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Combination of N-acetylcysteine, α-lipoic acid and α-tocopherol substantially prevents the brain synaptosomal alterations and memory and learning deficits of aged rats

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ABSTRACT

This study has compared several synaptosomal parameters in three groups of rats: young (46 months), aged (22–24 months) and antioxidant supplemented aged rats (antioxidant supplementation given with the diet as a combination of N-acetylcysteine, α-lipoic acid and α-tocopherol from 18 months onwards till 22–24 months). The synaptosomes from aged rat brain, in comparison to those of young animals, exhibit an increased membrane potential with altered contents of Na^+ and K^+ under basal incubation condition and in the presence of depolarizing agents. The intrasynaptosomal Ca^{2+} is also higher in aged than in young rat. These age-dependent changes in synaptosomal parameters are prevented markedly in the antioxidant supplemented group. When examined on T-maze, the aged animals are noticeably impaired in learning and memory functions, but the deficit is remarkably prevented in the antioxidant supplemented aged animals. It is suggested that the synaptosomal alterations partly contribute to the cognitive deficits of aged animals, and both are rescued by long-term antioxidant supplementation.

1. Introduction

The aging of the brain is associated with ultrastructural modifications, biochemical deficits, inflammatory changes and impairment of memory and cognition, and many of these alterations have been linked to oxidative stress (Chakrabarti et al., 2011; Pizza et al., 2011; Riddle, 2007). In different systems, oxidative stress is also known to alter the properties of biomembranes and inactivate membrane-bound enzymes, receptors and channels (Cai and Sesti, 2009; Halliwell and Gutteridge, 2007; Kourie, 1998; Stark, 2005; Yang et al., 2010). In the aging brain, the oxidative modification of membrane bound Na^+, K^+ ATPase, Ca^{2+} ATPase, sodium, potassium or calcium channels or pre and post synaptic receptors could affect neuronal resting membrane potential, excitability, calcium signaling, and neurotransmitter release and may also induce cell death pathways like apoptosis (Annunziato et al., 2002; Chakraborty et al., 2003; Cotella et al., 2012; Sesti et al., 2010; Zaidi and Michaelis, 1999). Since oxidative stress is an overwhelming phenomenon in the aged brain, it is conceivable that many of the age-associated deficits of brain functions including the impairment of learning and memory could result from the oxidative inactivation of ion channels, receptors and transport enzymes like Na^+, K^+ ATPase and Ca^{2+} ATPase.

The brain synaptosomes are good experimental systems to study the effects of various pharmacological agents on neurotransmitter release, cation homeostasis, and calcium influx, and such studies have corroborated the results of electro-physiological experiments (Chinopoulos et al., 2000; Garcia-Sanz et al., 2001; Nicholls, 2010). In the present study, we have used rat brain cortical synaptosomes to identify the age-related alterations of presynaptic resting membrane potential and ionic homeostasis, and further to establish the involvement of oxidative stress in these processes, we have examined the effects of long-term antioxidant treatment to aged rats in rescuing the age-dependent synaptosomal alterations. Separately, we have also examined if this antioxidant treatment regimen has any effect on learning and memory functions of aged rats. There are not many reports which have addressed these issues of altered presynaptic functions during brain aging and their modulation by chronic dietary antioxidant supplementation.

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In a series of earlier studies in this lab, it has been shown that a cocktail of antioxidants comprising of N-acetylcysteine, α-tocopherol and α-lipoic acid can prevent the mitochondrial dysfunction, proinflammatory state of the aged brain and inactivation of synaptosomal Na^+, K^+ ATPase (Bagh et al., 2008, 2011; Thakurta et al., 2012). The same combination of drugs has been retained also in this study to examine its potential beneficial effects on age-dependent changes in brain presynaptic functions. The justifications for using multiple antioxidants instead of a single one have been elaborately described in earlier publications, but one compelling reason is that it ensures interference with oxidative damage network at different levels through different mechanisms (Bagh et al., 2011; Thakurta et al., 2012). We presume that multiple oxidative stress related changes are responsible for the functional deterioration of the brain in the aged animals and human beings, and similar processes may also contribute to the impairment of memory and cognition in age-related neurodegenerative diseases like Alzheimer's disease. Thus, the principal objective of this ongoing series of study in our lab is to identify the various age-dependent alterations in metabolic pathways, membrane functions, gene expressions and cell signaling mechanisms in the brain that can be rescued by a prolonged dietary administration of an antioxidant combination of pure compounds. Several antioxidants e.g. vitamin E, α-lipoic acid, acetyl-L-carnitine, and coenzyme Q have been used individually or in combination to prevent one or a few specific biochemical or functional deficits of the aged brain in different studies (Chakrabarti et al., 2011; Liu et al., 2002a,b; Navarro et al.; 2005). On the other hand, we are trying to evaluate the efficacy of a specific combination of antioxidants in ameliorating the multiple and varied age-related biochemical and functional alterations of the brain caused by the oxidative stress through an ongoing series of studies. Such a combination of antioxidants in principle can then be exploited for its therapeutic potential in preventing cognitive decline and memory impairment of normal aging as well as Alzheimer's disease.

2. Materials and methods

2.1. Materials

All common chemicals and reagents were of analytical or HPLC grade and purchased from Sisco Research Laboratories, India. PBFI-AM (1,3-benzenedicarboxylic acid, 4,4′-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis tetrakis [(acetyloxy)methyl] ester) and SBFI-AM (1,3-benzenedicarboxylic acid, 4,4′-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyl)]bis tetrakis[(acetyloxy)methyl] ester) were obtained from Molecular Probes, USA. DiOC(5)3 (3,3′-dipentyloxacarbocyanine iodide), DL-dithiothreitol (DTT), (±)-α-lipoic acid, phenylmethanesuphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA), Fura-2AM, gramicidin, glutamate dehydrogenase, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), ionomycin, leupeptin hydrochloride, N-acetyl-L-cysteine, Percoll, butylated hydroxytoluene (BHT), protease inhibitor cocktail, valinomycin, and veratridine were purchased from Sigma-Aldrich Chemical Company, USA.

2.2. Animals and treatment

Albino rats of Charles-Foster (CF) strain kept on laboratory chow with water ad libitum were used for this study. The animals were maintained as per the guidelines of the animal ethical committee of the institute. The animals (average life span 28 months under our lab conditions) were divided into 3 groups: young (4–6 months), aged (22–24 months) and antioxidant-supplemented aged (22–24 months) rats. The antioxidant-supplemented aged rats were fed daily with oral antioxidants in the form of a combination of N-acetylcysteine (50 mg/100 g body wt), α-lipoic acid (3 mg/100 g body wt) and α-tocopherol (1.5 mg/100 g body wt) from 18 month onwards till they were used for experiments at 22–24 months of age. The rationale for the selection of antioxidant dosage and duration and the method of oral antioxidant supplementation was described in our earlier publication (Bagh et al., 2008).

2.3. Preparation of synaptosomes by density gradient centrifugation

Rat brain synaptosomes were prepared by density gradient centrifugation following an earlier published method (Sihra, 1997). Briefly, the cerebral cortices were homogenized in 5 volumes of ice cold buffer containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol (DTT), pH 7 and centrifuged at 3000 *g* for 2 min at 4 °C. The supernatant was centrifuged at 14,500 *g* for 12 min and the resulting pellet (P2) resuspended in 4 ml of homogenizing buffer to obtain crude mitochondrial–synaptosomal suspension. An aliquot (2 ml) of this suspension was loaded on a discontinuous gradient of Percoll (23%, 10% and 4%) in homogenizing buffer and spun at 32,500 *g* for 7 min at 4 °C to obtain the synaptosomal fraction at the 23% and 10% Percoll interface. The synaptosomal fraction was collected and washed twice in homogenizing buffer at 27,000 *g* for 10 min in order to remove the contaminating Percoll. The synaptosomes were resuspended in appropriate isotonic buffer for further experiments.

2.4. Determination of synaptosomal Na^+ and K^+ contents

Aliquots of synaptosomal fraction (2 mg protein/ml) were incubated in KRH (Krebs Ringer HEPES) medium (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, 16 μM BSA adjusted to pH 7.4) for an hour at 37 °C in the presence of 10 μM SBFI-AM (fluorescent dye for Na^+) or 10 μM PBFI-AM (fluorescent dye for K^+) in a total volume of 200 μl as published earlier (Deri and Adam-Vizi, 1993; Kasner and Ganz 1992). At the end of the incubation dye-loaded synaptosomes were pelleted out, and the pellet resuspended in either Na^+ free KRH buffer (for Na^+ measurement experiments) or K^+ free KRH buffer (for K^+ measurement experiments) without BSA. The pellet in each case was washed twice in the corresponding buffer and then resuspended in the same buffer in appropriate dilution for fluorescence measurements. The intra-synaptosomally trapped SBFI or PBFI fluorescence was measured at dual excitation wavelengths of 340/380 nm and 510 nm emission wavelength in a JASCO (FP 6300) spectrofluorometer. A calibration curve for Na^+ was prepared by incubating synaptosomes in the presence of 3 μM gramicidin in a medium containing different concentrations of Na^+ with choline chloride added to adjust the osmolarity.

A similar calibration curve was prepared for K^+ in which synaptosomes were incubated in the presence of valinomycin (3 μM) in a medium containing different K^+ concentrations.

2.5. Measurement of synaptosomal membrane potential

The measurement of the synaptosomal membrane potentia1 was performed using the voltage sensitive fluorescent probe DiOC(5)3 as adapted from the published methods (North and Fleischer, 1983). In short, the synaptosomes were incubated in KHS (Krebs HEPES Sucrose) medium (130 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 0.5 mM EGTA, 0.2 M sucrose, 10 mM HEPES, 10 mM glucose, pH 7.4) at 37 °C for 10 min in the presence of 2 μM DiOC(5)3. For some experiments, synaptosomes were incubated in a similar manner but in the presence of 65 mM K^+. The fluorescence intensity of the synaptosomal suspension was measured in a JASCO (FP 6300) spectrofluorometer (λex 470 nm/λem 510 nm).

2.6. Intrasynaptosomal Ca^{2+} measurements

Intrasynaptosomal calcium concentration was measured using the fluorescent dye Fura-2-acetoxymethyl ester (Fura-2AM) as published earlier (Wang and Chen, 2007). Synaptosomes (0.5 mg/ml)