANOVA) for the three factor experiment, with factors diet (AL vs. DR), dose (0, 10, 20) and age (6, 16, 25 months), was constructed. If an overall significance was established for a particular factor, then post hoc comparisons between relevant cells was made. Values of $p < .05$ were considered significant.

RESULTS

Effect of age, diet, and succinate on oxy-radical production

The mitochondrial electron transport chain is the major source of $O_2^{\cdot-}$, and H_2O_2 generation,^{37–43} primarily under state 4 conditions (no exogenous ADP, but excess amount of substrate). The rates of state 4 respiration of heart and kidney mitochondria show a strong positive correlation with the rate of $O_2^{\cdot-}$ generation.⁶ Several previous studies have shown that ox-

Table 1.

Age (months)	Diet	Number of Animals (<i>n</i>) for each Succinate Concentration		
		Control	10 mM	20 mM
6	AL	6	6	6
6	DR	6	6	6
16	AL	7	7	7
16	DR	5	5	5
25	AL	8	5	5
25	DR	8	5	5

idation of succinate by the electron transport chain during the resultant mitochondrial respiration and its inhibition with mitochondrial inhibitors like antimycin and azide lead to EPR-detectable oxygen-derived radicals.^{44,45} In our study, mitochondrial respiration in a mixed population of synaptosomes and mitochondria was stimulated with increasing concentrations of succinate, a complex II substrate. A highly significant decrease ($p < .0001$) in 5-NS signal amplitude was observed as a graded function of an increasing dose of succinate (Fig. 2). Thus, a clear consequence of stimulation of succinate dehydrogenase, a part of complex II of the mitochondrial electron transport chain, is to decrease the $M_I = 0$ central resonance line signal amplitude (B_0) of the 5-NS spin probe. This observation is consistent with the generation of oxy-radicals that reduce the nitroxide moiety of the 5-NS spin probe to the corresponding hydroxyl amine.^{34–36} Consistent with this notion, addition of 2 mM potassium ferricyanide ($K_3Fe(CN)_6$) to the succinate-stimulated samples regenerated nearly 100% of the original EPR signal intensity (Fig. 3). Potassium ferricyanide is a known oxidizer of hydroxylamines to their corresponding nitroxides.³⁴ The results in Fig. 2 are also consistent with a peroxidative attack on the lipid membrane bilayer. Figure 2 also shows the effect of the animal's age on mitochondria in terms of its propensity to generate oxy-radicals upon metabolic stimulation. Our results demonstrate a significant overall age effect in the decrease of 5-NS signal amplitude following succinate-induced state 4 mitochondrial respiration ($p < .0001$). Thus, metabolic stimulation of mitochondria with succinate demonstrates a significant difference in the level of possible lipid peroxidative changes with age. The most significant differences were found between the 16- and 25-month-old animals ($p < .03$). Interestingly, at 10 mM succinate stimulation, the generation of oxy-radicals, as evidenced by a decrease in 5-NS amplitude in 6-month-old animals, was similar in magnitude to the 25-month-old animals. The capacity to generate oxy-radicals increased with increasing concentrations

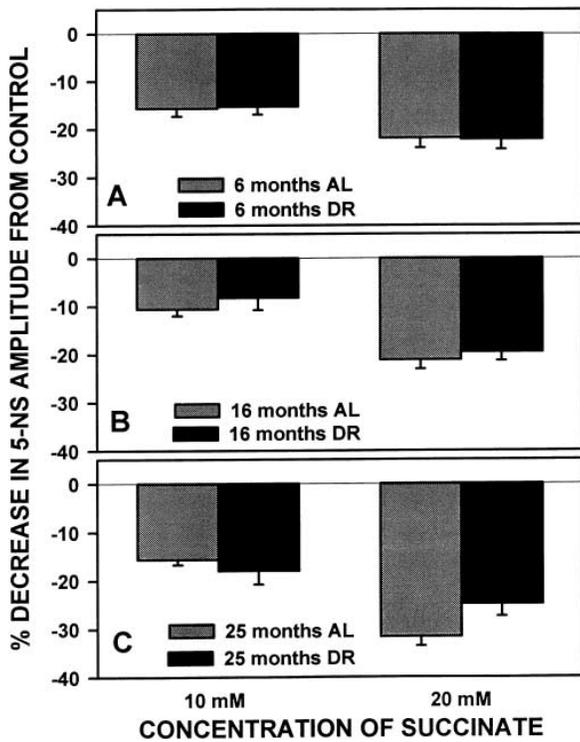


Fig. 2. Effect of age and caloric restriction changes in 5-NS signal amplitude (B_0) of spin-labeled lipid membranes following succinate-induced mitochondrial respiratory stimulation. There is an overall significant decrease in B_0 when 10 mM and 20 mM succinate stimulated preparation is compared with control ($p < .0001$). The amplitude changes in 5-NS-labeled mixed population of synaptosomes and mitochondria isolated from rats cortices is indicative of possible peroxidation-induced changes in the synaptosomal lipid membrane. A significant decrease in 5-NS signal amplitude is observed with increasing age ($p < .0001$) of animal following metabolic stimuli. No overall significant protective effect was observed with caloric restriction under similar conditions of metabolic stimulation. (A) A significant decrease in 5-NS amplitude, which is consistent with oxyradical generation, was observed between control and 10 mM succinate ($p < .0001$) and 10 mM vs. 20 mM ($p < .05$). (B) Significant decreases in B_0 in 10 mM ($p < .0001$) and 20 mM succinate treatments when compared to controls as well as when compared with each other ($p < .0005$). (C) Significant decrease in B_0 between control vs. 10 mM succinate ($p < .0001$) and 20 mM ($p < .0001$). Although statistically nonsignificant ($p < .06$), there was a protective trend due to caloric restriction in terms of oxyradical generation following stimulation with 20 mM succinate.

of succinate as evidenced by a loss in 5-NS amplitude following respiratory stimulation of mitochondria. At 20 mM succinate stimulation, a highly significant age effect was observed ($p < .001$). Succinate stimulation of mitochondria resulted in greater membrane damage in 25-month-old animals compared younger age groups, suggesting increased mitochondrial leakage with age.

Caloric restriction has been suggested to play a major role in modulation of the aging process.^{17,24,46-47} Some of our studies were designed to investigate

whether caloric restriction plays any role in decreasing oxy-radical generation upon mitochondrial complex II stimulation with succinate. Studies with animals of ages 6, 16, and 25 months on either a calorically restricted diet or fed ad lib showed significant age ($p < .0001$) and succinate concentration ($p < .0001$) effects on the ability of the mitochondria to generate oxy-radicals upon succinate stimulation. There was no significant effect of dietary restriction in altering the mitochondrial ability to generate oxy-radicals under identical metabolic stimulation conditions (Fig. 2). However, in the 25-month-old animals following succinate stimulation of mitochondrial respiration a statistically nonsignificant trend for a decline in oxy-radical generation with caloric restriction was observed ($p < .06$, Fig. 2C).

Effect of age, diet, and succinate on membrane fluidity

The order parameter is a measure of the fluidity of the membrane. The changes in the anisotropic motion of the 5-NS spin probe allow this lipid-specific spin label to report any changes that may occur in the microenvironment of the paramagnetic oxazolidine moiety in the membrane bilayer. Membrane fluidity changes were monitored as a function of age, caloric restriction, and metabolic stimulation of mitochondria with succinate. Figures 4 and 5 depict the changes in membrane order with age caloric restriction and met-

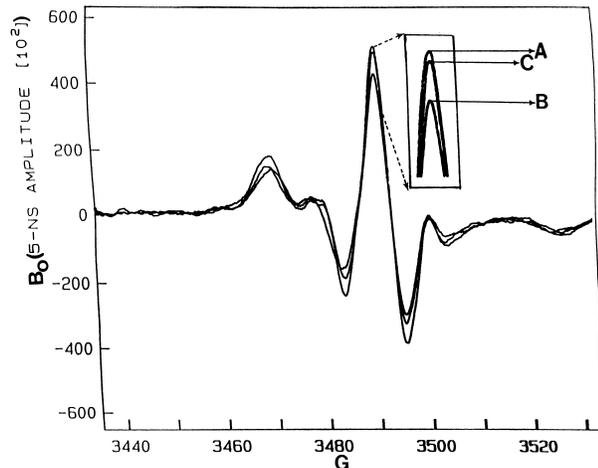


Fig. 3. Evidence for generation of hydroxylamine by reduction of 5-NS following succinate-stimulation of mitochondria. Spectrum A represents vehicle-treated control sample ($B_0 = 53,648$) and spectrum B depicts a 3-h succinate-stimulated sample ($B_0 = 42,756$; 80% of control sample). Spectrum C represents sample of reoxidized succinate-stimulated sample with 2 mM potassium ferricyanide for 1 h ($B_0 = 51,784$; 97% of control value).

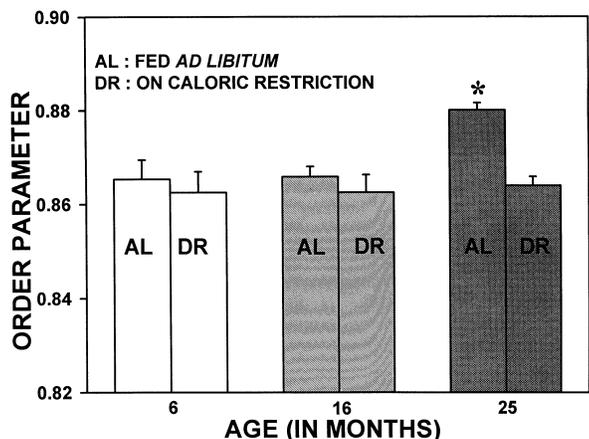


Fig. 4. A significant increase in membrane order indicative of membrane rigidization was found with increasing age of the animal ($p < .0007$). Significant increase in order of the mixed population of synaptosomal and mitochondrial membranes was found in 25-month rats fed ad lib when compared to age-matched rats on caloric restriction ($p < .0014$). No such protection from membrane rigidization was observed in the 6- or 16- month rats.

abolic stimulation. The membrane order (decreased fluidity) of the mixed population of synaptosomes and mitochondria, isolated from animals fed AL and without any succinate stimulation, increased with age ($p < .007$), consistent with earlier findings (Fig. 4).^{24,27} A highly significant lowering in the order parameter was observed with increasing succinate stimulation in animals of all age groups ($p < .0001$) (Fig. 5). There were significant increases in membrane fluidity in all animals upon mitochondrial respiratory stimulation with 20 mM succinate ($p < .007$) as compared to the unstimulated mixed population of mitochondria and synaptosomes (Fig. 5).

The influence of diet on membrane order among 6-, 16-, and 25-month-old animals was also investigated with respect to age, diet, and dose. Caloric restriction modulated membrane order only in the 25-month-old animals when not metabolically stimulated with succinate (Fig. 4; $p < .0014$). This result suggests that diet does play a role in decreasing the membrane rigidization in animals of the older age group. Following metabolic stimulation of the mitochondria with 20 mM succinate in animals fed either ad lib or a calorically restricted diet, there was a significant decrease in membrane rigidity in animals of all age groups. Thus, the role of diet as a means of protection against membrane damage in the face of mitochondrial oxy-radical generation upon respiratory stimulation may be a consequence of the decreased endogenous substrate concentration seen in caloric restriction.

DISCUSSION

Oxidation of lipid membranes in biological systems has been implicated in cellular structure damage and age-related cellular dysfunction.^{24,47} Reactive oxygen species (ROS) in particular can initiate lipid peroxidation.^{48,49} Subsequent by-products of the interaction of ROS with lipids like lipid peroxides and aldehydes (e.g., 4-hydroxy 2-nonenal) are involved in the propagation of lipid molecule disruption in biomembranes.⁴⁸ These oxy-radical reactions in biological systems are known to increase during aging and the consequent damage is found to accumulate with age.¹⁷⁻¹⁹

Several studies in the past 2 decades have shown

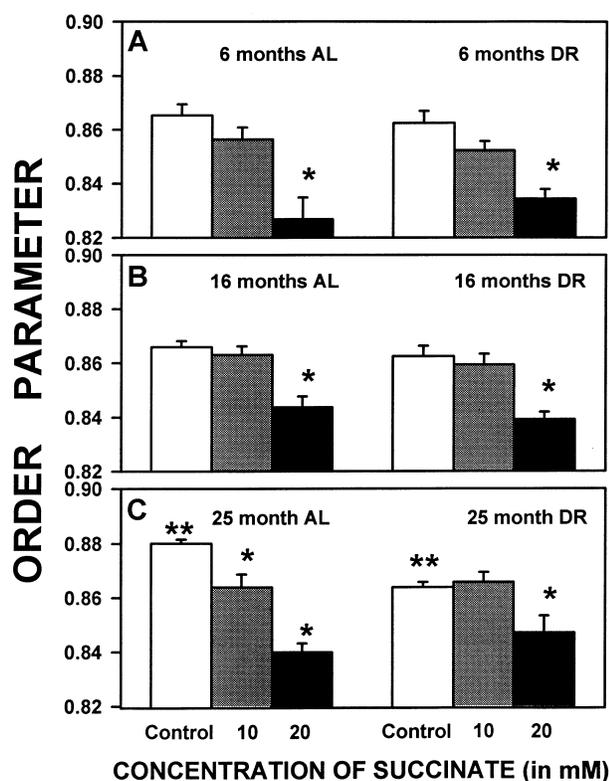


Fig. 5. Effect of age and caloric restriction on the order parameter, an inverse indicator of lipid membrane fluidity. A significant overall decrease in order was observed upon succinate stimulation of mitochondrial respiration in case of all three age groups and the two dietary groups ($p < .0001$). *Indicates significance when compared with controls. (A) There is a significant decrease in order in AL and DR groups with 20 mM succinate stimulation ($p < .0007$). (B) Homogenates treated with 20 mM succinate showed a greater increase in membrane fluidity ($p < .001$) in both dietary groups. (C) Significant attenuation in membrane rigidity by caloric restriction is observed in 25-month AL vs. 25-month DR groups (** $p < .0014$). Succinate (20 mM) stimulation caused increased membrane fluidity in both dietary groups. Stimulation of 10 mM caused a significant increase in membrane fluidity only in the 25-month AL group of animals ($p < .0007$).

that the main source of oxy-radicals in mammalian systems is the mitochondria.^{29,38,50-52} It has also been well established that the ubisemiquinone-cytochrome *b* region of the mitochondrial electron transport chain is the major source of $O_2^{\cdot-}$, and H_2O_2 generation.^{50,53} Generation of these ROS occur primarily under state 4 conditions when no exogenous ADP, but an excess amount of a respiratory chain substrate, is present. Respiratory stimulation with succinate, a complex II substrate, results in sequential reduction of the lipid-soluble electron shuttle coenzyme Q to the semiquinone and then to the dihydroubiquinone (Fig. 6). Oxy-radical production in mitochondria occurs mainly due to the semireduced coenzyme Q donating its electron to oxygen, which is in abundance in the mitochondria.³⁸ This leads to the generation of superoxide ($O_2^{\cdot-}$). Subsequent one-electron reduction of superoxide by another semireduced coenzyme Q results in production of hydrogen peroxide. Generation of these ROS and their interaction with iron in classical Fenton chemistry re-

sults in hydroxyl radical (OH^{\cdot}) production. This species reacts with biomolecular components at diffusion-controlled rates and can initiate lipid peroxidation in membranes of the mitochondria and other organelles of cells, resulting in their dysfunction.

The brain is particularly vulnerable to oxidative damage because it contains relatively high concentrations of easily peroxidizable polyunsaturated fatty acids, and the brain is not highly enriched with protective antioxidant enzymes or small molecule antioxidants. Apart from the peroxidizable polyunsaturated fatty acids, the likelihood of oxidative insult to normal neurons relative to other cells is a possible result of catalytically active metals (i.e., iron and copper), amyloid β -peptide derived from amyloid precursor protein, and particular ROS-generating enzymes (e.g., nitric oxide synthase, xanthine oxidase, etc.) present in the brain. Another factor that results in greater oxidative insult to brain relative to other tissues is that this organ consumes one-fourth of the total O_2 intake, and consequently, gener-

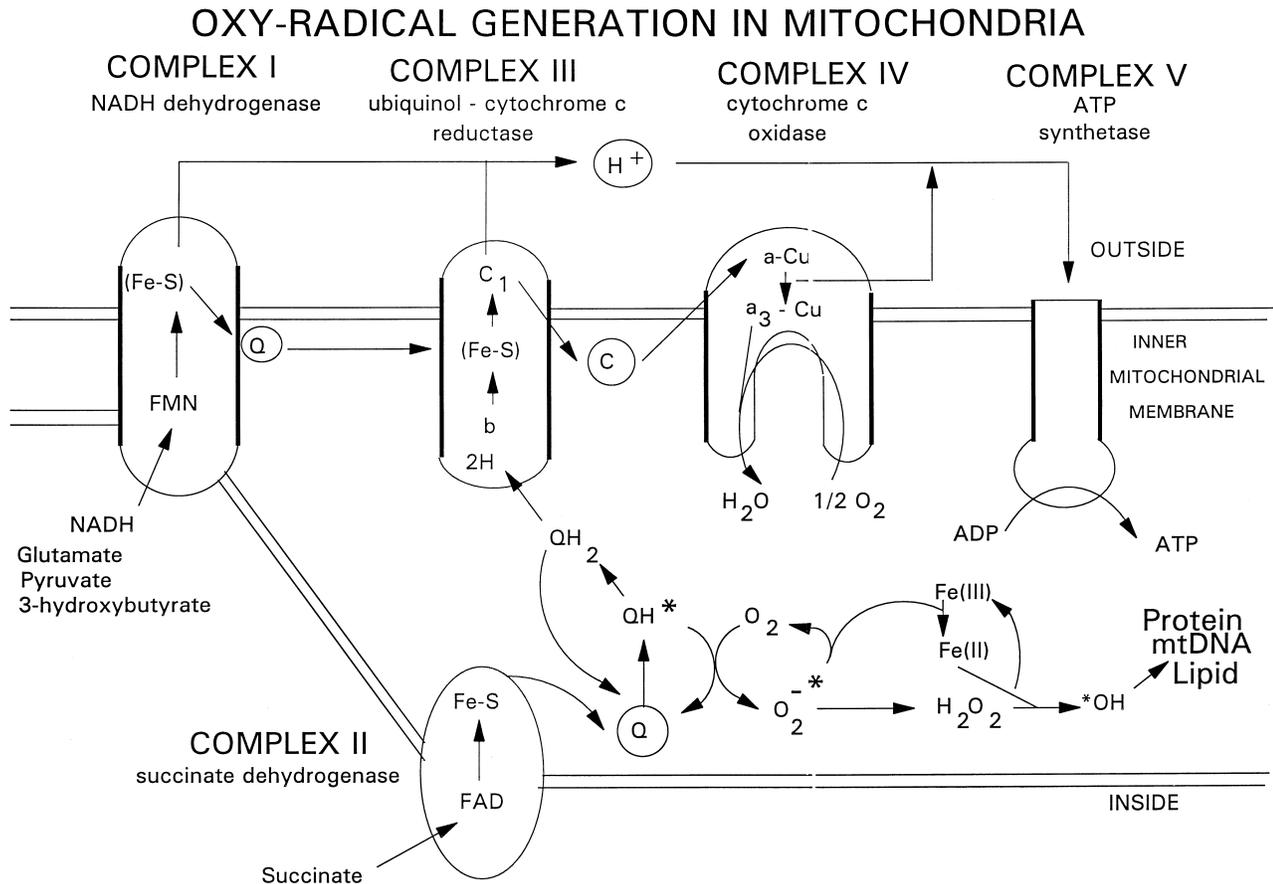


Fig. 6. Model for succinate-induced oxy-radical generation in the mitochondrion. Reducing equivalents generated by conversion of succinate to fumarate by complex II in the inner mitochondrial electron transport chain causes a sequential reduction of the lipid-soluble electron carrier ubiquinone. A one-electron reduction of oxygen by the semireduced ubiquinone (QH^*) results in production of superoxide ($O_2^{\cdot-}$), which leads to subsequent reactive oxygen species generation.

ates more oxy-radicals than most other organs in the body based on weight. Finally, due to the postmitotic neuronal cellularity of the brain, further differentiation and/or cellular repletion does not occur. As a consequence, the brain's organelles are more likely to accumulate more oxidatively damaged biomolecules, as compared to cells that undergo mitosis, resulting in a loss of function.⁵⁴ Thus, the brain neurons and their organelles must survive for longer periods with oxidatively damaged dysfunctional organelles that occurs as a function of age and long-term metabolic stress.⁵⁴

In our study, the overall succinate concentration effects observed with respect to loss in 5-NS signal amplitude and a decrease in order of synaptosomal and mitochondrial lipid membranes imply that an induced metabolic stress to mitochondria may cause an increased production of oxy-radicals through the complex II–ubiquinone–cytochrome *b* region of the electron transport chain. The reactive oxygen species conceivably cause initiation of lipid peroxidative changes in the membrane. Based on these findings, we hypothesize that an increasing metabolic stimuli results in the mitochondrial generation of oxy-radicals causing peroxidative damage to membrane lipids. It should be recalled that the 5-NS EPR signal reflect an average of all the labeled membranes present in the mixed population of synaptosomes and mitochondria and is influenced by membrane lipid composition.

With increasing age of the animal, the oxy-radical-generating capacity of the mitochondria increases, as evidenced by decreases in 5-NS signal amplitude and the order parameter for specific succinate concentrations. Thus, as the brain ages, it is likely that the mitochondria ‘leak’ increased ROS upon stimulation. This could pertain to a lack of tighter coupling in the electron transport chain with increasing age. At 10 mM succinate stimulation, the observation that 6-month-old rats showed similar decreases in comparison to 25-month-old animals may have to do with their higher basal metabolic rate resulting in a larger flux of oxy-radicals generated by the mitochondria. Differences in lipid composition with age may also contribute to the comparable decreases in B_0 of 5-NS. Mitochondrial respiratory stimulation with 20 mM succinate illustrates the increased leakage of oxy-radicals with age (Fig. 2).

As stated previously, our preparation consisted of a mixed population of synaptosomes and mitochondria isolated from the neocortex of male Brown Norway rat brains of differing ages and fed different levels of caloric intake. It is important to recognize that events that occur during normal brain aging within this rat model can influence the membrane lipid composition. Further, the restriction in caloric intake can potentially control

the constitution of synaptosomal and mitochondrial membranes. These factors could conceivably affect mitochondrial fragility or enrichment in our preparation. It must be noted that the conclusions based on our results do not take into consideration these possible changes in mitochondria and the effect, if any, it may have in terms of oxyradical generation and changes in order parameter in response to metabolic stimulation.

The ROS production depends on the mitochondrial metabolic state, which determines the rate of electron transport and the oxidation state of the electron carriers. Evidence provided by the Chance *et al.* and Sohal *et al.*^{29–30} suggest stimulation of mitochondrial respiration, specifically under state 4 conditions, causes oxyradical production. Under these conditions, the electron carriers are in a highly reduced state and conceivably there is an increased level of the ubisemiquinone intermediate to reduce oxygen causing generation of superoxide. The model selected was utilized to investigate the impact of mitochondrial electron transport chain stimulation by succinate on the possible generation of oxyradicals, under state 4 conditions, using established EPR measures of membrane damage as evidenced by membrane order changes. Because an assessment of oxygen uptake would not provide an insight into the mechanism of radical production and potential lipid peroxidative damage, an analysis of the respiratory control ratio (RCR) was not considered necessary.

Previous studies have elaborated the use of nitroxides as a means to measure the magnitude of oxyradical generation. Reduction of the nitroxide moiety of the probe with free radicals can result in a decrease in 5-NS amplitude.^{34–36} Further, the 5-NS probe, due to its intercalation in membrane lipids, is used to assess molecular motion. A significant feature of this study is the utilization of the 5-nitroxyl stearate probe for a relative quantitative assessment of oxyradical generation and changes in the order of the membrane lipids following mitochondrial respiration. Potassium ferricyanide and perdeuterated tempone (¹⁵N-PDT) have been used to elucidate the generation of hydroxylamine due to reduction of 5-NS.^{55,56} We used this methodology to demonstrate that we indeed had generated the corresponding EPR-silent hydroxylamine from 5-nitroxyl stearate following metabolic respiratory stimulation of mitochondria.

A significant decline in order (i.e., an increase in membrane fluidity) in all age groups upon mitochondrial respiratory stimulation with 20 mM succinate was observed when compared with 10 mM succinate treatment or no metabolic stimulation at all (Fig. 5). Because molecular oxygen and hydrogen peroxide are hydrophobic, these molecules tend to accumulate in the

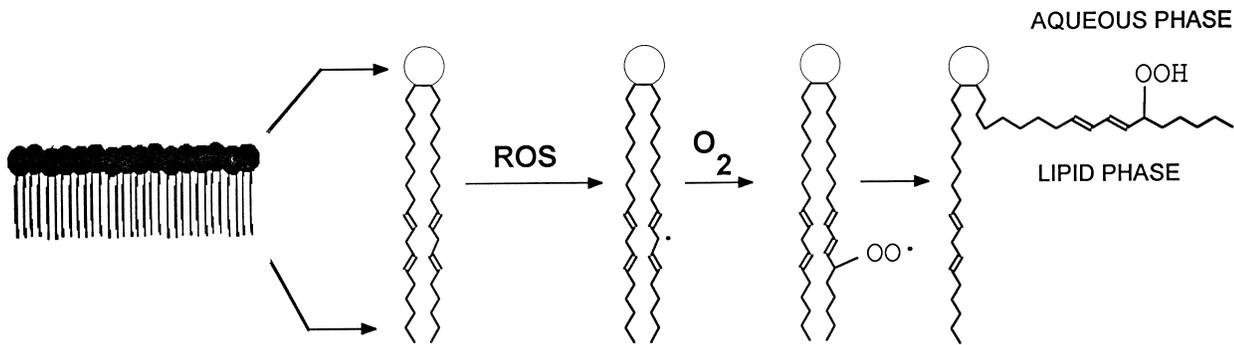


Fig. 7. Model for changes in lipid spin-label motion in neocortical membrane bilayers damaged by oxidants during metabolic stimulation of mitochondria. Attack of oxygen free radicals leads to formation of lipid peroxides in the membranes. This leads to a rearrangement of the acyl side chains as more polar hydroperoxides move toward the membrane surface (hydrophilic end), increasing the spin label motion. (Adapted from Hall et al.⁵⁵.)

hydrophobic center of the lipid bilayer, and it is conceivable that oxy-free radicals generated by mitochondrial respiration upon stimulation with succinate can generate lipid hydroperoxides within the membrane (Fig. 7).⁵⁷ The lipid hydroperoxides are hydrophilic in nature and tend to migrate towards the aqueous environment. This creates a gap in the lipid membrane and causes an apparent increase in membrane fluidity. The lack of damage to membranes by oxy-radicals at 10 mM as compared to 20 mM stimulation may be due to sufficient levels of antioxidant protection in both systems to contend with the oxy-radical surge.

The effect of these free radicals on membrane fluidity during aging and its possible attenuation by dietary restriction has been of recent interest.²⁷ In our study, diet did not seem to play a protective role in the ability of the mitochondria to generate ROS in vitro. We have provided evidence in vitro that succinate-stimulated brain mitochondria, isolated from animals on a calorically restricted diet, generate equal amounts of oxy-radicals as compared to animals fed ad lib. Caloric restriction did modulate rigidization of the membrane with age under control conditions, as found in previous studies,²⁷ but did not influence the increase in membrane fluidity following mitochondrial stimulation with succinate. These findings suggest our hypothesis that caloric restriction by itself may not ameliorate the damage to mitochondrial processes that occur with age. These dysfunctional mitochondria, upon subsequent metabolic stimulation, induce lipid membrane damage to the cell and organelle lipid membranes resulting in their dysfunction.

From our studies we conclude that there is a dose-dependent decrease in 5-NS signal amplitude consistent with an increase in generation of oxy-radicals upon mitochondrial respiratory stimulation with succinate. This oxy-radical flux is able to cause lipid peroxidative dam-

age to both the mitochondrial and synaptosomal membranes as evidenced by the changes in membrane fluidity. It should be qualified that these extreme metabolic conditions caused by high succinate concentrations do not reflect a situation present in vivo. The role of caloric restriction in attenuating the mitochondrial ability to generate radicals and prevent lipid peroxidation was found to be limited. Brain mitochondria isolated from calorically restricted animals, when subjected to similar metabolic stress conditions as mitochondria from animals fed ad lib, generate approximately the same flux of radicals and subsequent oxidative damage. A further evaluation will be required to determine whether the defects in the mitochondria are similar in animals from both sets of dietary conditions, and the effect of oxy-radical leakage is masked until the stimulation of succinate dehydrogenase with high levels of substrate.

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