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EFFECTS OF AGE, DIETARY AND BEHAVIORAL ENRICHMENT ON BRAIN MITOCHONDRIA IN A CANINE MODEL OF HUMAN AGING

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Abstract

Dogs develop cognitive decline and a progressive accumulation of oxidative damage. In a previous longitudinal study, we demonstrated that aged dogs treated with either an antioxidant diet or with behavioral enrichment show cognitive improvement. The antioxidant diet included cellular antioxidants (Vitamins E, C, fruits and vegetables) and mitochondrial co-factors (lipoic acid and carnitine). Behavioral enrichment consisted of physical exercise, social enrichment and cognitive training. We hypothesized that the antioxidant treatment improved neuronal function through increased mitochondrial function. Thus, we measured reactive oxygen species (ROS) production and bioenergetics in mitochondria isolated from young, aged and treated aged animals. Aged canine brain mitochondria show significant increases in ROS production and a reduction in NADH-linked respiration. Mitochondrial function (ROS and NADH-linked respiration) was improved selectively in aged dogs treated with an antioxidant diet. In contrast behavioral enrichment had no effect on any mitochondrial parameters. These results suggest that an antioxidant diet improves cognition by maintaining mitochondrial homeostasis, which may be an independent molecular pathway not engaged by behavioral enrichment.

Keywords

beagle; carnitine; carbonyls; cognitive training; environmental enrichment; lipoic acid; vitamin E

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INTRODUCTION

Oxidative damage and mitochondrial dysfunction may be critical events leading to neuronal dysfunction and contribute to the development of age-associated neurodegenerative disorders such as Alzheimer disease (AD) (Ames, 1993). In humans, some epidemiological studies have shown a positive effect of antioxidant supplementation on cognition and reduction of risk for developing AD (Engelhart, 2002; Morris, 2002), but other studies have failed to report beneficial effects (Masaki, 2000; Luchsinger, 2003). In some cases, epidemiological studies indicate that combinations of antioxidants are superior to single compound supplementation (Zandi and Group., 2004) and further reduce the risk of developing AD. Moreover, dietary intake has been shown to be superior to supplements in human studies (Morris, 2002). Difficulties in interpreting human studies stem from differences in the amount of supplements taken, the form and source, duration and regularity of use, and the challenges of determining the exact intake of antioxidants. However, recent reviews of the human literature have emphasized that antioxidants may be a promising approach for preventing AD (Rutten, 2002). Studies in animal models have provided strong evidence that oxidative damage contributes to neuronal and behavioral dysfunction. Reducing oxidative damage in aged rodents, by providing antioxidants either in supplements or in fruits and vegetables can improve learning, memory and motor function (e.g.(Socci et al., 1995; Joseph, 1998, 1999; Bickford et al., 2000b)).

Dogs develop cognitive decline in measures of learning and memory as a function of age (Milgram et al., 1994; Tapp et al., 2003b; Christie et al., 2005; Studzinski et al., 2006). In parallel, the aged dog brain progressively accumulates oxidative damage to proteins and lipids (Kiatipattanasakul et al., 1997; Papaioannou et al., 2001; Head et al., 2002; Skoumalova et al., 2003a; Rofina et al., 2004; Rofina et al., 2006). Further, endogenous antioxidant activity decreases with age in dogs (Kiatipattanasakul et al., 1997; Opii et al., 2008; Opii et al., In press). These age-effects suggest a link between oxidative damage and cognitive decline. Oxidative damage may be a consequence of mitochondrial dysfunction and the production of damaging free radicals (Shigenaga, 1994; Zhu et al., 2007). As a test of this hypothesis we treated aged dogs for over 2 years with a diet enriched in a broad spectrum of antioxidants and mitochondrial cofactors in the dose range typically used in human clinical trials. One of the advantages to using canines in diet studies is that absorption of nutrients from food, including antioxidants and mitochondrial co-factors, is similar to that in humans (Roudebush et al., 2005).

We have previously reported that this antioxidant enriched diet significantly improves cognition in aging dogs (Cotman et al., 2002b; Milgram et al., 2002b; Milgram et al., 2002a; Tapp et al., 2003a; Milgram et al., 2005; Siwak et al., 2005). In the same study, we also treated a group of animals with behavioral enrichment (physical exercise, social enrichment and cognitive training) and observed similar improvements in cognition. Interestingly, the combination of the treatments resulted in larger learning and memory improvement than either treatment alone (Milgram et al., 2005). In the current study, we hypothesized that the antioxidant treatment improved cognitive function through increased mitochondrial function.

MATERIALS AND METHODS

Subjects

Twenty-four beagles ranging in age at the start of the study from 8.05-12.35 yrs (Mean = 10.69 yrs SE=.25, 12M/12F) were obtained from the colony at the Lovelace Respiratory Research Institute. The typical median lifespan for animals in this colony is 13.2 years (Lowseth et al., 1990). Animals were born and maintained in the same environment and all had documented dates of birth and comprehensive medical histories. A second group of 5 young beagle dogs

(3M,2F) from the same colony were also obtained to serve as untreated controls (Mean = 4.1 yrs SE = 0.19). All studies were conducted in compliance with approved IACUC protocols, consistent with the National Research Council's Guide for the care and use of laboratory animals.

Group Assignments and Study Timeline

All dogs underwent extensive baseline cognitive testing as has been described previously (Milgram et al., 1994). Based on cognitive test scores, animals were ranked in order of cognitive ability and placed into one of four treatment groups such that each group contained animals with equivalent ranges of cognition (e.g. poor to good): C/C - control environment/control diet; E/C - enriched environment/control diet; C/A - control environment/antioxidant diet; E/A - enriched environment/antioxidant diet. One aged animal did not complete the baseline phase of the study and thus, 23 animals were treated for a period of 2.4-2.8 years. Over the duration of the study, 5 additional animals were not available or not included in the current experiments and included 1 C/C, 2 E/C, 2 C/A and 1 E/A animals. Four of these animals including 1 C/C dog (age=13.5 years), 2 E/C dogs (12.5 and 12.2 years), 1 C/A dog (13.8 years) had been euthanized prior to the end of the study for health reasons and one animal (E/A, 13.0 years) was used to pilot the mitochondrial assays (data not included). Thus at the end of the study and for the mitochondrial studies described in the current experiments there were a total of 18 animals available: N=5 (Mean age 14.05 years, SD=0.79) C/C animals, n=4 (Mean age 13.58 years, SD = 0.60) in the E/C group, N=4 (Mean age 12.75, SD=1.37) in the C/A group and N=5 (Mean age = 12.61 SD=1.72) in the E/A group. Age differences at the end of the study were not statistically significantly different ($F(3,17)=1.46$ $p=0.27$).

Environmental Enrichment

The environmental enrichment protocol consisted of housing animals in pairs (social enrichment), providing 2-20 minute outdoor walks per week (physical exercise) and continuous cognitive testing (cognitive enrichment). The cognitive enrichment consisted of a landmark discrimination task (Milgram et al., 2002b), an oddity discrimination task (Cotman et al., 2002b), and a size discrimination learning and reversal task (Head et al., 1998; Milgram et al., 2005).

Diet

The two foods were formulated to meet the nutrient profile for the American Association of Feed Control Officials recommendations for adult dogs (AAFCO 1999). Control and test diets were identical in composition, other than inclusion of a broad-based antioxidant and mitochondrial cofactor supplementation to the test diet. The control and enriched foods had the following differences in formulation on an as fed basis respectively: dl-alpha-tocopherol acetate, (120 ppm vs 1050 ppm), l-carnitine (< 20 ppm vs 260 ppm), dl-alpha-lipoic acid (<20 ppm vs 128 ppm), ascorbic acid as Stay-C (< 30 ppm vs 80 ppm), and 1% inclusions of each of the following (1 to 1 exchange for corn): spinach flakes, tomato pomace, grape pomace, carrot granules and citrus pulp.

Cortical Biopsy Procedure

Prior to euthanasia, 18aged dogs and all 5 young dogs underwent a cortical biopsy procedure. Animals were sedated with 0.2 mg/kg acepromazine given subcutaneously 20 min before induction of general anesthesia. General anesthesia was induced with 5% isoflurane by inhalation. An endotracheal tube was then placed in each dog and surgical level of anesthesia maintained with 2-3% isoflurane in oxygen. An incision was made just to the right of the sagittal crest over the parietal and frontal bones to the external frontal crest and then down on each end to form a flap. The muscles were then dissected away from the bones covering right parietal

cortex region. A bone saw was used to cut a small 3 by 3 cm piece of the parietal bone and this was removed to expose the underlying meninges and brain. Cautery was used to cut the meninges and to provide hemostasis in the sample site. A sterile metal "scoop" was used to dissect out a 2 × 1× 1 cm block of parietal cortex tissue. Dogs were maintained under anesthesia until the final euthanasia procedure.

Mitochondrial Isolation

Isolated brain mitochondria were prepared as previously described with slight modifications (Sullivan et al., 1999; Sullivan et al., 2003). The fresh biopsied tissue was dissected and minced in ice-cold homogenization buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA, 20 mM HEPES, pH 7.2). All subsequent steps were conducted at 4°C. The tissue was rinsed with 10 ml homogenization buffer to remove residual blood and processed (8 strokes) using a hand-held tissue homogenizer (Thomas Scientific). The resulting homogenate was centrifuged for 5 min at 1300 x g, the supernatant was removed and centrifuged at 13,000 x g for 10 min and the pellet resuspended in 1 ml of isolation buffer. Synaptic mitochondria were then released by nitrogen cell disruption (1000 PSI, 10 min) as previously described (Sullivan et al., 2003). Following nitrogen decompression the sample was centrifuged at 13,000 x g and the pellet resuspended in ETGA-free isolation buffer and centrifuged at 10,000 x g and the final pellet collected for all subsequent experiments. Mitochondrial protein concentration was determined using a Pierce BCA kit. Purity of the preparations was assessed by Western blot analysis (data not shown) of the mitochondrial protein cytochrome oxidase subunit IV (Molecular Probes) and the synaptic protein postsynaptic density (PSD) 95 (Transduction Laboratories).

Mitochondrial Respiration

Respiration activity of the isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK), and approximately 100-150 μg of isolated striatal or nigral mitochondrial protein were suspended and constantly stirred in a sealed and thermostatically controlled chamber at 37°C in respiration buffer (215 mmol/L mannitol, 75 mmol/L sucrose, 0.1% BSA, 20 mmol/L HEPES, 2 mmol/L MgCl, 2.5 mmol/L KH_2PO_4 at pH 7.2). The rate of oxygen consumption was determined based on the response slope of the isolated mitochondria to oxidative substrates as previously described (Sullivan et al., 2000; Sullivan et al., 2003). State II respiration was initiated by the addition of 5 mmol/L pyruvate and 2.5 mmol/L malate. State III respiration was initiated by the addition of 150 μmol/L ADP followed by the addition of oligomycin (1 μg/mL) to inhibit the ATP synthase and induce state IV respiration. The mitochondrial uncoupler carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (1 μmol/L) was added to the chamber to induce maximum NADH-linked state V respiration (complex I driven). Rotenone (1 μmol/L) was added to the chamber to inhibit complex I of the electron transport system. Then, FADH maximum respiration (complex II driven) was assessed by the addition of succinate (10 mmol/L) to the chamber. Three runs were performed for each sample and the average was used for statistical analysis. All rates are expressed as nmoles of O_2 consumed per minute per milligram of protein (O_2/min/mg).

Mitochondrial ROS production

Mitochondrial ROS production was measured under state II conditions using the indicator dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) as previously described (Sullivan et al., 1999; Sullivan et al., 2003). Briefly, 100-150 μg of isolated mitochondrial protein was incubated in a total volume of 200 μl respiration buffer including 5 mmol/L pyruvate and 2.5 mmol/L malate as substrate at 37°C for 15 min in the presence of 10 μM H_2DCFDA, which was made fresh before each use. Negative controls in which mitochondrial samples were omitted were used for non-specific sources of ROS were background subtracted

from all experimental samples. The relative amount of mitochondrial H_2O_2 and free radical production was measured using a CytoFluor 4000 fluorometric plate reader (excitation 490 nm, emission 526 nm). Wells containing known amounts of H_2O_2 were used as positive controls and for linear calibration of each plate.

Data Analysis

To measure age effects on mitochondrial function, untreated young (n=5) and aged (n=5) were compared. A repeated measures analysis of variance was used to detect age effects in ROS production and mitochondrial respiration (ETC). A repeated measures analysis of variance (ROS-5,10,15 min) with two independent factors - diet (aox, ctl) and behavioral enrichment (enr, ctl) was used to test for treatment effects on ROS production and on ETC components. All analyses were conducted using SPSS 14.0 for Windows and an alpha level of 0.05.

RESULTS

Mitochondrial ROS production increased as a function of time (5,10,15 min) for both young and aged animals ($F(2,16)=90.9$ $p<.0005$)(Figure 1A). Overall, mitochondria isolated from aged canine brain produced higher levels of ROS relative to young animals ($F(1,8)=17.6$ $p=0.003$)(Figure 1A). Note that young animals show minimal individual variability and error bars in the graph cannot be seen with the scaling of the y-axis. Further, aged animals produced higher levels of mitochondrial ROS at a more rapid rate than young animals ($F(2,16)=14.9$ $p<.0005$) (Figure 1A) and thus the slopes of the two curves are significantly different (Young slope = 31 OD/min and aged = 55 OD/min when fitted to a straight line). To detect age effects a repeated measures ANOVA was used to compare mitochondrial respiration rates for components of the ETC between young (n=5) and aged untreated control animals (n=5). There was a significant main effect of age ($F(1,8)=37.3$ $p<.001$) and an age by ETC component interaction ($F(4,32)=37.2$ $p<.001$)(Figure 1B). NADH-linked, complex I-driven respiration rate was higher in young relative to old animals ($t(8)=6.1$ $p<.0005$)(Figure 1B). No significant differences were detected in complex-II driven respiration as a function of age.

To test the hypothesis that mitochondrial ROS production decreases in aged treated animals we used a repeated measures 2-way ANOVA. As with the age analysis, a significant rise in mitochondrial ROS was observed across time ($F(2,28)=99.67$ $p<.0001$)(Figure 2A). A significant main effect of diet ($F(1,14)=5.0$ $p=0.042$) but not behavioral enrichment ($F(1,14)=0.17$ $p=0.69$) nor a two way interaction between the treatment conditions was observed in terms of mitochondrial ROS production ($F(1,14)=1.35$ $p=0.265$) (Figure 2A). Further, a significant interaction between the rate of mitochondrial ROS production and diet treatment indicates that those fed an antioxidant diet produce less ROS ($F(2,28)=5.6$ $p=0.009$) (Figure 2B). A repeated measures ANOVA was used to compare different treatment conditions within the aged group on measures of mitochondrial bioenergetics. A significant main effect of diet ($F(1,14)=4.9$ $p=0.044$) but not of behavioral enrichment ($F(1,14)=0.16$ $p=0.695$) was observed (Figure 2C). However, the antioxidant diet selectively increased NADH-linked, complex I-driven respiration ($F(4,56)=5.47$ $p=0.001$)(Figure 2C). In comparison, aged treated dogs showed an increase in the maximum NADH-linked respiration, assessed using the uncoupler FCCP, relative to untreated aged animals but was still lower than younger animals (Figure 2D) ($F(2,22)=41.9$ $p<.0005$).

DISCUSSSION

The current study tested the hypothesis that mitochondrial dysfunction increases with age and can be reduced with appropriate antioxidant dietary intervention using a dog model of human brain aging. The function of isolated mitochondria from 5 young and 20 aged beagles that were provided with either or both dietary and behavioral modifications for a period of over 2.5 years

was examined. Our results show an age-dependent decrease in mitochondrial function and an increase in ROS production from aged dogs that could be reduced selectively with an antioxidant enriched diet but not through behavioral enrichment.

In dog brain, the accumulation of carbonyl groups, which is a measure of oxidative damage to proteins, increases with age (Head et al., 2002; Skoumalova et al., 2003b) and is associated with reduced endogenous antioxidant enzyme activity or protein levels such as in glutamine synthetase and superoxide dismutase (SOD) (Kiatipattanasakul et al., 1997; Head et al., 2002; Hwang et al., 2008; Opii et al., 2008). In several studies, a relation between age and increased oxidative damage has been inferred by measuring the amount of end products of lipid peroxidation (oxidative damage to lipids) including the extent of 4-hydroxynonenal (4HNE) (Papaioannou, 2001; Rofina et al., 2004; Rofina et al., 2006; Hwang et al., 2008), lipofuscin (LF) (Rofina et al., 2006), lipofuscin-like pigments (LFP) (Papaioannou, 2001; Rofina et al., 2004), or malondialdehyde (Head et al., 2002). Last, evidence of increased oxidative damage to DNA or RNA (8OHdG) in aged dog brain has been reported (Rofina et al., 2006). The results of the current study suggest that mitochondrial ROS production may be a contributor to increased oxidative damage in the dog brain. More specifically, our studies with isolated mitochondria now also show ROS production is significantly higher in aged animals relative to young controls suggesting impaired mitochondrial function.

Mitochondrial dysfunction can be partially reversed by providing aged animals with a diet rich in antioxidants and mitochondrial co-factors (Liu et al., 2002b; Liu and Ames, 2005) thereby reducing cognitive dysfunction (Cotman et al., 2002a). Fruits and vegetables are rich in flavonoids and carotenoids and other antioxidants (Joseph et al., 1998; Bickford et al., 2000a). Vitamin E is lipid soluble and acts to protect cell membranes from oxidative damage and vitamin C is essential in maintaining oxidative protection for the soluble phase of cells as well as preventing vitamin E from propagating free radical production (Butterfield et al., 2002). Alpha-lipoic acid is a cofactor for the mitochondrial respiratory chain enzymes, pyruvate and alpha-ketoglutarate dehydrogenases, as well as an antioxidant capable of redox recycling other antioxidants and raising intracellular glutathione levels (Packer et al., 1997a; Pocernich and Butterfield, 2003). In addition, l-carnitine is a precursor to acetyl-l-carnitine and is involved in mitochondrial lipid metabolism and maintaining efficient function (Rebouche, 1992; Calabrese et al., 2006).

As we hypothesized, aged animals treated with the antioxidant diet showed significant decreases in ROS levels compared to the control group implying the involvement of oxidative stress in mitochondrial dysfunction. It is also possible that age-related ROS increases that are subsequently decreased with treatment are generated by mitochondria themselves and implies that ROS are a byproduct of mitochondrial failure. In contrast, animals receiving the behavioral enrichment alone, levels of ROS production were comparable to untreated aged animals. One interpretation of these results is that cognition benefited from treatment independently of improved mitochondrial function. However, it is also possible, and likely, that multiple different pathways could be modified in dogs provided with either or both behavioral enrichment and the antioxidant diet leading to improved cognition. Thus, an alternative explanation is that reduced mitochondrial ROS production that is selective to animals fed the anti-oxidant enriched diet may also lead to cognitive improvements.

It is proposed the mitochondrial enzymes are particularly susceptible to oxidative damage and lose their enzymatic activities with age that may be countered by high doses of anti-oxidants and/or substrates and co-factors. Complex I or NADH-ubiquinone oxidoreductase of the ETC is the major acceptor of electrons from NADH and transfers them to coenzyme Q (ubiquinone), accompanied by pumping of protons from matrix into the inter-membrane space. Components of Complex I are also a major site for free-radical generation within the ETC. NADH driven

respiration was found to be significantly higher in young compared to old animals. In the current study only NADH-linked, complex-I driven respiration was demonstrated to be reduced in the aged animals indicated by the absence of any respiratory decline after circumventing complex I and driving respiration via complex II of the ETC. However the current paradigm cannot distinguish between specific complex I enzyme dysfunction or damage upstream in components of the Kreb cycle. Regardless, NADH-linked respiration was improved in aged animals provided with a diet rich in a broad spectrum of cellular antioxidants as well as mitochondrial co-factors. Thus, the addition of mitochondrial co-factors either alone or in combination with the cellular antioxidants may have resulted in improved mitochondrial function by increasing NADH-linked respiration. However, it is not clear in the present study if the increase in mitochondrial NADH-linked, complex I driven respiration itself was the cause or effect of the decrease in ROS production.

Significant improvements in mitochondrial function in aged treated animals may be due to the addition of mitochondrial co-factors into the antioxidant diet. Targeting mitochondria specifically may be another way in which to reduce the production of reactive oxygen species and consequently, reduce oxidative damage. Thus, antioxidant strategies reported over the last several years also have been directed toward improving mitochondrial function (Liu et al., 2002c; Liu et al., 2002d; Liu et al., 2002a). While antioxidants such as vitamin E, the spin-trapping agent alpha-phenyl-N-tert-butylnitrone, and blueberry extracts (containing powerful antioxidants in polyphenolics) all have positive effects on behavior and brain pathology, these compounds are generally not specifically directed at the mitochondria but to cytoplasmic targets. Mitochondrial cofactors, particularly alpha-lipoic acid, carnitine and coenzyme Q, all have been shown to be effective in targeting mitochondria and reducing ROS production both in tissue culture and rodent models (Packer et al., 1997b; Hagen, 1998; Hagen et al., 1999; McGahon, 1999; Liu et al., 2002c; Liu et al., 2002d; Liu et al., 2002a). However, rodents do not naturally develop some forms of neuropathology observed in human brain aging, suggesting the need for extension (Kawarabayashi, 2001; Oddo et al., 2003) higher mammalian model systems (Price, 1985; Head, 2001) such as the canine (Cummings et al., 1996; Head, 2001).

When the results of the current study are taken in combination with our previous reports of improved cognition in aged antioxidant-treated dogs, we may now suggest that one of the neurobiological mechanisms underlying improved cognition, in antioxidant and mitochondrial co-factor fed animals is through improved mitochondrial function. These mechanisms may converge to improve neuronal, cortical and cognitive function.

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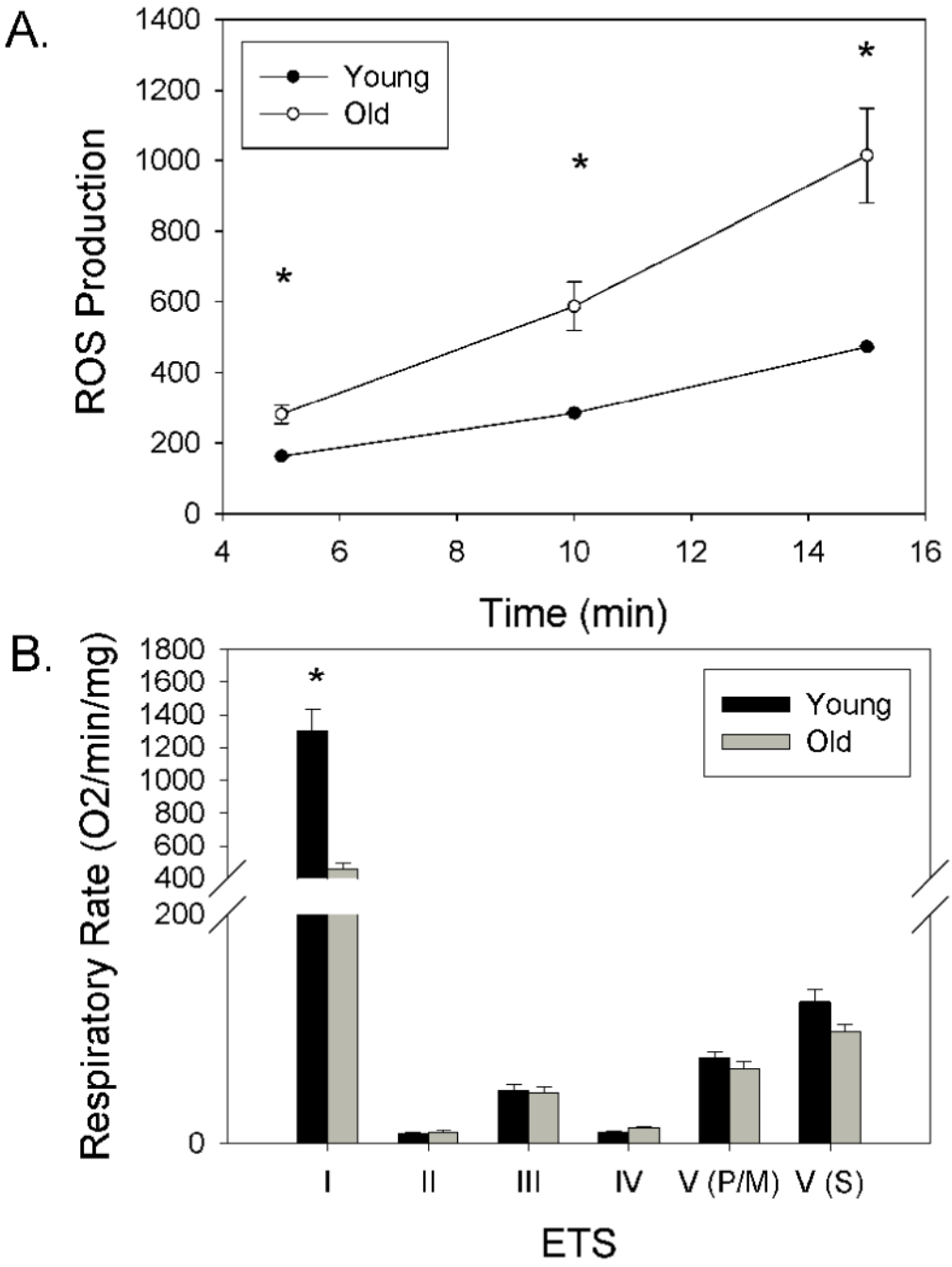

Figure 1.

Mitochondrial dysfunction occurs with age in dogs. A. Reactive oxygen species production was higher overall in aged dogs and was further increased over time. B. Age effects were seen selectively as a decrease in NADH-linked, complex I-driven respiration rate but not in complex II, III, IV, or in complex V in the presence of the mitochondrial uncoupler FCCP and pyruvate/malate (V(P/M)) or following the addition of rotenone and succinate treatment (V(S)). Bars represent group means, error bars represent standard error of the mean. * $p<.05$ between young and old dogs at each time point measured. A. Note that young animals showed minimal individual variability and error bars in the graph cannot be seen with the scaling of the y-axis

but the standard errors were as follows: ROS at 5 min = 0.87, at 10 min = 1.7 and at 15 min = 2.73.

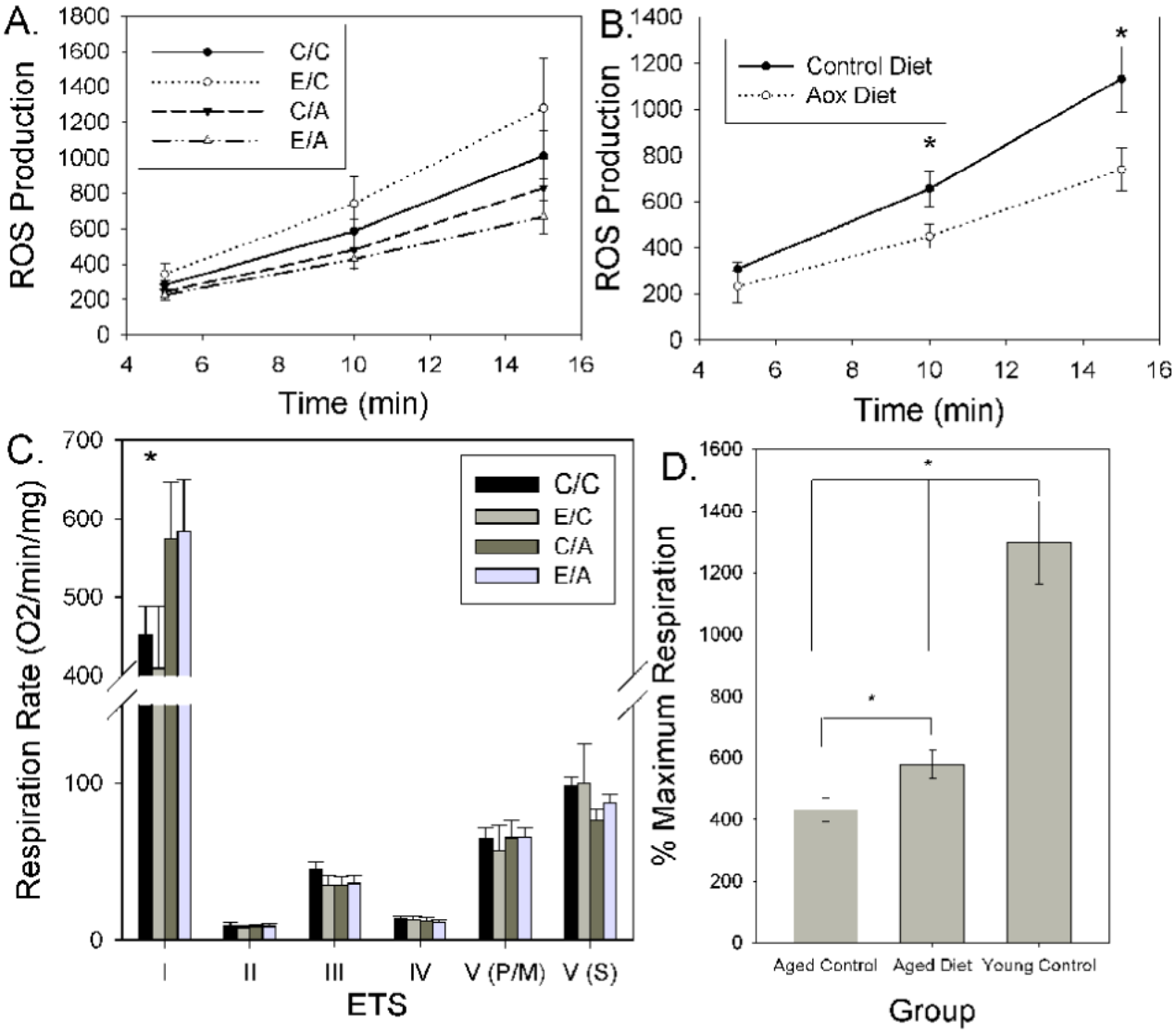

Figure 2.

Mitochondrial dysfunction in aged dogs can be reduced with an antioxidant enriched diet. A. ROS production by mitochondria of aged dogs was reduced with treatment with either an antioxidant diet alone or with a combination of an antioxidant diet with behavioral enrichment. B. Reduced ROS production was selectively due to aged animals fed an antioxidant enriched diet. C. Respiration rates were significantly improved in the two treatment groups that received the antioxidant enriched diet in contrast to little change in complex II, III, IV and complex V respiration in the presence of the mitochondrial uncoupler FCCP and pyruvate/malate (V(P/M)) or following the addition of rotenone and succinate treatment (V(S)). * $p<.05$. D. For comparison, the % maximum respiration (state V/state IV) associated with NADH substrates shows that treated aged animals were significantly improved relative to untreated controls ($p<.05$) but not to the extent of untreated young dog levels. Bars represent group means, error bars represent standard error of the mean. In D, lines represent group differences. C/C - control environment/control diet, E/C - enriched environment/control diet; C/A - control environment/antioxidant diet; E/A - enriched environment/antioxidant diet.