ELSEVIER

Neurochemistry International 40 (2002) 123–130

NEUROCHEMISTRY International

www.elsevier.com/locate/neuint

Dietary modulation of age-related changes in cerebral pro-oxidant status

Stephen C. Bondy [a,*], Y. Ellen Yang [a], Thomas J. Walsh [a,1], Yuan-Wen Gie [b], Debomoy K. Lahiri [b]

[a] *Center for Occupational and Environmental Health, Department of Community and Environmental Medicine, University of California, Irvine, Irvine, CA 92697-1820, USA*

[b] *Institute of Psychiatric Research, Indiana University School of Medicine, 791 Union Drive, Indianapolis, IN 42602-4887, USA*

Received 11 April 2001; received in revised form 28 June 2001; accepted 10 July 2001

Abstract

It has been proposed that senescence may be associated with changes associated with oxidative damage to macromolecules. Levels of cerebellar nitric oxide synthase (NOS) and rates of generation of cortical reactive oxygen species (ROS), have been determined in mice of various ages. Both of these parameters were significantly reduced in mice aged 9 months relative to 3-month-old mice. In order to determine whether dietary manipulation can modulate these changes, the effect of exposure of mice to differing diets incorporating various antioxidants, was examined. These diets were given to 3-month-old mice for a total period of 6 further months. The presence of melatonin (40 ppm) in the basal diet restored both NOS and ROS levels to the corresponding values found in the younger (3-month-old) group of mice while lipoic acid (1650 ppm) also restored levels of NOS to those found in 3-month-old animals. Addition of coenzyme Q (ubiquinone), 200 ppm or α-tocopherol (1000 ppm) to the basal diet had no effect on either NOS levels or ROS generation. These data suggest that dietary supplementation may aid in delaying onset of metabolic changes characteristic of the older brain. In behavioral testing, older (9-month-old) animals exhibited reduced motor activity and diminished recall ability on the second day of exposure to the test paradigm. While no diet altered motor activity or improved recall of older animals, lipoic acid or tocopherol treatment adversely affected place recall familiarity.

Keywords: Diet; Aging brain; Melatonin; Lipoic acid; Tocopherol; Coenzyme Q; Ubiquinone

1. Introduction

There is considerable evidence that age-related changes in cerebral function are in part attributable to an imbalance between pro-oxidant and antioxidant factors (LeBel and Bondy, 1992). The potential relevance of such changes to cerebral functioning is illustrated by the report that a free-radical spin trapping agent, phenbutyl nitrone, may reverse age-related loss of memory skills while attenuating indices of oxidative damage to proteins (Butterfield et al., 1997; Sastre et al., 2000). Delay of age-associated behavioral deficits have been reported by placing animals on long term diets supplemented with additions such as α-tocopherol as well as strawberry or spinach extracts (Joseph et al., 1993).

In the current report, age-related changes in two indices of oxidant activity have been studied. The effects of addition of four selected compounds, namely α-tocopherol, lipoic acid, melatonin and coenzyme Q, to the diet over an extended time have been investigated. The potential value of all these compounds in the mitigation of age-related neural changes has been documented (Ren et al., 1994; Reiter, 1995; Ma et al., 1996; Beal et al., 1998; Hagen et al., 1999). The intent of the present study was to identify the most promising dietary addition capable of attenuating age-related changes, prior to selection for a more detailed study. Behavioral consequences of such dietary treatments have also been examined. The ages that were compared

* Corresponding author. Tel.: +1-949-8248642; fax: +1-949-8242070.

E-mail address: scbondy@uci.edu (S.C. Bondy).

[1] Deceased 12 May, 2000.

0197-0186/01/$ - see front matter
PII: S0197-0186(01)00084-5

were intended to reflect (middle adulthood and) presenescent periods rather than stages of early development or extreme age.

2. Methods

2.1. Animals

Male B/6C3F1 mice, a hybrid between C57BL/6 and C3H from Harlan Labs, Indianapolis, IN, were housed four per cage and were maintained on a 12 h light/dark cycle in a temperature controlled (20 ± 1 °C) room. Specified diets and water were provided ad lib. This strain of mouse lives to around 22–24 months of age.

2.2. Diets

The minimal basal diet (No. 101101, Dyets Inc., Bethlehem, PA) consisted of 50% sucrose and 26% casein (w/w) as well as a minimal salt and vitamin mix including 0.011% (0.23 mM) α-tocopherol acetate. This was supplemented with either 0.1% (1.9 mM) DL-α-tocopherol succinate, 0.02% (0.23 mM) Coenzyme Q_{10} (ubiquinone), 0.165% (8.0 mM) DL-thioctic acid (lipoic acid) or 0.004% (0.17 mM) melatonin. The rationale behind these selected concentrations is based on earlier reports of neuroprotective properties of these agents following dietary administration (Bondy et al., 1996; Packer et al., 1997; Pierpaoli and Regelson, 1997). These diets were fed to mice at 3 (or 9) months age for a further 6 months.

2.3. Preparation of the cerebrocortical synaptosomal/mitochondrial fraction

Mice were decapitated, the brains were excised quickly on ice, and the entire cerebral cortex was dissected out. Tissue was weighed and homogenized in 10 vol of 0.32 M sucrose and centrifuged at $1800 \times g$ for 10 min. The resulting supernatant fraction was then centrifuged at $31\,500 \times g$ for another 10 min to yield the crude cerebral synaptosomal pellet (P2) and the supernatant S2. The P2 pellet was taken up in HEPES buffer to a concentration of 0.01 *g* Eq/ml. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10, at pH 7.4.

2.4. Assay for reactive oxygen species formation

ROS were assayed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is de-esterified within cells to the ionized free acid, dichlorofluorescin, DCFH. This is trapped within cells and thus accumulated. DCFH can be oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) by reactive oxygen (Bass et al., 1983). The utility of this probe in isolated subcellular cerebral systems has been documented (LeBel and Bondy, 1992). P2 suspensions which contain both mitochondria and synpatosomes are especially suitable for this assay since mitochondria are the main source of intracellular ROS generation. P2 fractions were diluted in 19 vol of HEPES buffer. The diluted fractions were then incubated with 5 μM DCFH-DA (added from a stock solution of 1.25 mM in ethanol) at 37 °C for a 15 min preincubation. After this loading with DCFH-DA, the fractions were incubated for a further 60 min. The production of ROS by the P2 fraction is linear over this period (LeBel and Bondy, 1990). Fluorescence was monitored on a Perkin–Elmer spectrofluorometer, with excitation wavelength at 488 nm and emission wavelength of 525 nm. The formation of reactive oxygen species was quantitated using a DCF standard curve and results were expressed as nmol DCF formed/mg protein/hour. The rationale for utilizing the P2 fraction for this study, is based on earlier work showing that ROS formation by this fraction was consistent between animals, responsive to various treatment paradigms, and has been characterized in detail (LeBel et al., 1992). The fraction can be rapidly prepared and the yield from animals of various ages, is more consistent than that of highly purified subfractions. Furthermore the use of synaptosomes permits the study of mitochondria within their natural milieu and possessing an unimpaired membrane potential (Bondy and McKee, 1991).

2.5. Nitric oxide synthase

Frozen cerebella were thawed and suspended in 10 mM Tris–HCl, pH 7.4, at 20-ml buffer per gram of tissue. The tissue was homogenized using a Polytron homogenizer (Brinkman, NY) and centrifuged at $11\,000 \times g$ for 10 min at 4 °C. The supernatant was collected and assayed for nitric oxide synthase (NOS). 2 μCi of [L-^{3}H]arginine (Amersham, NJ, specific activity 41 Ci/mM) was added to 10 mM Tris–HCl, pH 7.4, 1 mM NADPH, 0.6 mM $CaCl_2$ and 15 μg of tissue extracts from each sample of the treatment group in a total volume of 50 μl. After incubation for 1 h at 37 °C, the reaction was stopped with 400 μl buffer containing EDTA. 100 μl of an anion resin (Stratagene Inc., La Jolla, CA) equilibrated at pH 5.5, when it binds positively charged arginine and not neutral citrulline, was added to the samples, and then they were loaded to spin cups and centrifuged for 1 min at full speed in a microcentrifuge. The NOS activity was then determined by quantitating the radioactivity in the eluate. Reaction(s) mixtures containing 1 mM *N*-nitro L-arginine or mixtures devoid of NADPH and calcium were used as blanks. The ratio of citrulline formed to unconverted arginine remaining denotes the percent conversion and

thus reflects NOS activity. The cerebellum was chosen for this assay as this region is especially high in NOS. In addition cerebellar NOS contains a relatively high proportion of the neuronal rather than the endothelial or calcium-independent inducible glial enzyme (Arima et al., 1996).

2.6. Glutathione concentration

Glutathione (GSH) levels were determined within S2 fractions using a modification of the method of Shrieve et al. (1988). The principle behind the assay is that monochlorobimane (mBCl), a nonfluorescent compound, reacts with glutathione to form a fluorescent adduct. Protein thiols are not assayed by this procedure. mBCl was dissolved in ethanol to a concentration of 5 mM and stored at -10 °C in the dark. mBCl was added to 2 ml of a S2 suspension to a final concentration of 10 μM, after which the suspension was incubated for 15 min at 37 °C. The fluorescence of the supernatant was spectrofluorometrically determined at an excitation wavelength of 395 nm and an emission wavelength of 470 nm. The tissue GSH concentration was calculated using a GSH standard curve.

2.7. Measurement of locomotor activity

At the end of the dietary exposure, mice were tested for open field locomotor activity by using an Digiscan Animal Activity Monitor (Accuscan Inc., Columbus, OH). The testing apparatus consisted of a plastic cage ($40 \times 40 \times 30$ cm) with 16 photocell detectors along two perpendicular sides of the box and 16 light-emitting diodes (LEDs) along the other two sides. Total distance traveled was measured rather than horizontal activity as this is a more accurate indicator of ambulatory activity. Advantages of the optical beam technique for measuring activity include: (1) the test subjects are unaware of the invisible IR light beams so that behavior is unaffected by the monitoring instrument; (2) the Digiscan is insensitive to activity outside the narrow range of optical beams, unlike proximity or vibration type sensors; and (3) the output, collected and printed with a Digiscan Analyzer, is digital in nature and very repeatable. The apparatus was cleansed with 1 mM acetic acid to remove residual odors.

2.8. Protein determination

Protein was quantitated using the method of Bradford (1976).

2.9. Statistical analyses

Differences between groups were assessed by one-way analysis of variance followed by Fisher's least significant difference test. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution.

2.10. Materials

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR), and 2′, 7′-dichlorofluorescein (DCF) required for calibration, was obtained from Polysciences, Inc. (Warrington, PA). All other chemicals were obtained from Sigma (St. Louis, MO).

3. Results

3.1. Biochemical studies

Major age-related decreases in levels of both cerebellar nitric oxide synthase and the rate of generation of reactive oxygen species were apparent within cerebral cortex. However, following 6 months treatment with dietary melatonin, the levels of NOS and ROS formation were restored to those found in younger (3-month-old) animals (Figs. 1 and 2). A parallel prolonged exposure to either coenzyme Q or α-tocopherol succinate did not modulate the age-related decline of ROS and NOS. Lipoic acid also reversed the reduction of NOS found in animals receiving basal diet but had no effect on a similar decline of ROS. Cortical levels of glutathione were not significantly different between younger (3-month-old) and older mice (9-month-old) and were unchanged by any of the diets tested (data not shown).

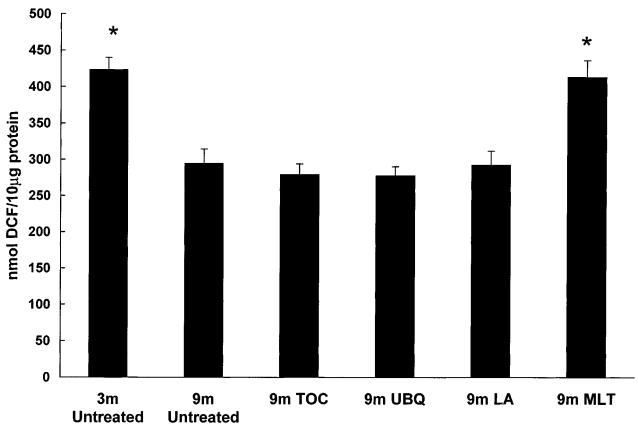

Fig. 1. Cerebral cortical rates of generation of reactive oxygen species in mice subjected to various diets for 6 months. Younger (3-month-old = 3m) mice were maintained on basal diet while four groups of older (9-month-old = 9m) animals received supplementation as described in the Experimental section. * Nine-month-old value differs significantly from corresponding value of untreated 3-month-old mice. TOC = α-tocopherol, UBQ = ubiquinone, LA = lipoic acid, MLT = melatonin.

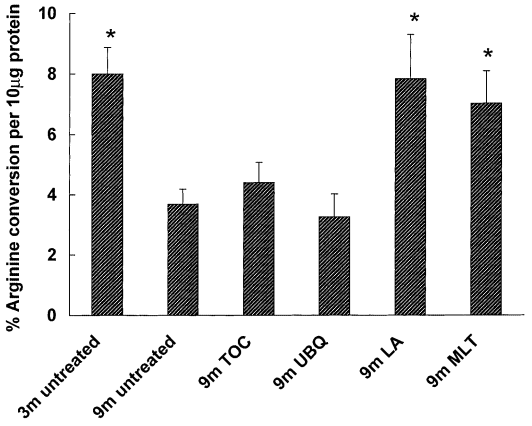

Fig. 2. Cerebellar nitric oxide synth[et]ase in mice subjected to various diets. Younger (3-month-old = 3m) mice were maintained on basal diet while four groups of older (9-month-old = 9m) animals received supplementation as described in the Experimental section. *Nine-month-old value differs significantly from corresponding value of untreated 3-month-old mice. TOC = α-tocopherol, UBQ = ubiquinone, LA = lipoic acid, MLT = melatonin.

In view of the effect of dietary melatonin in preventing the age related loss of NOS and ROS, the effect of a range of concentrations of melatonin upon ROS generation in an isolated synaptosomal P2 preparation, was examined (Fig. 3). At lower concentrations there was a minor antioxidant effect which is in accord with several prior reports of melatonin possessing antioxidant properties (see discussion). However, levels of melatonin above 5 μM, effected a pronounced dose-related increase in rates of ROS production. This latter effect was unexpected, and the possibility was considered that the pro-oxidant effect of melatonin might be due to binding of trace amounts of iron. Such incomplete chelation of iron can potentiate its ROS-promoting properties (Campbell and Bondy, 2001). The enhanced ROS generation observed at a melatonin concentration of 20 μM was not diminished in the presence of 20 μM deferoxamine (Fig. 4), indicating that this stimulation was intrinsic and not attributable to the formation of a pro-oxidant chelate between melatonin and traces of iron. This was supported by the finding that the simultaneous presence of melatonin and iron in the incubation mixture, showed essentially additive effects rather than a more complex interaction.

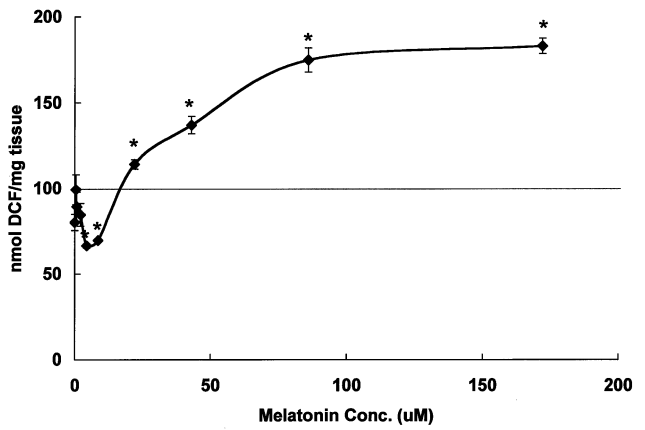

Fig. 3. Rates of generation of reactive oxygen species in an isolated cerebrocortical P2 fraction isolated from 3-month-old mice. Incubation was in the presence of various concentrations of melatonin. Value represent mean ± standard error (S.E.) from eight individual samples. *$P < 0.05$ that value differs from corresponding level in absence of added melatonin.

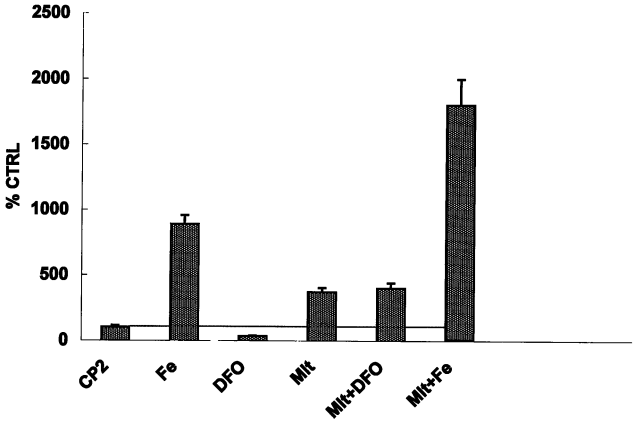

Fig. 4. Rates of generation of reactive oxygen species in an isolated cerebrocortical P2 fraction exposed to melatonin (MEL, 170 μM), deferoxamine (DFO, 10 μM) and $FeSO_4$ (20 μM). Values represent mean ± S.E. of three determinations.

3.2. Behavioral studies

In behavioral tests, following a 1 min adaptation period, locomotor activity of all groups was tested in three successive 10-min tests. These were repeated on the next day, for a total of six trials (Fig. 5). On the first day, activity declined with each further test. How-

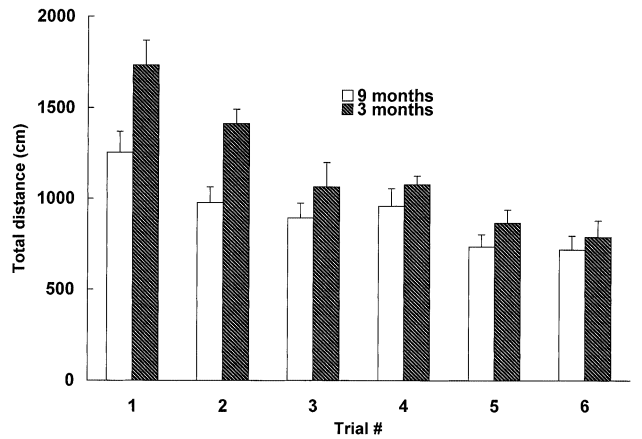

Fig. 5. Locomotor activity of 3- and 9-month-old mice receiving basal diet. Total distance traveled was quantitated over three successive 10 min intervals repeated on two consecutive days. Results are expressed as means ± S.E. from data from 7 animals.

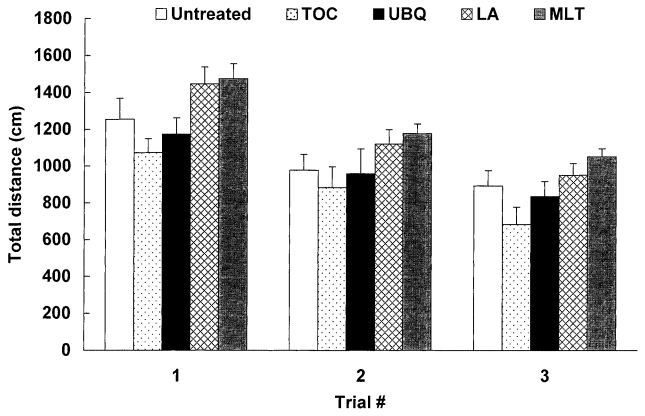

Fig. 6. Locomotor activity of groups of 9-month-old mice receiving differing diets for the preceding 6 months. Total distance traveled was quantitated over three successive 10 min intervals. TOC = α-tocopherol, UBQ = ubiquinone, LA = lipoic acid, MLT = melatonin. Results are expressed as means ± S.E. from data from 7 animals.

ever, younger animals (3-month-old) were consistently more active. On the second day of testing, all groups showed further reduced exploratory locomotion but this trend was less pronounced in older (9-month-old) animals. This suggested an age-related diminution of retention of the experience of the prior day's testing. On the first day of testing, the extent of locomotor activity in older animals (9-month-old) was not significantly altered by any of the four dietary additives tested (Fig. 6). On the second day of testing of older animals experiential recall was retained in animals receiving either coenzyme Q or melatonin but was lost in mice whose diets were supplemented with α-tocopherol or lipoic acid (Fig. 7).

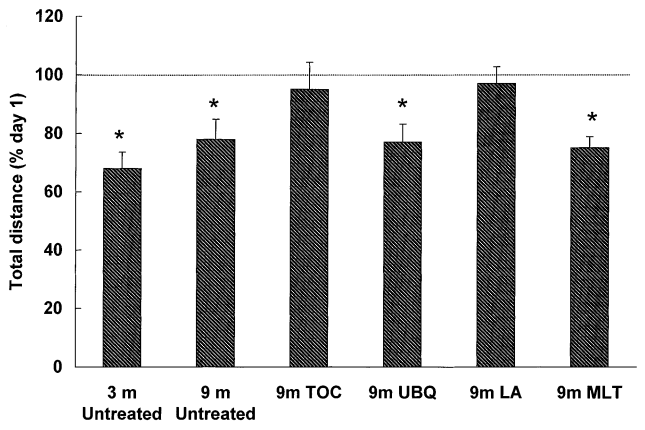

Fig. 7. Locomotor activity of 3- and 9-month-old mice receiving varying diets, on the second day of locomotor testing. Data are expressed as% of total distance traveled (± S.E.) on the previous day. Results were derived from 7 animals. * Value differs significantly from corresponding value for mice tested on day 1. TOC = α-tocopherol, UBQ = ubiquinone, LA = lipoic acid, MLT = melatonin.

4. Discussion

The current work was designed to avoid age-related extremes but rather to investigate the changes associated with transition from full maturity to early aging. The detection of subtle changes preceding major age-related decline in function is less likely to be confounded by epiphenomena than is the study of the multiplicity of changes seen in truly senescent animals. The avoidance of extremely young subjects, made the P2 fractions compared more consistent, since incomplete myelination could alter the composition of this fraction. Despite the relatively short age span covered, major reductions in both NOS levels and ROS production were evident with the transition from maturity to a period immediately prior to senescence, and these were temporally associated with a decline in locomotor activity and retention of experience. Due to the low level of NOS in cortex, this enzyme was assayed in the cerebellum. Since ROS were measured in cortex, these two parameters had to be measured in differing brain regions. There are other reports of age-related declines in both cerebral NOS and ROS (Mollace et al., 1995; LeBel and Bondy, 1991; Yamada et al., 1996). However, increased levels of oxidative damage to macromolecules in aging brain have been described (Smith et al., 1991; Sohal, 1997). These latter findings may represent a summation of events over an extended duration. In contrast, the assays used in this study reflect a snapshot of actual, ongoing dynamic pro-oxidant status in the animal at a single instant in time. Oxidative damage represents the sum of events over a longer time while the rate of generation of reactive oxygen species reflects the metabolic rate at the time of death. Age-related depression of the rate of production of short-lived oxidative species may thus be consonant with reports of accumulation of damage to macromolecules with age. Since most of the intracellular appearance of ROS has a mitochondrial origin, our findings may reflect a slowing of electron transport chain with age. There is other evidence suggesting such a reduction of respiratory intensity attributable to mitochondrial dysfunction during senescence (Feuers, 1998; Nishikawa et al., 1998; Sastre et al., 2000).

Despite reports of amelioration of cerebral aging and neurodegenerative events by all four compounds studied here, coenzyme Q and α-tocopherol have restricted access to the brain (Zhang et al., 1996; Vatassery et al., 1998) and this may limit their effectiveness. This may account for our finding that melatonin, which can readily traverse the blood–brain barrier (Oaknin-Bendahan et al., 1995), was most active in altering the biochemistry of the brain with time. Melatonin has been described as neuroprotective and this is reported to occur by antioxidant means (Melchiori et al., 1996; Miller et al., 1996; Yamamoto and Hsai-Wang, 1996;

Lin and Ho, 2000). In addition, recent cell culture studies have demonstrated that melatonin exerts a neuroprotective role, as well as accelerating neuronal differentiation (Pappola et al., 1997; Song and Lahiri, 1997). However, melatonin is a fairly poor inhibitor of lipid peroxidation, requiring higher than physiological concentrations for any free radical scavenging properties to become apparent (Longoni et al., 1998). In the current work, we have found melatonin to have pro-oxidant properties in an isolated system where ROS are directly measured, in contrast to reports of changes occurring in target lipids or proteins.

While some studies have also shown melatonin supplementation to effect inhibition of age-related behavioral changes (Oaknin-Bendahan et al., 1995; Araghi-Niknam et al., 1999), others have not found this to be the case (Kopp et al., 1999). Melatonin is an unusual neuroprotectant in that it has endocrine properties. The mechanisms underlying the biological actions of melatonin upon chronologically related changes, are thus unlikely to be by way of simple antioxidant effects. In fact melatonin may act primarily by enhancing gene expression of antioxidant enzymes (Antolin et al., 1996). In line with this concept is our finding that melatonin not only diminishes the enhanced production of cytokine mRNAs that typifies aged animals (submitted for publication) but also prevents age-related changes in complex IV of the mitochondrial respiratory chain (Sharman and Bondy, 2001). Thus the increase in ROS and NOS found in melatonin-treated animals may reflect heightened mitochondrial activity typifying the younger animal, rather than deleterious events. In this preliminary broad-based study surveying the effects of four potentially beneficial agents, the number of ages used and type of parameters investigated were necessarily limited. Having identified melatonin as the most promising of the agents tested, in future studies we also plan to utilize more sophisticated tests for cognitive ability such as the Morris water maze in order to better understand the global behavioral role of melatonin.

With all of the diets used, there was absence of improvement in behavioral indices declining with age. The behavioral test was relatively simple and more sophisticated paradigms remain to be applied. However, there was some suggestion of adverse effects of α-tocopherol and lipoic acid as these agents actually blocked what minor evidence of place familiarity was apparent in older (9-month-old) mice on the second day of testing. While there are many reports concerning the protective effect of lipoate against a range of toxicants, there can be a synergistic cytotoxic action between this agent and the pro-oxidant doxorubicin (Dovinova et al., 1999). Furthermore, lipoate may promote ROS-generating redox cycling by phenolic compounds (Stoyanovsky et al., 1995). Lipoic acid treatment has been reported to increase ambulatory activity and spatial memory in old rats (Dovinova et al., 1999). Thus the behavioral model we used, which is modified by both activity and recall in opposite directions, may not be optimal to detect ipsidirectional changes in activity and memory.

There is growing evidence concerning possibility of retarding the rate of progression of, or actually reversing, adverse age-related changes by nutritional supplementation. For example, dietary supplementation with crude blueberry extracts can improve behavioral and motor performance of aged mice (Joseph et al., 1999.). It is not clear whether the underlying commonality of such reports of amelioration, are merely general antioxidant effects or a more targeted physiological effect. In the above report, the complex blueberry preparation was marginally more effective than addition of α-tocopherol to the diet, suggesting that beneficial changes were not solely confined to altered intracellular redox.

In conclusion, this study provides evidence that indices of cerebral redox status of adult mice are age-related. These changes taking place in healthy animals progressing toward a more aged state, may be harbingers of more deleterious age-related changes. Furthermore, the use of specific dietary supplements can maintain oxidant parameters at levels characteristic of younger animals. This implies that such micronutrients may retard the imbalance of homeostatic cellular mechanisms underlying aging.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health (ES 7992, AG 16794 and AG 14882). We are grateful to Dr Arezoo Campbell for a critique of the manuscript.

References

Antolin, I., Rodriguez, C., Sainz, R.M., Mayo, J.C., Uria, H., Kotler, M.M., et al., 1996. Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J. 10, 882–890.

Araghi-Niknam, M., Lane, L., Watson, R.R., 1999. Modification of physical movement in old C57BL/6 mice by DHEA and melatonin supplementation. Proc. Soc. Exp. Med. Biol. 221, 193–197.

Arima, T., Kitamura, Y., Nishiya, T., Takagi, H., Nomura, Y., 1996. Kyotorphin (l-tyrosyl-L-arginine) as a possible substrate for inducible nitric oxide synthase in rat glial cells. Neurosci. Lett. 4, 393–402.

Bass, D.A., Parce, J.W., Dechatelet, L.R., Sjeda, P., Seeds, M.C., Thomas, M., 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded dose response to membrane stimulation. J. Immunol. 130, 1910–1917.

Beal, M.F., Matthews, R.T., Tielman, A., Shults, C.W., 1998. Coenzyme Q10 attenuates the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) induced loss of striatal dopaminergic neurons in aged mice. Brain Res. 783, 109–114.

Bondy, S.C., McKee, M., 1991. Disruption of the potential across the synaptosomal plasma membrane and mitochondria by neurotoxic agents. Toxicol. Lett. 58, 13–21.

Bondy, S.C., Guo, S.X., Adams, J.D., Klaidman, L.K., Edwards, J., 1996. Dietary alpha-tocopherol content modulates responses to moderate ethanol consumption. Env. Tox. Pharm. 1, 45–49.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. Anal. Biochem. 72, 248–254.

Butterfield, D.A., Howard, B.J., Yatin, S., Allen, K.L., Carney, J.M., 1997. Free radical oxidation of brain proteins in accelerated senescence and its modulation by N-tert-butyl-alpha-phenylnitrone. Proc. Natl. Acad. Sci. USA 94, 674–678.

Campbell, A., Bondy, S.C., 2001. Particulates as contributory factors in neurodegenerative disease. In: Bondy, S.C., Campbell, A. (Eds.), Inflammatory Events in Neurodegeneration. Prominent Press, Scottsdale, pp. 309–325.

Dovinova, I., Novotny, L., Rauko, P., Kvasnicka, P., 1999. Combined effect of lipoic acid and doxorubicin in murine leukemia. Neoplasma 46, 237–241.

Feuers, R.J., 1998. The effects of dietary restriction on mitochondrial dysfunction in aging. Ann. N.Y. Acad. Sci. 854, 192–201.

Hagen, T.M., Ingersoll, R.T., Lykkesfeldt, J., Lui, J., Wehr, C.M., Vinasksky, V., et al., 1999. (R)-α-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage and increased metabolic rate. FASEB J. 13, 441–448.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.V., Prior, R.L., Cao, G., Martin, A., 1993. Long term dietary strawberry, spinach or vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. J. Neurosci. 18, 8047–8055.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., et al., 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J. Neurosci. 19, 8114–8121.

Kopp, C., Vogel, E., Rettori, M.C., Delagrange, P., Guardiola-Lemaitre, B., Misslin, R., 1999. Effects of melatonin on neophobic responses in different strains of mice. Pharm. Biochem. Behav. 63, 521–526.

LeBel, C.P., Bondy, S.C., 1990. Sensitive and rapid quantitation of oxygen reactive species in rat synaptosomes. Neurochem. Int. 17, 435–440.

LeBel, C.P., Bondy, S.C., 1991. Persistent protein damage despite reduced oxygen radical formation in the aging rat brain. Int. J. Develop. Neurosci. 9, 139–146.

LeBel, C.P., Bondy, S.C., 1992. Oxidative damage and cerebral aging. Prog. Neurobiol. 38, 601–602.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin diacetate as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Lin, A.M.Y., Ho, L.T., 2000. Melatonin suppresses iron-induced neurodegeneration in rat brain. Free Rad. Biol. Med. 28, 904–911.

Longoni, B., Salgo, M.G., Pryor, W.A., Marchiafava, P.L., 1998. Effect of melatonin on lipid peroxidation induced by oxygen radicals. Life Sci. 62, 853–859.

Ma, X.Y., Su, Y.B., Zhang, F.R., Li, J.F., 1996. Effects of vitamin E on the blastogenic response of splenocytes and lipofuscin contents in the hearts and brains of aged mice. J. Env. Path. Tox. Oncol. 15, 51–53.

Melchiori, D., Rieter, R.J., Chen, L.D., Sewerynek, E., Nistico, G., 1996. Melatonin affords protection against kainate-induced in vitro lipid peroxidation in brain. Eur. J. Pharmacol. 305, 239–242.

Miller, J.W., Selhub, J., Joseph, J.A., 1996. Oxidative damage caused by free radicals during catecholamine autoxidation: protective effects of *o*-methylation and melatonin. Free Rad. Med. Biol. 21, 241–249.

Mollace, V., Rodino, P., Massoud, R., Rotiroti, D., Nistico, G., 1995. Age-dependent change of NO synthase activity in the rat brain. Biochem. Biophys. Res. Commun. 215, 822–827.

Nishikawa, T., Takahashi, J.A., Fujibayashi, Y., Fujisawa, H., Zhu, B., Nishimura, Y., et al., 1998. An early stage mechanism of the age-associated mitochondrial dysfunction in the brain of SAMP8 mice, an age-associated neurodegeneration model. Neurosci. Lett. 254, 69–72.

Oaknin-Bendahan, S., Anis, Y, Nir, I, Zisapel, N., 1995. Effects of long-term administration of melatonin and a putative antagonist on the aging rat. NeuroReport 6, 785–788.

Packer, L., Tritschler, H.J., Wessel, K., 1997. Neuroprotection by the metabolic antioxidant alpha-lipoic acid. Free Rad. Biol. Med. 22, 359–378.

Pappola, M.A., Sos, M., Omar, R.A., Bick, R.J., Hickson-Bick, D.L., Reiter, R.J., et al., 1997. Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide. J. Neurosci. 17, 1683–1690.

Pierpaoli, W., Regelson, W., 1997. Pineal control of aging: effect of melatonin and pineal grafting on aging mice. Proc. Natl. Acad. Sci. USA 91, 787–791.

Reiter, R.J., 1995. The role of the neurohormone melatonin against macromolecular oxidative damage. Neurochem. Int. 6, 453–460.

Ren, Z., Ding, W., Su, Z., Gu, X., Huang, H., Liu, J., et al., 1994. Mechanisms of brain injury with deep hypothermic circulatory arrest and protective effects of coenzyme Q10. J. Thoracic. Cardiovasc. Surg. 108, 126–133.

Sastre, J., Pallardo, F.V., de la Assuncion, J.G., Vina, V., 2000. Mitochondria, oxidative stress and aging. Free Rad. Res. Commun. 32, 189–198.

Sharman, E., Bondy, S.C., 2001. Effects of age and dietary antioxidants on cerebral electron transport chain activity. Neurobiol. Aging 22, 629–634.

Shrieve, D.C., Bump, E.A., Rice, G.C., 1988. Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. J. Biol. Chem. 263, 14107–14114.

Smith, C.D., Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., et al., 1991. Excess protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc. Nat. Acad. Sci. 88, 10540–10543.

Sohal, R.S., 1997. Role of mitochondria and oxidative stress in the aging process. In: Beal, M.F., Howell, N., Bodis-Wollner, I. (Eds.), Mitochondria and Free Radicals in Neurodegenerative Diseases. Wiley-Liss, New York, pp. 91–107.

Song, W., Lahiri, D.K., 1997. Melatonin alters the metabolism of beta amyloid precursor protein in the neuroendocrine cell line PC 12. J. Mol. Neurosci. 9, 75–92.

Stoyanovsky, D.A., Goldman, R., Claycamp, H.G., Kagan, V.E., 1995. Phenoxyl radical-induced thiol-dependent generation of reactive oxygen species: implications for benzene toxicity. Arch. Biochem. Biophys. 317, 315–323.

Vatassery, G.T., Fahn, S., Kuskowski, M.A., 1998. Alpha-tocopherol in cerebrospinal fluid of subjects on high dose vitamin E in the DATATOP study. Neurology 50, 1900–1902.

Yamada, K., Noda, Y., Komori, Y., Sugihara, H., Hasegawa, T., Nabeshima, T., 1996. Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats. Neurosci. Res. 24, 393–402.

Yamamoto, H., Hsai-Wang, T., 1996. Antagonistic effect of melatonin against cyanide-induced seizures and acute lethality in mice. Toxicol. Lett. 87, 19–24.

Zhang, Y., Turunen, M., Applekvist, E.L., 1996. Restricted uptake of dietary coenzyme Q is in contrast to the unrestricted uptake of alpha-tocopherol into rat organs and cell. J. Nutr. 126, 2089–2097.