PERSISTENT PROTEIN DAMAGE DESPITE REDUCED OXYGEN RADICAL FORMATION IN THE AGING RAT BRAIN

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Abstract—The relation between cerebral oxygen radicals and the aging process was investigated in crude synaptosomal (P2) fractions from rats. The rate of formation of oxygen radicals was measured using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is de-esterified and subsequently oxidized by oxygen radicals to a fluorescent product 2',7'-dichlorofluorescein (DCF). There was a significant age-dependent decrease in the formation rate of oxygen radicals, observed by decreased formation of DCF. No difference in oxygen radical formation was apparent between age groups following an in vitro challenge with an ascorbate/FeSO₄ mixture. This age-dependent decrease in cerebral oxygen radical generation coincided with age-dependent increases in superoxide dismutase. No age-related alterations in lipid order in either the hydrophilic or lipophilic membrane regions were observed using fluorescence polarization analysis. Age-dependent losses in cerebral P2 tryptophan fluorescence (a measure of overall proteolytic activity) were observed. Results suggest that aging does not proceed as a result of elevated rates of generation of oxygen radicals, a finding that does not support the proposed free radical theory of aging. The observed age-dependent decrease in the formation of oxygen radicals does not effect membrane lipid order. These findings implicate modifications in proteins and activated protein catabolic pathways as major contributing factors in the normal physiological process of senescence.

Key words: aging, free radicals, oxygen radicals, proteins, proteolysis.

Considerable attention has been given to the possible role of oxidative damage in the aging process as a consequence of the work of Harman on atherosclerosis. A general consensus is that the sum total of free radical reactions, primarily those involving oxygen, is the major contributing cause of aging. Such reactions generate oxygen radicals which are the initiators of phenomena such as peroxidation of polyunsaturated lipids, and scission and crosslinkage of macromolecules, events that alter the structure and function of cell components. 

The brain is particularly sensitive to oxidative damage for several reasons. Cerebral tissue contains a high concentration of polyunsaturated fatty acids which are easily peroxidizable, and has a high rate of oxygen consumption. The accumulation with age of the pigment lipofuscin in nerve cells is thought to result from the peroxidation of membrane lipids. Brain is also low in antioxidant protective agents such as catalase, glutathione peroxidase, α-tocopherol and glutathione. Furthermore, certain brain regions are high in total iron content, a transition metal well known to induce lipid peroxidation in cerebral and other tissues.

Despite the numerous reports on the involvement of oxygen radicals with aging processes, the relation between these events is unresolved. Numerous aging-oxygen radical studies exist regarding lipid peroxidation, antioxidant protectors and membrane fluidity, many of which have disparate results that have further confounded understanding of the aging process. Contributing to this problem is the fact that conclusions regarding the relation between oxygen radicals and aging are generally based on data obtained from events secondary (i.e. lipid peroxidation) to the formation of oxygen radicals.

The aim of the present study was to clarify the issue of oxygen radicals as a contributing factor in senescence. Several fluorescent techniques were employed to investigate this hypothesis. The chemical 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used as a means of direct quantitation of cerebral oxygen radicals. DCFH-DA is a stable, non-fluorescent molecule that crosses cell membranes and is subsequently de-esterified within the cell. The released DCFH can then be oxidized in the presence of oxygen radicals to highly fluorescent 2',7'-dichlorofluorescein.

Abbreviations: DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCFH, 2',7'-dichlorofluorescein; DCF, 2',7'-dichloro-fluorescein; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1,4-(trimethylamino)-phenyl-6-phenyl-1,3,5-hexatriene.
(DCF). Cerebral membrane order was investigated by fluorescence polarization analysis using 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1,4-(trimethylamino)-phenyl-6-phenyl-1,3,5-hexatriene (TMA-DPH) as probes for membrane order. Protein degradation was evaluated by tryptophan destruction, where levels of this amino acid within protein were assessed by fluorometric detection. The overall rate of cerebral proteolysis was determined using $^{14}$C[protein as substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**

2',7'-Dichlorofluorescein diacetate (DCFH-DA), 1,6-diphenyl-1,3,5-hexatriene (DPH), and 1,4-(trimethylamino)-phenyl-6-phenyl-1,3,5-hexatriene (TMA-DPH) were obtained from Molecular Probes (Eugene, OR), and 2',7'-dichlorofluorescein (DCF) was purchased from Polysciences, Inc. (Warrington, PA). $^{14}$C-Methylated proteins (5 µCi/ml) were purchased from Amersham (Arlington Heights, IL).

**Animals**

Male Fisher 344 rats of several ages obtained from the National Institute of Health Aging Colony, were generously donated by Dr Sue Duckles (UC Irvine, Department of Pharmacology). Animals were decapitated and the brains were rapidly excised, the cortex was dissected out and stored at -70°C until sample preparation.

**Tissue preparation**

A crude cerebrocortical P2 fraction consisting of mitochondria, synaptosomes and myelin fragments was employed for all studies. Each cortex was weighed and homogenized in 10 volumes of 0.32 M sucrose. The crude nuclear fraction (P1) was removed by centrifugation at 1800 g for 10 min. The resulting supernatant fraction (S1) was centrifuged at 32,500 g for 10 min to yield the crude synaptosomal pellet (P2). The P2 pellet was suspended in HEPES buffer to a concentration of 0.037 g equiv/ml. The composition of HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH$_2$PO$_4$, 1.2; MgCl$_2$, 0.1; NaHCO$_3$, 5.0; glucose, 6.0; CaCl$_2$, 1.0 and HEPES 10. After preparation the P2 pellets were first stored at -20°C for 24 hr, then stored at -70°C until analysis.

**Assay for oxygen radical generation**

The formation rate of oxygen radicals was determined as previously described. Briefly, cerebrocortical P2 fractions were diluted with 9 volumes of ice-cold 40 mM Tris buffer, pH 7.4, in a final volume of 2 ml. Fractions were then loaded with 5 µM DCFH-DA (from a stock solution of 1.25 mM DCFH-DA in methanol) for 15 min at 37°C. The initial fluorescence value of the dye-loaded samples was recorded and samples were then incubated in the presence or absence of ascorbate (10 µM)/FeSO$_4$ (1 µM) for 60 min at 37°C. These two conditions were taken to represent basal and induced oxygen radical formation. The formation of the fluorescent probe DCF was monitored at an excitation wavelength of 488 nm (bandwidth 5 nm), and an emission wavelength of 525 nm (bandwidth 20 nm). The cuvette holder was thermostatically maintained at 37°C. Autofluorescence was subtracted prior to DCF fluorescence calculations, and DCF formation was quantified from a standard curve in methanol (0.05–1.0 µM).

**Assay for superoxide dismutase activity**

Total superoxide dismutase activity was measured using the xanthine/xanthine oxidase, cytochrome c reduction assay. An aliquot of cerebrocortical P2 fractions (0.5 ml) was diluted with 0.5 ml 10 mM HEPES (mM: NaCl, 120; KCl, 2.5; NaH$_2$PO$_4$, 1.2; MgCl$_2$, 0.1; NaHCO$_3$, 5.0; glucose, 6.0; CaCl$_2$, 1.0 and HEPES, 10.0) containing 25 mM β-o-D-glucopyranoside. The samples were centrifuged at 12,500 g for 5 min, and 0.2 ml was added to HEPES buffer containing 100 µM xanthine, 10 µM cytochrome c, and 6 nM xanthine oxidase. One unit of superoxide dismutase activity was defined as a 50% decrease in the rate of reduction of cytochrome c at pH 7.8.
Assay for membrane order

Five ml of HEPES buffer was added to 1 ml aliquots of P2 fractions (0.027 g equiv./ml) which were centrifuged at 12,500 g for 8 min, and resuspended in 4 ml HEPES. Two ml of the resuspended P2 fractions were then incubated for 15 min at 37°C with either 5 µM DPH or TMA-DPH to determine basal membrane order. Following the measurement of basal membrane order, all samples were then oxidatively challenged with ascorbate (10 µM)/FeSO₄ (1 µM) for 10 min, and again membrane order was assessed. Fixed excitation and emission polarization filters were used to measure fluorescence intensity both parallel (I₁) and perpendicular (I₂) to the polarization phase of the exciting light. I₁ corresponds to both vertically polarized excitation and emission while I₂ corresponds to vertically polarized excitation and horizontally polarized emission. Excitation and emission wavelengths of 360 and 430 nm, respectively, were used with the bandwidth of both monochromators at 10 nm. Cuvette temperature was maintained at 37°C with a circulating water bath. Fluorescence anisotropy (r) was determined by the formula: \( r = \frac{(I₁) - (I₂)}{(I₁) + 2(I₂)} \).³⁰

Assay for protein degradation

Protein degradation was measured by the loss of tryptophan according to the method of Davies et al.⁹ Cerebrocortical P2 fractions were diluted with 9 volumes of 100 mM phosphate buffer (pH 7.4) containing 0.1% SDS in a final volume of 2 ml. Samples were incubated for 30 min at 37°C in either the presence or absence of ascorbate (10 µM)/FeSO₄ (1 µM) or hydrogen peroxide (10 µM), and the loss of tryptophan was determined by the measured fluorescence using excitation wavelength 280 nm (bandwidth 5 nm) and emission wavelength 345 nm (bandwidth 20 nm).

Assay for overall proteolysis

An aliquot of cerebral P2 fraction (0.4 ml) was placed into 0.6 ml of ice-cold deionized water. Next, 5 µl of [¹⁴C]protein was added to each tube, and the samples were allowed to incubate for 60 min at 37°C. Blanks contained 0.4 ml of bovine serum albumin (0.15 mg/ml) used as a carrier. Incubations were terminated and methylated proteins were precipitated by the addition of 0.4 ml 1.6 M perchloric acid, and the samples were vortexed and centrifuged at 12,500 g for 10 min. One ml of the resulting supernatant was then added to a 20 ml scintillation vial containing 5 ml Ready Solv EP (Beckmann Instruments, Inc., Fullerton, CA), and the amount of [¹⁴C] liberated from precipitated methylated proteins, a measure of overall protease activity, was assessed by liquid scintillation spectroscopy. Background radioactivity in the blanks was subtracted prior to the calculation of overall cerebral protease activity.

Assay for protein concentration

Protein content of P2 fractions was assayed by the method of Bradford³ using bovine serum albumin as a reference.

Statistical analysis

Analysis of variance (ANOVA) was used to determine statistical difference between treatments, followed by Duncan's new multiple range test (DNMRT) with \( P < 0.05 \) taken as significant.

RESULTS

The generation rate of oxygen radicals in cerebrocortical P2 fractions was studied in 1-, 6- and 20-month-old rats. Under basal conditions, there was a clear age-dependent decrease in the formation rate of oxygen radicals (Fig. 1). Basal oxygen radical formation in 20-month-old rats was significantly lower than that observed in 1-month-olds. Following treatment with ascorbate/FeSO₄ in vitro, there was an approximately 6–8-fold increase in the generation rate of oxygen radicals compared to basal conditions in all age groups (Fig. 1). However, subsequent to the oxidative challenge, no differences in oxygen radical formation rates were observed between any of the age groups.
To investigate whether age-related decreases in basal formation rates of oxygen radicals were regulated by the activity of antioxidant enzymes, total cerebrocortical superoxide dismutase activity was examined. The results demonstrate that 6- and 20-month-olds have significantly higher superoxide dismutase activities than young rats (Fig. 2).

Since it has been proposed that alterations in membrane order influence function of cell membranes and/or other cell components, cerebral membrane order was examined. The fluorescent probes DPH and TMA-DPH employed provided a measure of the lipid order in both the lipophilic (DPH) and hydrophilic (TMA-DPH) regions of membranes. No age-related alterations in membrane lipid order were observed under either basal or ascorbate/FeSO₄ challenge conditions (data not shown).

A role for oxygen radicals in the protein degradative process has been proposed. We were interested in determining whether such a relation existed in the nervous system. To verify the methodology employed, concentrations of ascorbate/FeSO₄ and hydrogen peroxide, previously shown to induce damage in cerebral proteins. Both ascorbate/FeSO₄ and hydrogen peroxide treatment caused decrease in tryptophan fluorescence in cerebral P2 fractions compared to untreated controls (Fig. 3). Analysis of tryptophan fluorescence in P2 fractions from different aged rats also demonstrated significant decreases in both mature and senescent animals (Table 1). However no age-related alterations were observed in total protein concentration of cerebral P2 fractions (Table 1).

The age-related decreases in tryptophan fluorescence within proteins may be related to an increase in overall cerebral proteolytic activity. To study this possibility, P2 fractions from 1- and 2-month-olds were lyzed in ice-cold water, and incubated with [14C]-methylated proteins. Results revealed that P2 fractions isolated from senescent rat brains liberated [14C]fragments to the acid-soluble fraction at a significantly higher rate (2.3-fold P<0.001) than that found in young rats (Table 1).
Persistent protein damage in aging rat brain

Fig. 3. Tryptophan fluorescence in cerebrocortical P2 fractions following in vitro exposure to ascorbate/FeSO₄ or hydrogen peroxide. Data are expressed as mean ± S.E. derived from three separate experiments. Asterisks denote statistical difference from 1-month-old rats (P<0.05).

Table 1. The effects of aging on protein degradation, protein concentration, and proteolytic activity in rat cerebrocortical P2 fractions

| Age (months) | Tryptophan (F.I./mg protein) | Protein (mg/g equiv.) | Proteolysis rate (cpm/mg prot./h) |
|--------------|----------------------------|-----------------------|----------------------------------|
| 1            | 176.8 ± 7.9                | 41.4 ± 1.1            | 403 ± 63                         |
| 6            | 146.8 ± 5.4*               | 43.4 ± 1.9            | 934 ± 67**                       |
| 20           | 148.8 ± 4.2*               | 42.2 ± 1.1            |                                   |

The data are expressed as the mean ± S.E. derived from six animals. Asterisks represent significant difference (*P<0.01; **P<0.001) from 1-month-old animals according to DNMRT. F.I. corresponds to total fluorescence intensity of tryptophan.

DISCUSSION

Aging has been defined as the progressive accumulation of those time-associated changes that are responsible for increased susceptibility to disease and death. Free (oxygen) radicals have been proposed as the etiological agents underlying these changes. However, few studies have directly measured oxygen radicals in the senescent brain.

The findings of the present study clearly demonstrated that young (1-month-old) rats generated oxygen radicals at significantly higher rates than do older (20-month-old) rats (Fig. 1). These data are in agreement with the work of Floyd et al. who measured superoxide anion generation in brain mitochondria via an oxygen electrode method. Our findings are however in conflict with the free radical theory of aging, and are not supported by a study that used electron spin resonance spectroscopy and observed age-dependent increases in superoxide anion formation in brain mitochondria. These divergent results illustrate a critical problem in the free radical-aging research area. While differences in the methodologies employed could explain the contradictory results observed, this explanation only further confounds the issue of whether increased oxygen radical generation is the driving force behind the aging process. Several key features must be addressed in order to clarify the issue: (1) various oxygen radicals have been implicated in a variety of physiological and pathological conditions, and the claim that one particular oxygen species (i.e. superoxide anion) is the major causative species of senescence has not taken this into account; (2) the separation and identification of the effects produced by a given oxygen radical has proved to be a formidable task in biological systems; (3) it is unclear whether the formation of oxygen radicals in an isolated organellar preparation (i.e. purified mitochondria) is indicative of events occurring in vivo.

The methodology employed in this study used a fluorescent probe (DCF) to provide a direct measure of the formation rate of several oxygen radicals such as hydroxyl radical, transition metal–peroxide complexes, and lipoperoxyl radicals. Therefore, the data generated using DCFH-DA in cerebrocortical P2 fractions provides an indication of the sum total of several, not individual oxygen radicals formed in a given system. As mentioned previously, the isolation of a specific oxygen radical in a biological system is a complex problem, handicapping the designation of a given radical as being responsible for a particular event. In addition, oxygen reactive species such as superoxide anion and hydrogen peroxide can co-react to form hydroxyl radicals via the...
Haber–Weiss reaction. Thus the estimation of a single species of oxygen radical in a brain mitochondrial preparation may not adequately depict the prooxidant status of the aging nervous system.

Another concern is that isolated mitochondrial preparations, while they are known to be a major source of oxygen radicals, may not contain all of the necessary defense mechanisms against oxygen radical formation. In the eukaryotes Cu,Zn superoxide dismutase is found primarily in the cytosol, while Mn superoxide dismutase is localized in the mitochondrial matrix.\(^\text{14}\) Rat cerebral cortex contains an approximately 4-fold higher activity of Cu,Zn superoxide dismutase than the Mn form.\(^\text{9}\) Therefore reported age-dependent increases in superoxide anion from isolated brain mitochondria\(^\text{37}\) do not take into account the protective effect of the major form of defense against the superoxide anion, the Cu,Zn superoxide dismutase.

The current study used cerebrocortical P2 fractions to determine whether age-related alterations existed in oxygen radical generation. This preparation contains pinched off nerve terminals with membranes that reseal to form entirely enclosed particles.\(^\text{17}\) The particles formed (synaptosomes) contain neurosecretory vesicles, mitochondria, and microperoxisomes. More importantly they are autonomous particles than synthesize ATP and accumulate all the necessary ions and substances to be viable markers for synaptic mechanisms. Komulainen and Bondy\(^\text{21}\) have evidence that extrasynaptosomal mitochondria are not likely to be active in low K\(^\text{+}\) buffers, findings that suggest mitochondrial function is compromised in isolated preparations. The use of a crude synaptosomal preparation in the study of oxygen radical formation thus provides an indication of the overall oxidant status in nerve terminals. Therefore, the age-dependent decrease in oxygen radical generation rates described here (Fig. 1) in cerebrocortical P2 fractions, taken together with the likelihood that most of the antioxidant defense mechanisms are present in the synaptosomes, may more closely resemble the in vivo situation than would an isolated mitochondrial preparation.

Superoxide dismutase, as a major antioxidant defense enzyme, is involved in the regulation of superoxide anion and overall oxygen radical turnover. Therefore, it was relevant to determine the aging pattern of superoxide dismutase in cerebrocortical P2 fractions. Superoxide dismutase activity from partially purified cortical synaptosomes was significantly higher in 6- and 20-month-old rats when compared to 1-month-olds (Fig. 2), a finding that appears to have considerable support in the literature.\(^\text{11,13,18}\) However, disparate findings have been reported in an isolated mitochondrial preparation from aged rat brain.\(^\text{42}\) In general, reports demonstrating age-dependent increases in superoxide dismutase employed more complete tissue preparations (i.e. whole homogenates), while those in disagreement\(^\text{42}\) have essentially only shown data on Mn superoxide dismutase, the less active form of the enzyme. The concentration and distribution of antioxidant enzymes and oxygen radical scavengers (\(\alpha\)-tocopherol, \(\beta\)-carotene, glutathione) will be factors that determine the overall rate at which oxygen radicals are formed. It has been shown that another antioxidant enzyme, glutathione peroxidase increases with age in the rat.\(^\text{48,44}\)

The peroxidation of membrane lipids as a result of an age-related increase in oxygen radicals\(^\text{22}\) is the central dogma of the free radical theory of aging.\(^\text{19}\) However, the data reported in this study (Figs 1 and 2) would suggest that the aged brain has a decreased prooxidant/antioxidant ratio, and therefore increases in lipid peroxidation should not occur. In support of this concept, decreased cerebral peroxidative potential during aging has been demonstrated and proposed to be related to lower levels of oxygen radicals, decreased lipid substrate, and decreased iron availability.\(^\text{11,13}\)

The effects of aging on cerebral membrane order were investigated since a direct relation had been shown between increases in lipid peroxidation and membrane order.\(^\text{24}\) and increased oxygen radical formation rates.\(^\text{24}\) Employing the fluorescent probes DPH and TMA-DPH, no age-related alterations in either lipophilic or hydrophilic membrane domains (data not shown) were observed, results in agreement with studies done in rat synaptosomes.\(^\text{44}\) The lack of age-related effects on cerebral membrane order described in the present study may be related to decreased oxygen radical generation rates (Fig. 1), and decreased lipid peroxidative potential.\(^\text{11,12}\) Our findings suggest that age-related decreases in oxygen radical generation and peroxidative activity do not affect membrane order.

While the evidence continues to mount against age-related increases in oxygen radical generation and consequent increased lipid peroxidation, the accumulation of lipofuscin with age appears to be a consistent parameter that holds true over a wide range of species.\(^\text{12}\) The mechanism of
lipofuscin accumulation is believed to be via oxidative insult with either a concomitant increased rate of pigment formation or a decreased rate of destruction. Since the aged brain may tend towards decreased peroxidative activity, a decrease in the ability to degrade lipofuscin with age may account for its accumulation. By this means altered protein structure and function may be a contributing factor to senescence.

It has been proposed that oxidatively modified proteins are rapidly degraded via proteolysis.9,10,13 For this reason, protein modification was studied in the aging rat brain. It was found that concentrations of ascorbate/FeSO₄ and hydrogen peroxide known to increase oxygen radical generation rates 6–8-fold (Fig. 1),26 induced a modest decrease in tryptophan fluorescence (Fig. 3). Such a loss in tryptophan fluorescence has previously been utilized as an indication of protein damage.9 In cerebrocortical P2 fractions, tryptophan fluorescence was significantly lower in 6- and 20-month-old as compared to 1-month-old rats (Table 1). This age-dependent decrease in free tryptophan content in brain has previously been reported,26,36 but while the underlying mechanism for this age-dependent decrease remains unknown, it does not appear to result from elevation of oxygen radicals or augmented lipid peroxidation. Agents that stimulate lipid peroxidation have been shown to increase the rate of degradation of proteins and the formation of protein aggregates.21,35 This degradation will only occur after oxygen radical scavengers and antioxidant enzyme systems are overwhelmed.21 Since we have demonstrated age-dependent decreases in oxygen radical generation rates and increases in superoxide dismutase, our findings suggest that adaptation of the aged brain involves reduction of adverse oxidative processes.

It is evident that an appreciable amount of oxygen radicals are formed and lipid peroxidation occurs under normal homeostatic conditions in the brain and other organs. It was recently shown that age-dependent reductions in cerebral mitochondrial proteins of molecular weights 20 and 16 kDa occur.33 The present study demonstrated that cerebrocortical P2 fractions from aged rats liberated more [¹⁴C]protein fragments to the acid-soluble fraction than that observed in young rats (Table 1), suggesting higher basal proteolytic potential. This may be a result of increased cytosolic free Ca²⁺.16 While this age-related increase in proteolytic potential could be a consequence of subcellular fractionation techniques, similar increases have been observed in human red cells.17 In addition, other studies have not found age-related differences in total protein concentrations.9 It may be that total protein assays lack the sensitivity required to observe subtle effects in protein turnover dynamics during senescence. The present study suggests that an increased extent of protein degradation and increased rates of proteolytic activity may be a component of the normal physiological aging process.

In conclusion, the generation rate of oxygen radicals decreases with age in the brain, an event that appears to result from increases in antioxidant enzyme activities. These fluxes in the intensity of oxidative events do not effect lipid order within membranes. It may be that irreversible age-dependent modifications in proteins occur as a consequence of normal oxidative metabolism together with accelerated rates of protein catabolism.

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