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Biochimica et Biophysica Acta 1372 (1998) 163–173

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Effects of mitochondrial respiratory stimulation on membrane lipids and proteins: an electron paramagnetic resonance investigation

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Received 17 February 1998; accepted 2 March 1998

Abstract

Previous studies have implicated mitochondria-derived reactive oxygen species (ROS) in both the aging process and age-related diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease etc. The current study, utilizing electron paramagnetic resonance (EPR) spectrometry, was designed to determine if mitochondrial respiratory stimulation, under state 4 conditions, caused extensive oxidative modifications to membrane cytoskeletal proteins and lipids in the brain. A mixed population of cortical synaptosomes and mitochondria, prepared by centrifugation techniques using rat brain cortex from adult (4–6 months) female Brown Norway rat brains, were labeled with the lipid-specific spin probe, 5-nitroxyl stearate (5-NS). Stimulation of the mitochondrial electron transport chain was accomplished using 20 mM succinate at 25°C for 3 h. Mitochondrially derived free radicals, when reacted with the paramagnetic center of the spin probe, result in a loss of paramagnetism resulting in loss of intensity. A significant lowering (23%, $P < 0.0001$) in the signal amplitude (B_0) of 5-NS, indicative of generation of oxyradicals, was found. The order parameter, an inverse EPR-measure of membrane fluidity of the 5-NS spin labeled mitochondrial and synaptosomal membranes, also decreased following mitochondrial respiratory stimulation ($P < 0.005$). Changes in the physical state of cytoskeletal and transmembrane proteins due to succinate oxidation were measured using MAL-6 (2,2,6,6,-tetramethyl-4 maleimidopiperdin-1-oxyl), a thiol-specific nitroxide spin label. The ratio of the amplitudes of the weakly to strongly immobilized spin label reaction sites (*W*/*S* ratio) in the low-field region of the spectrum was used to determine any alteration in protein conformation. Previous studies in our laboratory have established that increased protein oxidation is associated with a decreased *W*/*S* ratio. In the current study, our results indicated significant lowering of the *W*/*S* ratio in cortex (30%, $P < 0.0001$) upon stimulation of the mitochondria with 20 mM succinate. Thus, we conclude that respiratory stimulation of mitochondria, due to a hypermetabolic stress with succinate, caused significant oxidative modifications of cortical membrane lipids and proteins.

Keywords: Brain; Mitochondrion; Electron paramagnetic resonance; Protein oxidation; Lipid peroxidation; MAL-6; 5-Nitroxyl stearate

1. Introduction

Multiple lines of evidence have shown that an inevitable repercussion of living in an aerobic environ-

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0005-2736/98/$19.00
PII: S0005-2736(98)00040-6

ment is the lingering production of oxygen free radicals. Mitochondria are principal endogenous sources of free radicals accountable for the most oxidants produced by cells during normal aerobic respiration, which consumes greater than 80% of the oxygen in the cellular milieu. The free radical theory of aging [1,2] postulates that oxygen-derived free radicals result in a cumulative damage to critical cellular components, eventually leading to many age-related disorders. An increase in the metabolic rate could lead to a substantial production of endogenous oxidants, like superoxide (O_2^{-*}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH*) as a by-product of normal oxygen metabolism in the mitochondria. Studies corroborating this suggestion have demonstrated that consequent damage in terms of the level of oxidative DNA damage are roughly related to metabolic rate in a number of mammalian species [3–6]. Apart from normal brain aging, it is hypothesized that there is a free radical-mediated deterioration of neuronal membrane components leading to age-related neurodegenerative disorders like Alzheimer's disease, Parkinsonism, amyotrophic lateral sclerosis and Huntington's disease. Harman was the first to propose that the mitochondrion was involved in the aging process [7]. The dysfunctional mitochondrion is a cellular organelle which has also been implicated in several neurodegenerative disease states [8]. Evidence suggests that biomolecular components of the mitochondria, like mitochondrial DNA (mtDNA), electron transport chain enzymes (e.g. cytochrome oxidase), lipid components (e.g. cardiolipin), undergo possible free radical-mediated deterioration, resulting in a compromise of the associated bioenergetic processes [8,9]. The tightly coupled process of oxidative phosphorylation during mitochondrial respiration utilizes the 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 [8,10].

Although, the mechanism underlying the age-related increases in mitochondrial production of O_2^{-*} and H_2O_2 is unknown, it has been well established that mitochondrial macromolecules undergo damage by self-generated ROS [11]. 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 [3,4,12–15]. These ROS can contribute to oxidative damage of mitochondrial lipids, proteins and DNA.

In the current study, electron paramagnetic resonance (EPR) spectrometry was used in conjunction with site-specific spin labels to investigate: (a) whether succinate stimulation of mitochondrial respiration results in oxidative modification of membrane lipids; (b) whether there are changes in the conformational structure of brain membrane proteins caused by oxidative processes induced following heightened state 4 metabolic stimulation of mitochondrial respiration.

2. Materials and methods

2.1. Chemicals

Ultrapure sucrose, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) buffer (ultrapure grade), and other standard chemicals were obtained from Sigma (St. Louis, MO). Protease inhibitors (leupeptin, pepstatin, aprotinin) were obtained from Calbiochem (San Diego, CA). The protein specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and the lipid bilayer-specific spin label 5-doxylstearic acid (5-NS) were obtained from Aldrich (Milwaukee, WI).

2.2. Animals and diet

Brown Norway (BN) 6-month-old adult female rats and 4-month-old male Sprague–Dawley rats were used in this study. All procedures involving animals used in these studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. The rats were housed in $21 \times 10 \times 8$ in. solid bottom polycarbonate cages with wire tops for placement of food and water. IACUC Purina Ag chow rat food and water were provided ad libitum. The rats were maintained on a 12-h light, 12-h dark cycle with light portion beginning at 06.00 h. Following a 2- to 3-week adaptation period, the rats were used for experiments.

2.3. Isolation of synaptosomes and mitochondria

The brain of the animal was removed after decapitation and the cerebellum and the neocortex were dissected free; and immediately, the remaining brain material was suspended in separate containers of ice-cold isolation buffer suspended in separate containers of ice-cold isolation buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM ethylenediamine tetraacetic acid (EDTA), 2 mM ethylene glycol-bis(amino-ethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and the protease inhibitors: 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.2 mM PMSF. Samples were homogenized with 12 passes through a Wheaton motor Teflon-coated homogenizer 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 desferrioxamine, an iron chelator. This P-2 fraction containing the mitochondria and synaptosomes was used for the lipid membrane and protein oxidation study. The protein concentration of mixed population of mitochondria and synaptosomes was measured using the Lowry method [16].

2.4. Succinate-induced mitochondrial stimulation and ESR spin labeling

2.4.1. 5-NS lipid spin labeling

5-NS is a compound which belongs to the class of so-called doxyl nitroxide-substituted stearic acid analogs. It contains an oxazolidine ring, with resident nitroxide functionality, tethered covalently to a specific position on the stearic acid hydrocarbon tail. 5-NS has the oxazolidine moiety at the 5-position of the hydrocarbon tail, and exhibits an anisotropic motion, unlike the isotropic motion exhibited by non-oriented spin probes like MAL-6, the protein sulfhydryl-specific spin label [17,18]. A typical spectrum of 5-NS intercalated in to the lipid bilayers of a mixed population of synaptosomes and mitochondrial membranes is shown in Fig. 1. The signal amplitude of the $M_I = 0$ central line EPR spectrum is designated by B_0. The spectrum in Fig. 1 reflects an average of all the labeled membranes present in the mixed population and is influenced by membrane lipid composition. In order to measure changes in the membrane fluidity, the model of 5-NS intercalation into the lipid bilayer is such that its long alkyl chain lies parallel to the alkyl chains of the membrane lipids (Fig. 2) [17,18]. 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:

$$S = \frac{T_{\parallel'} - T_{\perp'}}{T_{\parallel} - T_{\perp}} \times \frac{TrT}{T'rT}$$

$$T'rT = T_{\parallel'} + 2T_{\perp'}.$$

where the primed values are spectroscopically measured (Fig. 1) and the unprimed values are known constants obtained from single crystal data [19]. Lower values of S indicate an environment of lower order and increased motion, suggesting increased fluidity.

The P-2 pellet preparation derived from brain cortices of adult male brown Norway rats at a protein concentration of 4 mg/ml were labeled with the lipid-specific 5-nitroxyl stearate spin probe. The lipid bilayer of membranes of this homogenate were spin labeled by incubation for 30 min with gentle shaking at 25°C using 0.6 μg of the 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 two Eppendorf tubes, and then treated with either the vehicle (Kreb's buffer) or 20 mM succinate in order to stimulate mitochondrial respiration. After a 3-h incubation at 22°C, EPR measurement of the amplitude 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.

2.4.2. Regeneration of 5-NS by oxidation of hydroxyl amine

Following stimulation of mitochondria with succinate and measuring the effect on the amplitude (B_0),

an experiment designed to regenerate the hydroxylamine product of the reduction of 5-NS was performed. The mixed population of synaptosomes and mitochondria labeled with 5-NS and stimulated with 20 mM succinate was apportioned into Eppendorf tubes and one was treated with vehicle (Kreb's buffer) and the other with potassium ferricyanide (final concentration of 2 mM). After incubating at 22°C for half an hour, the EPR parameters B_0 and S were determined. Instrument operating parameters for the regenerated 5-NS spectrum were same as given above.

2.4.3. MAL-6 protein spin labeling

As a means to measure the changes in conformation of cytoskeletal proteins in synaptosomes, the thiol-specific, isotropic spin label, MAL-6 was utilized. The mixed population of synaptosomes and mitochondria was adjusted to a 4 mg/ml protein concentration. The preparation was then aliquoted and either treated with 20 mM succinate or with equal volume of vehicle (Kreb's buffer). For the cytoskeletal protein modification analysis, synaptosomes were isolated via ultracentrifugation techniques using the succinate-stimulated and vehicle-treated P-2 pellet suspensions. The synaptosomes were prepared by utilizing a discontinuous sucrose gradient method using standard methods adapted in our laboratory [20,21]. The resulting synaptosomes were resuspended in 20 ml ice-cold lysing buffer, containing 10 mM HEPES, 2 mM EGTA, and 2 mM EDTA, at pH 7.4 and the resulting suspension was then spun down at $32\,000 \times g$ for 10 min. This process was repeated two more times to remove any residual cytoplasm. The resulting pellet was resuspended in 2.0 ml of the lysing buffer and assayed for total protein concentration by the method of Lowry et al. [16]. Ice-cold MAL-6 spin label was added to synaptosomal membranes containing 5.0 mg of protein to give final concentration of MAL-6 of 20 μg/mg protein [20,21]. The resulting mixture was shaken by hand and incubated at 4°C for 16–18 h. Samples were then centrifuged and washed six times in 10–15 volumes of lysing buffer to remove excess spin label. On the last wash, the samples were brought to a volume of ca. 400 μl in lysing buffer. Samples were then allowed to equilibrate to room temperature for 30 min prior to electron paramagnetic resonance analysis. The relevant spectral parameter (W/S ratio) was then determined. All the EPR spectra of labeled samples were acquired on a Bruker 300 EPR spectrometer equipped with computerized data acquisition and analysis capabilities located in climate-controlled room of constant temperature (22 ± 1°C) and humidity. Instrumental operating parameters were: incident microwave power = 18 mW, modulation amplitude = 0.4 G, time constant = 1.28 ms, conversion time = 10 ms.

3. Results

3.1. Reduction of 5-NS labeled membrane lipids following stimulation of mitochondrial respiration

In order to determine if changes in the physical state of cortical synaptosomal lipid bilayer occurred after metabolic stimulation, the lipid-specific spin probe 5-nitroxyl stearate was utilized. The 5-NS 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

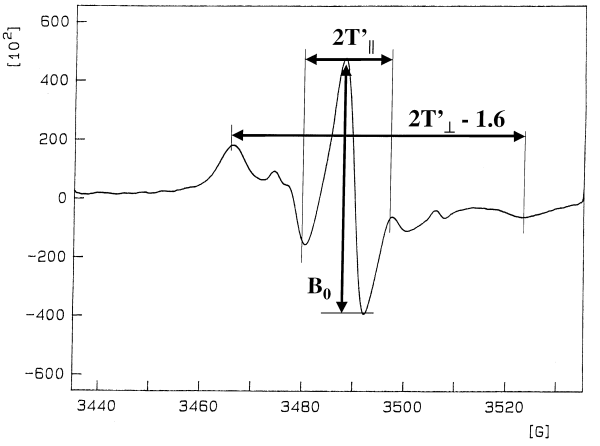

Fig. 1. Representative EPR spectrum of 5-nitroxyl stearate (5-NS). T-tensor measurements from which the order parameter is calculated and the signal amplitude of the central resonance line (B_0) are indicated. The concentration of the spin probe is 1.6 μM in the mitochondrial plus synaptosomal suspended in calcium-free Krebs buffer.

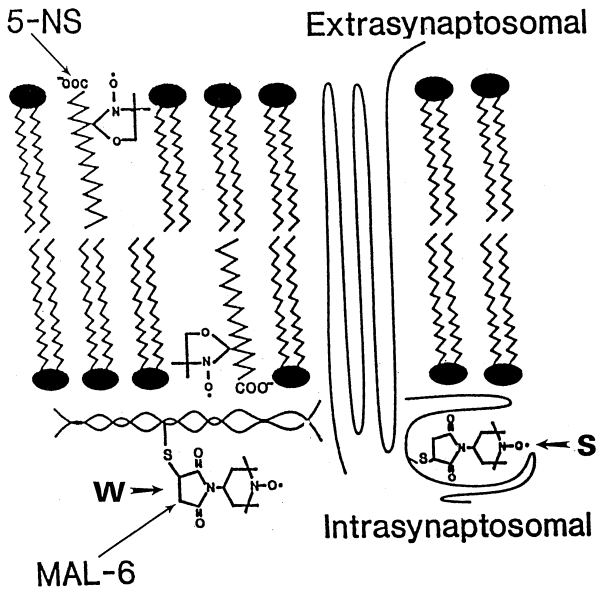

Fig. 2. Pictorial location of the spin labels, 5-NS and MAL-6, in synaptosomal membrane lipid bilayer domain and membrane proteins, respectively. The lipid specific spin label, 5-NS, intercalates between the fatty acid chains of the lipid membrane, with the alkyl chains parallel to each other. MAL-6 binds to thiol groups of the synaptosomal membrane proteins in weakly (*W*) or strongly (*S*) immobilized sites. The ratio of the amplitudes of the weakly to strongly immobilized spin label reaction sites (*W*/*S* ratio) is used to determine alterations in protein conformation.

lipids, while, under normal conditions, the hydrophobic tail is free to undergo rapid anisotropic motion in the interior of the bilayer [17,18]. Since 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. The oxazolidine moiety, being attached to the 5-position on the stearic acid molecule, allows the spin probe to be proximal to the lipid/water bilayer. This facilitates the spin probe to report oxidative events occurring closer to lipid/water bilayer rather than processes taking place deep within the membrane.

A parameter of interest is the amplitude (B_0) of the 5-NS signal. This value is a measure of the amplitude of the 5-NS signal and, with a constant line width, a decrease in its magnitude is indicative of direct reduction of the nitroxide head group by reactive oxygen species like superoxide (O_2^{-*}) and hydroxyl radical (OH*) to the corresponding EPR-silent hydroxyl amine [21–28]. As shown in Fig. 3, our results indicate that metabolic stimulation of mitochondrial respiration, using succinate as the substrate, leads to a highly significant decrease in the magnitude of the B_0 value of the 5-NS spin label ($P < 0.0001$). Simultaneously, there is a significant increase in the fluidity of the membrane lipids following succinate stimulation of the mitochondria ($P < 0.001$, Fig. 4).

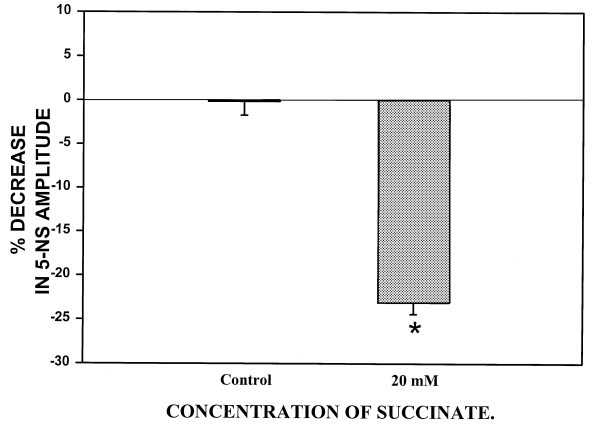

Fig. 3. Changes in the signal amplitude (B_0) following induction of mitochondrial respiration utilizing succinate, a complex II substrate for the electron transport chain. A significant lowering in B_0 was observed ($^*P < 0.0001$). This decrease was indicative of an oxyradical flux, generated by the mitochondria, which likely can interact with the cortical membranes, initiating lipid peroxidation.

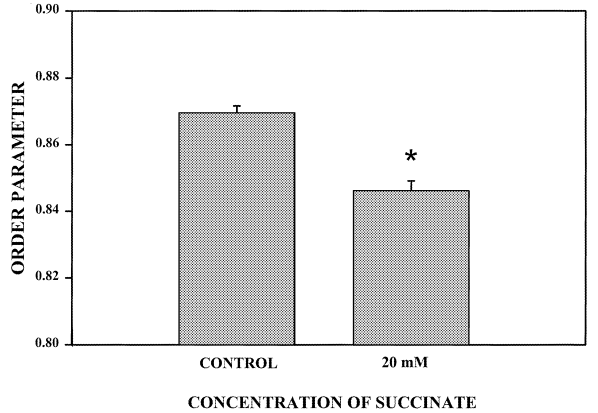

Fig. 4. Order parameter changes in cortical lipid membranes following stimulus of state 4 mitochondrial respiration with 20 mM succinate. A significant decrease in order, i.e. increase in membrane fluidity, was observed ($^*P < 0.001$).

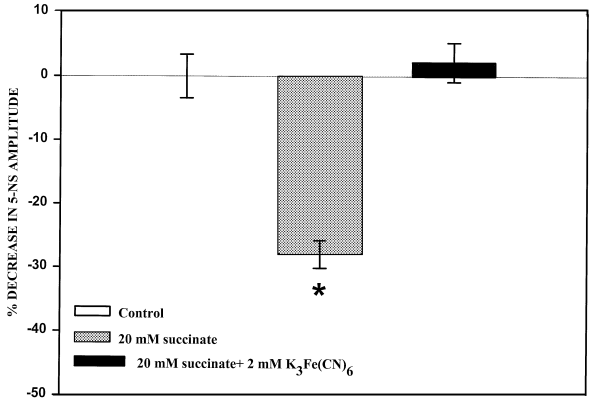

Fig. 5. Regeneration of lost 5-NS signal by potassium ferricyanide. A significant decrease in the signal amplitude, B_0, was observed with 20 mM succinate stimulation of mitochondrial respiration (*$P < 0.0001$). As shown on the right, the reduced 5-NS signal was completely regenerated following re-oxidation with 2 mM $K_3Fe[CN]_6$.

3.2. Evidence for reduction of 5-NS to corresponding hydroxyl amine

The reduction of the 5-NS signal amplitude is conceivably caused due to its chemical reduction by reactive oxygen species, generated following succinate oxidation in the mitochondrial electron transport chain, to form the EPR-silent hydroxylamine. Chemical studies demonstrate that a re-oxidation of the hydroxyl amine to the nitroxide is considered evidence in favor of hydroxylamine generation following reduction of nitroxides [29]. Potassium ferricyanide is a known oxidant of hydroxylamines to the corresponding EPR-detectable nitroxide [29]. In order to investigate whether the hydroxylamine is indeed generated following mitochondrial respiration, a re-oxidation of the succinate-treated preparation was performed. As shown in Fig. 5, 2 mM $K_3Fe[CN]_6$ addition leads to the 5-NS signal amplitude (B_0) being completely regenerated following oxidation of the succinate-stimulated, spin-labeled membranes. Predictably, although there was a near-complete regeneration of the 5-NS signal the increase in membrane fluidity caused by mitochondrial respiration was unaffected by the re-oxidation of the hydroxylamine (Fig. 6). Thus, our results indicated no significant attenuation of the decreased membrane order following re-oxidation by $K_3Fe[CN]_6$ of the 5-NS labeled, succinate-stimulated preparation.

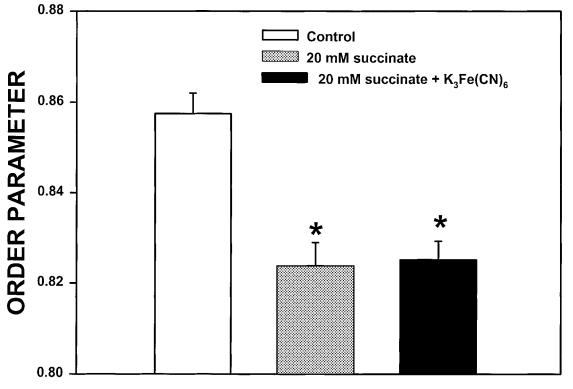

Fig. 6. The decrease in order parameter is not attenuated by re-oxidation of EPR-silent hydroxylamine to the corresponding EPR-detectable 5-NS. No significant difference between succinate-treated and succinate-treated followed by potassium ferricyanide re-oxidized samples were observed.

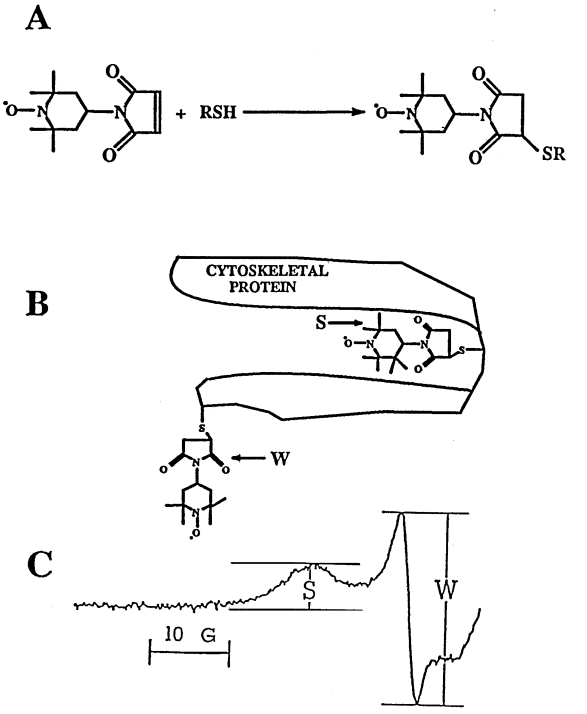

Fig. 7. MAL-6 binding to cytoskeletal proteins of the synaptosomes. (A) Reactivity of MAL-6 with sulfhydryl groups of the proteins in a 1,4-Michael-type addition. (B) Binding of MAL-6 to exterior sites on proteins resulting in a weakly immobilized resonance signal (*W*) or to sterically hindered protein sites deep within a protein pocket causing a strongly immobilized spin label resonance signal (*S*). (C) Low-field EPR spectrum of MAL-6 labeled synaptosomal cytoskeletal proteins indicating weakly and strongly immobilized spin labeled signal amplitudes.

3.3. Mitochondrial respiration-induced changes in MAL-6 labeled cytoskeletal protein

As a means to measure the changes in conformation of cytoskeletal and transmembrane proteins in synaptosomes, the thiol-specific MAL-6 spin label was utilized. A typical EPR spectrum of MAL-6 covalently attached to membrane proteins in cortical synaptosomes is shown in Fig. 7. As indicated in Fig. 2, at least two distinct populations of spin label binding sites, characterized by their ability to restrict spin label motion, are observed. The weakly immobilized site indicates covalent binding of the spin label to thiols closer to the exterior of the proteins allowing for greater degree of motional freedom to the label. The strongly immobilized binding site is a result of covalent linking of MAL-6 to free thiols present in sterically hindered deep pockets of the protein resulting in motional restriction of the spin label. This differential binding of the MAL-6 label results in line broadening of the low-field region of the spectrum (Fig. 7). The relevant EPR parameter measured is the ratio of the spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) to that of the $M_I = +1$ low-field strongly immobilized line (S), which is referred to as the W/S ratio. Changes in the W/S ratio are known to be strong indicators of perturbations in the normal interaction of cytoskeletal proteins and reflect characteristic physical modifications of membrane protein structure, typically arising from chemical perturbations [21,30–33]. Previous studies in our laboratory have shown that increased protein oxidation is associated with decreased values of the W/S ratio of MAL-6 [20,21,30–33].

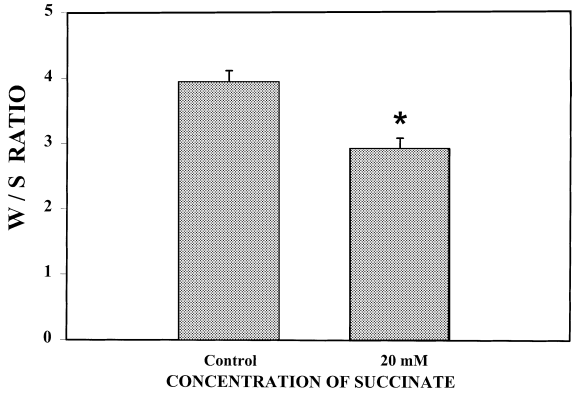

Fig. 8. A significant decrease in W/S ratio was observed following stimulation of mitochondrial respiration with 20 mM succinate for 3 h ($^*P < 0.0001$). This is consistent with oxidative modification of membrane protein conformations.

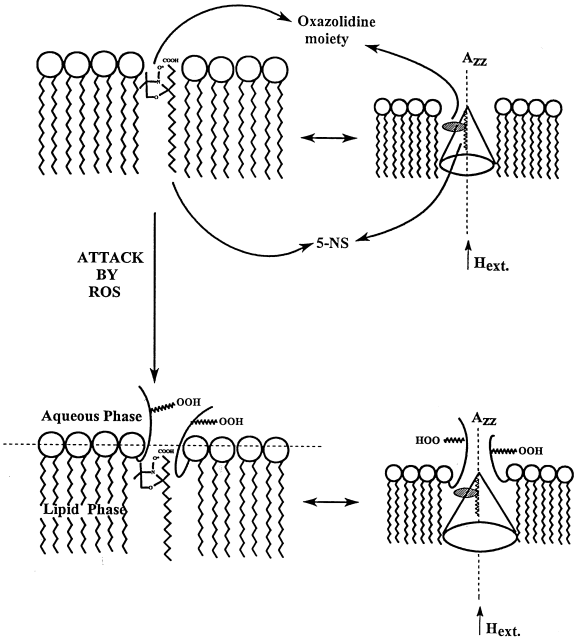

Fig. 9. Model for increase in fluidity as assessed by the 5-NS spin probe following oxyradical attack on membranes. The lipid bilayer, with the 5-NS spin probe intercalated within it, is susceptible to attack by ROS. The resultant more polar lipid hydroperoxides, formed due to interaction with ROS, move towards the aqueous phase creating a void in the membrane. This allows for increased lateral motional freedom for the 5-NS spin probe and is reflected by an increase in the order parameter.

Metabolic stimulation of the mitochondria with succinate, a complex II substrate of the electron transport chain, was found to result in a highly significant decrease in the W/S ratio ($P < 0.0001$). A 30% decrease in the W/S ratio was found to occur in cortical homogenates from the rat brain following mitochondrial stimulation for 3 h with 20 mM succinate (Fig. 8).

4. Discussion

A decrease in the signal amplitude (B_0) of the 5-NS labeled lipid membranes, following stimulation of mitochondrial respiration under state 4 conditions, is consistent with an oxygen-derived free radical surge capable of a peroxidative attack. Based on

similar results by others under different oxidative conditions [22–26], a complete regeneration of the signal amplitude by $K_3Fe[CN]_6$ after the loss in B_0 following succinate stimulation suggests that 5-NS, intercalated in the lipid membranes, are reduced by oxyradicals (like O_2^{-*} and OH*) generated by metabolic stress to the corresponding EPR-silent hydroxylamine.

The damage to the lipid membranes of the mixed population of synaptosomes and mitochondria can be assessed by changes in the order or rigidity of the membrane. From our results, we conclude that there is a significant reduction in membrane order, i.e. an increase in membrane fluidity ensues following succinate-induced mitochondrial respiration. This observation is consistent with the conclusion that specific changes in the membrane allow the 5-NS spin probe to have an increased lateral motion in the lipid membrane microenvironment [14]. Fig. 9 is an illustration depicting one possible chain of oxidative events thought to occur in the membrane following ROS generation. Following the onslaught of the oxyradicals on the fatty acid chains of the lipid molecule, the formation of a lipid hydroperoxide ensues. The creation of a peroxide moiety on the fatty acid chain results in the emergence of a hydrophilic side chain. The lipophobic property of this fatty acid molecule compels it to move towards the aqueous phase at the membrane periphery creating a 'gap' in the membrane. The remaining fatty acid side chains now have more latitude to wobble and thus, an increase in the fluidity of the membrane results. The intercalated 5-NS spin probe finds itself in an increasingly capacious lipid microenvironment (Fig. 9) and reflects this event by changes in *T*-tensor values, the determinants of the order parameter of the lipid membrane. Unlike the signal amplitude (B_0), the order of lipid membranes is not restored to control values when the 5-NS-labeled, succinate-stimulated samples are subjected to reoxidation with $K_3Fe[CN]_6$. This signifies a permanent damaging change in the biophysical state of the membrane causing an increase in membrane fluidity, as assessed by the 5-NS spin probe.

In some recent studies done in our laboratory, a different model accounting for the changes in membrane fluidity has been proposed. As is known the neuronal membrane contains a high level of unsaturated fatty acids which easily succumb to lipid peroxidative events caused by oxyradical processes. The deterioration of the membrane can result in a disruption in the ability of the membrane to prevent an elevated influx of calcium in to the cell. This alone can potentially turn on several noxious stimuli within the cell resulting in cell death. Damage to mitochondrial lipid membranes can also cause a defect in the sequestration of calcium in the mitochondria. This, in turn, can set off a chain of secondary free radical processes inside the cell and cause increased generation of lipid peroxidation products [34]. Some of the typical toxic aldehydic lipid peroxidation products of arachidonic acid are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [35]. Recent evaluations in our laboratory of the *W*/*S* ratio of MAL-6-linked synaptosomal membrane following 4-HNE treatment have shown that this toxic aldehyde forms covalent adducts and changes the conformation of membrane proteins, as monitored by a reduced *W*/*S* ratio of MAL-6-spin labeled synaptosomal membranes [36]. Amyloid β-peptide (Aβ) addition to brain cells leads to the formation of 4-HNE, consistent with Aβ-induced lipid peroxidation [27,28]. This 4-HNE-caused conformational change in membrane proteins [36] might explain the Aβ- or 4-HNE-induced loss of function of critical cellular proteins [36,37]. Since HNE adducts protrude from proteins into the hydrophobic domain of bilayers, a consequence of 4-HNE modified membrane-linked protein could be to create pockets of less tightly packed lipids. This would likely result in an overall increase in membrane fluidity as reflected by an increased ability of the 5-NS spin probe to wobble within the lipid bilayer, consistent with the findings of this research.

This study also investigated the effect of ROS, as generated by metabolic stimulation of mitochondria, on cytoskeletal and transmembrane proteins. MAL-6, a thiol-specific protein spin label, was utilized to assess covalent modifications in the cytoskeletal proteins. Based on previous studies of oxidative stress conditions, including Fenton chemistry-associated hydroxyl generation [21], in which the *W*/*S* ratio of MAL-6-labeled neocortical or erythrocyte membranes was reduced [21,30–33], we conclude that there was significant oxidative modification in cytoskeletal and transmembrane proteins due to

an onslaught by ROS generated following succinate-induced stimulation of mitochondrial respiration.

Oxidation of proteins can be a result of direct interaction with ROS like OH* radicals or possibly by interaction with toxic aldehydic products [38,39]. The result of this increased oxidative inactivation of proteins and is an overall inability of the cell to maintain ionic homeostasis. This triggers several mechanisms leading to eventual cell death. Many neurodegenerative disease conditions including Alzheimer's disease (AD), amyotrophic lateral sclerosis, and Parkinson's disease have implicated ROS in their pathogenesis [37,40–42]. Indeed, investigations have shown that brain autopsy samples from AD patients show significantly higher oxidized protein levels as compared to brain tissue from normal healthy elderly controls [43,44].

The brain is particularly vulnerable to oxidative damage since 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. Oxidative insult to normal neurons also results from catalytically active redox metal ions (i.e. iron and copper) and particular ROS-generating enzymes and peptides (e.g. nitric oxide synthase, xanthine oxidase, β-amyloid etc.) present in the brain. Another factor which results in greater oxidative insult to brain relative to other tissues is that this organ consumes one-quarter of the total O_2 intake, and, consequently, generates more oxyradicals 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 which undergo mitosis, resulting in a loss of function [38,39]. Thus, the brain neurons and their organelles must survive for longer periods with oxidatively damaged dysfunctional organelles which occurs as a function of age and long-term metabolic stress [38,39].

In the cellular milieu of the brain, the mitochondria provides energy for the sustenance and maintenance of overall organ function. Indeed, several lines of evidence implicate that energy depletion in the brain due to mitochondrial dysfunction can conceivably result in neurodegeneration [8–10]. The mitochondria are capable of producing and meeting the energy demands of the cell by coupling the cell's respiratory function and generation of ATP by a tightly coupled process commonly known as oxidative phosphorylation. The electron transport chain present in the inner mitochondrial membrane functions to relay electrons, derived from reducing equivalents, to oxygen generating water through a four-electron reduction concurrently generating a proton flux. Concomitant with this energy generation, partially reduced oxygen species are also generated [45–48]. Previous work in our laboratory suggests that oxidation of succinate by the mitochondria causes lipid peroxidation of synaptosomal and mitochondrial membrane, as assessed by EPR spin labeling [14].

Thus, our investigations using a mixed population of synaptosomes and mitochondria demonstrate an increase in lipid and protein oxidation, as assessed by specific EPR spin labels, following a metabolic stress, under state 4 conditions, induced by a high concentration of succinate. It must be noted that such high concentrations of succinate do not reflect the circumstances in vivo. It is thus conceivable that an increased metabolic stress on the mitochondria causing an increased production of oxyradicals can result in damage to biomolecular components, like proteins and lipids, in a nerve cell. Oxidant-induced mitochondrial damage resulting in energy depletion has been implicated in several neurodegenerative diseases [8–10]. It is indeed possible to envisage that respiration-derived oxyradicals following a high metabolic challenge can lead to loss in the functional integrity of lipids and membranes of the mitochondria leading to such a damage.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health (AG-10836, AG-05119).

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