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Rapid Report

Membrane protein alterations in rodent erythrocytes and synaptosomes due to aging and hyperoxia

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Abstract

We have applied the technique of electron paramagnetic resonance (EPR) protein-specific spin labeling to the study of membrane protein alterations occurring during age and exposure to isobaric hyperoxia. Cortical synaptosomes and erythrocyte membranes (ghosts) were isolated from young rodents (Fisher 344 rats or mongolian gerbils, 3–4 months of age) and aged rodents (age 22–27 months for rats, greater than 15 months for gerbils). Membrane proteins were spin labeled with the thiol-specific spin label MAL-6 (2,2,6,6,-tetramethyl-4-maleimido-piperdin-1-oxyl). The relevant EPR spectral parameter of MAL-6 labeled membranes, the W/S ratio, decreased significantly with age of animal in both synaptosomes and ghosts ($P < 0.001$). As a paradigm for accelerated oxidative stress, young and aged gerbils were exposed to an atmosphere of 90–100% O_2 for 0–48 h. In both young and aged gerbils, the W/S ratio decreased significantly with hyperoxic stress ($P < 0.003$). The W/S ratio of synaptosomes isolated from aged gerbils decreased continually from 0–48 h hyperoxia, whereas the W/S ratio of synaptosomes from young animals demonstrated a pronounced rebound effect from 24–48 h. The results are discussed with reference to membrane protein oxidation in aging.

Keywords: Membrane protein; Spin labeling; Hyperoxia; Protein oxidation; Aging; Free radicals

The free radical theory of aging proposes that the slow generation of reactive oxygen-free radicals, an unavoidable consequence of life in an aerobic environment, results in cumulative damage to critical cellular components, and eventually leads to age-related pathology [1]. A consensus is emerging that free radical processes do play an important role in the etiology of many age-related disorders [2], although the specific mechanisms of free radical generation differ among pathologies and are often subjects of great contention. Free radical-mediated damage to neuronal membrane components has been implicated in many neurodegenerative diseases, especially in Alzheimer's disease [3,4] in which the most dominant risk factor is subject age [5,6].

The study of free radical toxicity is often hindered by the difficulty of quantifying in vivo tissue oxidation. We have recently developed protocols to quantify free radical oxidation of membrane cytoskeletal proteins by the technique of EPR protein-specific spin labeling [7]. These techniques have been used to study in vitro $^{\cdot}OH$ (hydroxyl radical)-mediated oxidation of synaptosomes [7] arid menadione-induced erythrocyte oxidation [8], as well as in vivo neuronal oxidation induced by brain ischemia-reperfusion injury [9]. In these studies, isolated membrane preparations (synaptosomes or erythrocyte ghosts) were spin labeled with the protein thiol-specific spin label 2,2,6,6-tetramethyl-4-maleimido-piperdin-1-oxyl (MAL-6). A decrease in the relevant EPR spectral component (the W/S ratio of the $M_I = +1$ low-field resonance line) of MAL-6 labeled membranes was sensitively correlated with degree of plasma membrane oxidation [7].

We report here that in both gerbils and Fisher 344 rats, aging alone can induce changes in spin-labeled synaptosomal and erythrocytic membranes which are indicative of oxidative protein modification. The decrease in the W/S ratio of MAL-6 labeled synaptosomes induced by aging can be mimicked or accelerated by in vivo exposure to isobaric hyperoxia (90-100% O_2 tension). The time course

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of hyperoxia-induced synaptosomal membrane alteration differs significantly between young and old animals, implying a deficiency of the antioxidant defense/repair capacity in the neocortex of the aged brain.

MAL-6, sucrose (ultrapure grade), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (ultrapure grade) were obtained from Sigma Chemical (St. Louis, MO). Protease inhibitors (leupeptin, pepstatin, aprotinin) were obtained from Calbiochem (San Diego CA). Medical grade oxygen was obtained locally. All other chemicals were highest available commercial purity.

Male mongolian gerbils and male Fisher 344 rats were obtained from Tumblebrook Farms (West Brookfield, MA) and subsequently housed in the University of Kentucky Central Animal Care Facility and fed standard Rodent Laboratory Chow (Purina) ad libitum in the home cage. 'Young' rodents were 3–4 months old. 'Aged' rats were 22–27 months of age. All 'aged' gerbils used in hyperoxia experiments were retired breeder animals 15–17 months of age; 2 of the 9 aged gerbils used for old v. young comparisons were 30 months of age, the remainder being 15–17 months of age. For each experiment, pairs of young and old animals were randomly assigned and tissue from each animal prepared in parallel. Animals were killed by decapitation.

Animals, in their home cage, were placed inside a 1 m × 1.5 m × 0.5 m clear polycarbonate hyperoxia chamber ventilated with O_2 (maintained at slightly positive pressure) and containing exhaust ducts to ensure isobaric conditions. O_2 tension inside the chamber was constantly monitored by means of an electronic O_2 metering system (Hasdorf/Entronics #5577) and maintained at between 90–100% O_2. No animal mortality occurred in the hyperoxia chamber during the 0–48 h exposure periods reported in this study. Age-matched control animals were maintained outside the oxygen chamber, in their home cages, simultaneous with treatment groups maintained within the oxygen chamber.

Blood was collected by exsanguination into a heparinized receptacle and immediately placed on ice. Ghost preparation and spin labeling were performed as previously described [10,11]. Samples were diluted with 10 parts phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM K_2HPO_4/KH_2PO_4 (pH 8.0)). Whole blood was washed three times in PBS, pelleted by centrifugation at 600 × g for 5 min at 4° C, and supernatant and buffy coat removed by aspiration. Erythrocyte membranes were then isolated by hypotonic lysis in 5P8 buffer (5 mM $KHPO_4/KH_2PO_4$ (pH 8.0)). Hemoglobin was removed by 5 consecutive washes in 5P8 buffer, followed each time by centrifugation at 27 000 × g for 10 min at 4° C.

Protein concentration in ghost suspensions was measured by the Lowry assay [12] and adjusted to 3.0 mg/ml. 1 vol. of the 3.0 mg/ml suspension was incubated with 9 vol. of 51 μM MAL-6 in 5P8 buffer for 14–16 h. Excess spin label was then removed by 6 cycles of 27 000 × g centrifugation/5P8 resuspension, after which time supernatant was free of EPR-detectable quantities of spin label. After the final centrifugation, the ghost pellet was resuspended in a minimum volume of buffer, allowed to equilibrate to 22° C, and aliquoted into a 300 μl quartz EPR flat cell for spectral acquisition using a Bruker 300 EPR spectrometer (relevant instrumental parameters: microwave powers = 18 mW; modulation amplitude = 0.4 G; time constant = 1.28 msec.; conversion time = 10 msec).

Spin labeling was performed as previously described [7,13]. The neocortex was dissected free and immediately suspended in ice-cold isolation buffer (0.32 M sucrose, 10 mM Hepes (pH 7.4), and the protease inhibitors: 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, 0.2 mM PMSF, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bis(aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA)). Samples were homogenized by 8–12 passes through a Wheaton motorized Teflon-coated homogenizer with 0.25 mm clearance. Crude homogenate was centrifuged at 1500 × g for 10 min at 4° C, after which the supernatant was collected and recentrifuged at 20 000 × g for 10 min. The resulting pellet was dispersed and layered upon discontinuous sucrose gradients (10 ml of 1.18 M sucrose (pH 8.5); 10 ml 1.0 M sucrose (pH 7.4); 10 ml 0.85 M sucrose pH 7.4, each buffered by 10 mM Hepes and containing 2 mM EDTA and 2 mM EGTA). Sucrose gradients were centrifuged at 4° C and 82 500 × g for 120 min in a Beckman L2-65B refrigerated ultracentrifuge using an SW28 swinging-bucket rotor. Synaptosomes were removed from the 1.18/1.0 M interface, washed in 10 vol. of lysing buffer (10 mM Hepes, 2 mM EDTA, 2 mM EGTA (pH 7.4)), and protein concentration determined by the Lowry method [12]. Synaptosomal membranes were spin labeled by incubation for 14 h at 4° C with 20 μg MAL-6/mg protein in lysing buffer. For each animal, duplicate samples were spin labeled. After incubation with spin label, synaptosomal membranes were pelleted in a Beckman table-top microcentrifuge, supernatant was discarded, and pellet resuspended in fresh lysing buffer. After 6 cycles of centrifugation/resuspension, the supernatant was free of EPR-detectable spin label. The pellet was then resuspended in a minimal amount of lysing buffer and allowed to equilibrate to 22° C. 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 msec., conversion time = 10 msec.

Data were expressed as % change in W/S parameter between paired treatment and control animals, and analyzed by analysis of variance (AN0VA) and subsequent Student's t-tests. A $P < 0.05$ was considered significant.

The relevant EPR spectral parameter of MAL-6 labeled membrane proteins is the ratio of $M_I = +1$ low-field weakly- and strongly-immobilized components, termed the W/S ratio (Fig. 1). This parameter has been extensively studied in relation to cytoskeletal perturbations in both

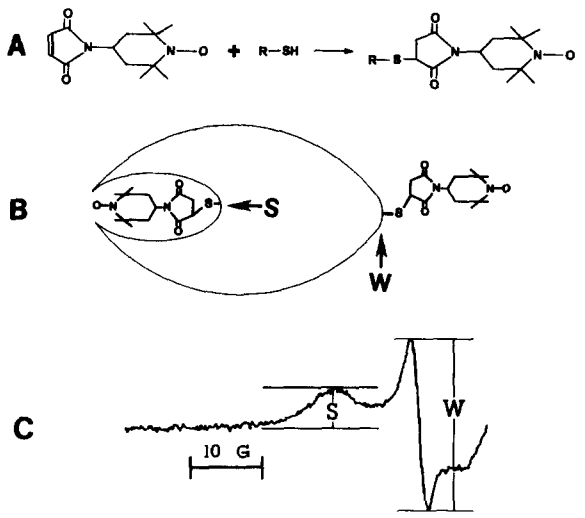

Fig. 1. (A) Reaction of the MAL-6 spin label with membrane protein thiols. (B) Schematic showing strongly (S) and weakly (W) immobilized MAL-6 binding sites. (C) $M_I = +1$ low-field EPR line of MAL-6 labeled synaptosomal membrane proteins showing W and S spectral components.

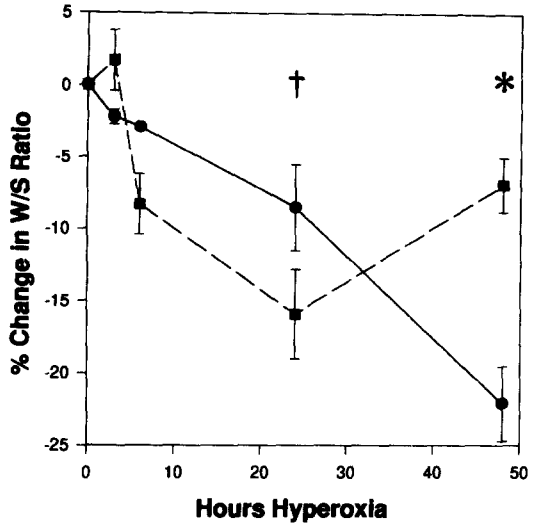

Fig. 2. Effect of 90–100% O_2 exposure on W/S ratio of MAL-6 labeled gerbil cortical synaptosomes. Solid curve: aged gerbils (15–17 months of age). Dashed curve: young gerbils (3–4 months of age). Data represent % Δ W/S between age-matched animals maintained inside and outside the hyperoxia chamber for the given exposure period. Error bars indicate standard error about the mean (S.E.M.) for 3 animals (duplicate samples per animal). † $P < 0.07$; * $P < 0.001$ between young and old animals at the indicated time point (by Student's t-test). For both old and young gerbils, depressions of the W/S ratio at time > 6 h hyperoxia are significant. Note the monotonic decrease in the W/S ratio of aged gerbil synaptosomes over the entire time course, and the significant rebound of the W/S ratio of young gerbil synaptosomes between 24–48 h ($P < 0.03$ for 24 vs. 48 h time points).

erythrocytes and synaptosomes [7–11]. A decrease in the W/S ratio indicates increased steric hindrance to spin label mobility and hence indexes protein tertiary and quaternary structure about the spin label binding sites. The W/S ratio of MAL-6 labeled synaptosomes decreases predictably with in vitro exposure to H_2O_2 and other oxidants [7,8], and in fact the response of the W/S ratio to increasing Fe^{2+} concentration is sensitive enough to allow accurate extrapolation to endogenous oxidizing equivalents [7].

Table 1 shows that both ghost and synaptosomal membrane proteins of gerbils and Fisher 344 rats differ as a function of animal age. The W/S ratio of ghosts and synaptosomes isolated from old animals is significantly lower than corresponding tissue isolated from young animals ($P < 0.009$ by ANOVA), consistent with the hypothesis that old animals are subject to a greater level of oxidative stress.

The response of the gerbil neocortical membrane to isobaric hyperoxia differs quantitatively between young and old animals. As Fig. 2 shows, exposing the animal to a hyperoxic environment (90–100% O_2) results in a significant decrease in the W/S ratio of MAL-6 labeled synaptosomes ($P < 0.003$ for both groups). Although both young and old animals demonstrate a decreased W/S ratio after exposure to hyperoxia, the time course of the effect is markedly different between the two groups. At time points less than 24 h, the magnitude of the decrease in the synaptosomal W/S ratio is greater for the young animals than for the old animals (Fig. 2), as would be expected since the absolute W/S value of the old animals is less than that of the young (i.e., the inherent level of membrane protein oxidation is inferred to be greater in old animals; Table 1). Within the first 24 h hyperoxia, the separation between the time course curves for young and old gerbils is approx. 7–8%, which is the difference in baseline W/S ratio between the groups (Table 1). In old animals, the W/S ratio declines monotonically with duration of hyperoxia over the entire hyperoxia time course 0–48 h. In young animals, however, the W/S ratio declines over the time course 0–24 h, then recovers significantly between 24–48 h (ΔW/S between 24 and 48 h time points = 9.1%, $P < 0.03$).

It should be noted that no animal death occurred in the hyperoxia chamber at exposure times less than 50 h; however, considerable mortality occurred in old animals exposed to greater than 50 h hyperoxia (data not shown). Therefore, 48 h was the practical limit to the experimental

Table 1
Decrease in W/S ratio with aging [a]

	Cortical synaptosomes	Erythrocyte ghosts
Gerbil	-6.84 ± 4.27 ($n = 9$, $P < 0.001$)	-
Fisher rat	-5.57 ± 0.75 ($n = 4$, $P < 0.009$)	-4.51 ± 0.34 ($n = 3$, $P < 0.001$)

[a] Data represented are mean % Δ W/S ± S.D. between randomly assigned pairs of young and aged animals. P-values computed by Student's t-test for pairwise comparisons. Young animals = 3–4 month old for gerbils and rats, aged animals = 15–30 months old for gerbils, 22–27 months old for rats.

hyperoxia time course. The 22% decrease in the W/S ratio of old animals at 48 h hyperoxia also, therefore, approximately represents the maximum change observable in this parameter prior to hyperoxia-induced mortality.

There is a considerable literature to suggest that both brain and peripheral tissue proteins become progressively oxidized with age in both non-human models and humans [14–16] and that brain protein oxidation is correlated with cognitive dysfunction in rodents [15]. Unfortunately, there are relatively few empirical techniques used to infer brain protein oxidation. Protein carbonyl content and changes in glutamine synthetase (GS) activity have been effectively used to quantify brain protein oxidation [14–16], but these biomarkers do not directly discriminate oxidative alterations in the physical state of cell membrane proteins. The MAL-6 protein spin labeling technique, however, provides a means of monitoring membrane biophysical perturbation induced by oxidative stress.

The trends in the W/S ratio reported in this work corroborate previous demonstration of increased protein carbonyl content in aged- and hyperoxia-stressed rodents [15,16] and aged human tissue [14]. The monotonic decline of the synaptosomal W/S ratio in the aged, but not the young, hyperoxic gerbil brain is an intriguing phenomenon which would seem to indicate an age-related loss of physiologic plasticity toward oxidative membrane stress. The synaptosomal rebound we observe in young animals exposed to prolonged hyperoxia bears a striking similarity to a phenomenon observed in rodent hepatocytes [16]. Protein oxidation products and glutamine synthetase/glucose-6-phosphate dehydrogenase immunologic cross-reactivity increase steadily-in hepatocytes of rats exposed to 48 h continuous hyperoxia, then decline during the interval 48–56 h, apparently due to upregulation of proteases which specifically degrade oxidized proteins [16]. Levels of neutral proteases are decreased in aged brain [16], possibly indicating a deficiency in the ability of the aged physiology to remove or replace oxidatively damaged proteins.

Another possible explanation for the rebound effect in young animals is upregulation of antioxidant defense mechanisms. Cortical levels of superoxide dismutase (SOD) have been reported to increase marginally in hyperoxic rats [17] at time points beyond 36 h O_2 exposure; in the same study, however, catalase activity was found to decline irreversibly. SOD activity is variously reported to increase [18] or remain unchanged [19] with age in rodent brain; baseline levels of the antioxidant enzyme glutathione peroxidase and reduced glutathione levels are reportedly unchanged in aged Fisher 344 rat brain [18] but decreased in the brains of senescence-accelerated mice [19], in the absence of additional oxidative stress. Antioxidant enzyme activity varies with species, sex, age, organ system, and tissue section (brain region) in rodents [18,19], which may partially account for the variation in reported enzyme activity trends within the literature. The inefficiency in the response of aged animals to oxidative stress may also arise as a result of age-induced damage to DNA and/or transcriptional and translational enzyme systems. Such an hypothesis is supported by the observation that certain enzymes involved with gene expression (e.g., ribonuclease) are readily inactivated by oxidatively damaging events [20].

The data reported in this study indicate that old and young rodents differ in baseline levels of neuronal and peripheral cell membrane protein oxidation, as well as in the physiological response to further oxidative insult. Further study of these biophysical differences may provide valuable insight into human neurodegenerative disorders. Such studies are currently underway in our laboratory.

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