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Late Onset Administration of Oral Antioxidants Prevents Age-related Loss of Motor Co-ordination and Brain Mitochondrial DNA Damage

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We have studied the effect of aging on brain glutathione redox ratio, on brain mitochondrial DNA damage and on motor co-ordination in mice and the possible protective role of late onset administration of sulphur-containing antioxidants. Glutathione redox ratios change to a more oxidized state in whole brain with aging but the changes are much more pronounced when this ratio is measured in brain mitochondria. The levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine in mitochondrial DNA are much higher in the brain of old animals than in those of young ones. Late onset oral administration of sulphur-containing antioxidants partially prevents oxidation of mitochondrial glutathione and DNA. There is an inverse relationship between age-associated oxidative damage to mitochondrial DNA and motor co-ordination in old mice.

Keywords: Aging, brain, oxidative stress, motor co-ordination

INTRODUCTION

According to the free radical theory of aging[1,2] oxygen-derived free radicals are responsible for cellular aging and age-associated diseases. The free radical theory of aging assumes that cellular antioxidant systems are not able to cope with the reactive oxygen species generated continuously throughout cell life. Thus, cellular aging would be associated with a "chronic" oxidative stress, which was defined by Sies as a disturbance in the balance between pro-oxidants and antioxidants, in favour of the former.[3]

At present, a great deal of experimental evidence supports the free radical theory of aging.[4,5] It is well known that free radicals are involved in the pathogenesis and development of many degenerative diseases associated with aging. A clear-cut evidence in favour of the free radical theory of aging is that simultaneous overexpression of copper-zinc superoxide dismutase and catalase genes in transgenic *Drosophila* extends their mean and maximum lifespan.[6] Furthermore, overexpression of these enzymes slows the aging process in flies, since transgenic flies exhibit

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a delayed loss of physical performance and a lower amount of protein oxidative damage.[6]

In the eighties, Miquel and co-workers proposed the mitochondrial theory of cell aging.[7,8] This theory suggests that senescence is a by-product of oxy-radical attack to the mitochondrial genome (mtDNA) of fixed postmitotic cells.[7] So far, many studies have shown oxidative damage to mitochondrial DNA, protein and lipids, as well as changes in mitochondrial function and morphology upon aging.[9,10]

Recently, Sohal and co-workers have found that age-related losses of cognitive function and motor skills are associated with oxidative protein damage in brain.[11] The free radical theory of aging is specially attractive because it provides a rationale for intervention, i.e. administration of antioxidants that might retard the impairment in performance associated with aging. However, a major question is whether administration of antioxidants must be given during the whole lifespan or it can be started late in life. We have reported previously that oral administration of glutathione[12] and other sulphur-containing antioxidants[13] are effective against age-associated free radical damage *in vivo*.

Thus, the aim of the present work was to find out whether late onset administration of sulphur-containing antioxidants is able to prevent not only the age-related damage to mitochondrial DNA, but also the impairment in physiological performance, particularly motor co-ordination, that occurs upon aging.

METHODS

Reagents

Reduced glutathione was purchased from Sigma Chemical Co., Madrid (Spain). The thiazolidine carboxylate derivative N-(2R)-3-acetyl-2-methylthiazolidine-4-carbonyl-β-alanine methyl ester were obtained from Laboratorios Zambon España S.A. (Barcelona, Spain). All the other reagents were from Sigma Chemical Co., Madrid (Spain) or Boehringer Mannheim (Germany).

Animals

Male mice of the C57BJ strain were used. They were subjected to a 12 h light–12 h dark cycle, and had free access to food and water. Mice were divided into five groups: a young control one (6 months old), an old control one (18 months old), and two old treated groups (18 months old) – one treated with reduced glutathione and another with N-(2R)-3-acetyl-2-methylthiazolidine-4-carbonyl-β-alanine methyl ester (abbreviated as AMTC). These antioxidants were administered with standard powdered food (Vara de Quart, Valencia) at a dose of 0.67 mmol/kg body wt per day for 6 months, from the age of 12 up to 18 months, when mice were sacrificed. The amount of food intake was not affected by antioxidant administration.

Mice were anaesthesized with penthobarbital just before sacrifice. Brain was quickly removed and homogenized as described in Ref. [14]. Brain mitochondria were isolated as described by Rickwood *et al.*[15] We have used this method in the past[10,14] and it has proved suitable to obtain preparations of viable mitochondria with negligible contamination by other components, as judged by mitochondrial enzyme activities (citrate synthase, cytochrome oxidase, succinate cytochrome c-reductase activities), flow cytometric analysis (mitochondrial morphology and membrane potential), electron microscopy and a negligible contamination with cytosolic enzymes (lactate dehydrogenase activity).

We studied the neuromuscular co-ordination using a motor co-ordination test,[16] which has proved useful in gerontological research to test physiological performance. Briefly, mice were placed on a thin string (cotton string of 1 mm in diameter and 60 cm in length) tied up on each side to the rod of a chemical stand. The string was suspended above a mouse cage at 60 cm of its bedding of wood shavings. Mice passed the test

when they are able to reach a side pole in the allowed time of 1 min.[16] The score for motor coordination is the transported biomass, which is calculated by adding the body weights of all mice in an age group which passed the test and dividing this number by the number of mice tested (including those which failed the test).[16]

Analysis of Metabolites

Reduced glutathione (GSH), glutathione disulphide (GSSG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine levels were measured as described in.[14]

Statistics

Results are expressed as means ± SD for the number of experiments in parentheses. Statistical analysis was performed in two steps. Analysis of variance was performed first, and then the sets of data in which F was significant were examined by the unpaired Student's t-test.

RESULTS

Effect of Aging and Sulphur-containing Antioxidants on Glutathione Redox Status in Whole Brain

GSH/GSSG ratio decreased with age in brain (see Figure 1). This change was due to a decrease ($P < 0.01$) in brain GSH levels together with an increase ($P < 0.01$) in brain GSSG levels. Indeed, brain GSH levels were 1.4 ± 0.6 μmols/g ($n = 12$) in young mice vs. 1.1 ± 0.2 μmols/g ($n = 13$) in old mice; whereas brain GSSG levels were 32 ± 13 nmols/g ($n = 9$) in young mice vs. 57 ± 18 nmols/g ($n = 13$) in old mice.

GSH administration resulted in an increase ($P < 0.01$) in GSH levels in brain. Brain GSH levels were 1.6 ± 0.4 μmols/g, $n = 12$ (compared with 1.1 ± 0.2 μmols/g ($n = 13$) in old untreated mice). However, GSSG levels did not change significantly. Administration of GSH increased slightly, but not significantly, the GSH/GSSG ratio when compared to that of old controls (see Figure 1).

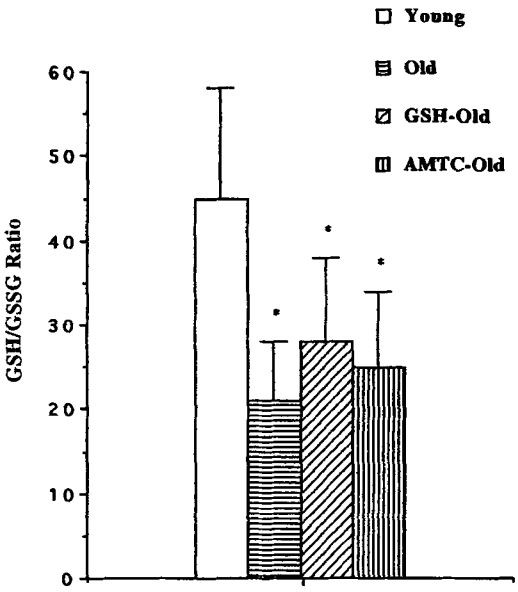

FIGURE 1 Effect of aging and sulphur-containing antioxidants on glutathione redox status in brain. Values are mean ± SD for $n = 9$–12. Statistical difference is indicated as follows: *$P < 0.05$ vs. the young group.

AMTC treatment caused a decrease ($P < 0.05$) in brain GSSG levels, however, the GSH/GSSG ratio did not change significantly when compared to that of old controls (see Figure 1). Brain GSH levels were 0.9 ± 0.2 μmols/g ($n = 8$) and brain GSSG levels were 39 ± 13 nmols/g ($n = 10$) in old mice treated with AMTC.

Effect of Aging and Sulphur-containing Antioxidants on Glutathione Redox Status in Brain Mitochondria

GSH/GSSG ratio decreased sharply with age in brain mitochondria (see Figure 2). This change was due to a decrease ($P < 0.05$) in mitochondrial GSH levels together with an increase ($P < 0.05$) in mitochondrial GSSG levels. Indeed, mitochondrial GSH levels were 36 ± 12 nmols/g ($n = 5$) in

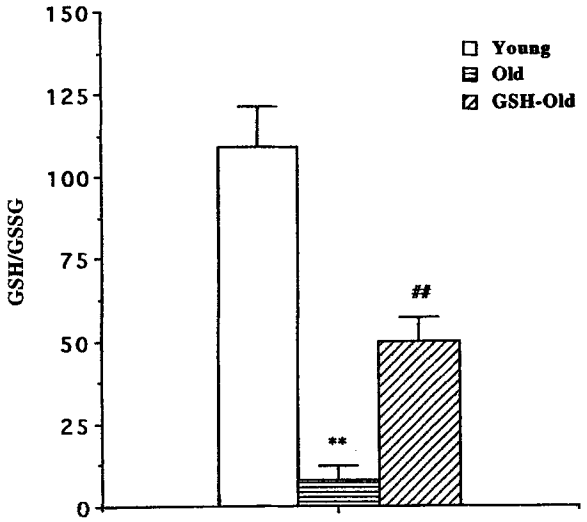

FIGURE 2 Effect of aging and sulphur-containing antioxidants on glutathione redox status in brain mitochondria. Values are mean ± SD for $n = 3$–4. Statistical difference is indicated as follows: $^{**}P < 0.01$ vs. the young group; $^{\#\#}P < 0.01$ vs. the old control group.

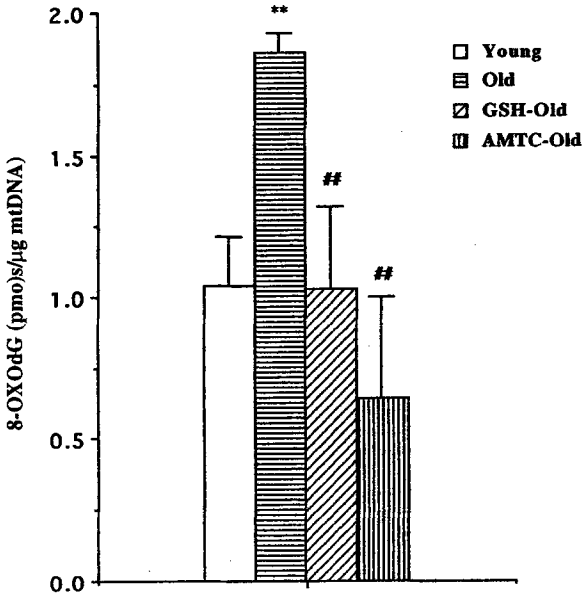

FIGURE 3 Effect of aging and sulphur-containing antioxidants on oxidative damage to brain mitochondrial DNA. Values are mean ± SD for $n = 3$–6. Statistical difference is indicated as follows: $^{**}P < 0.01$ vs. the young group; $^{\#\#}P < 0.01$ vs. the old control group.

brain of young mice vs. 16 ± 4 μmols/g ($n = 3$) in brain of old mice; whereas mitochondrial GSSG levels were 0.33 ± 0.13 nmols/g ($n = 4$) in brain of young mice vs. 1.9 ± 0.2 nmols/g ($n = 3$) in brain of old mice.

Oral administration of glutathione prevented the age-related decrease in mitochondrial GSH levels as well as the increase in mitochondrial GSSG levels in brain ($P < 0.05$). Thus, GSH administration prevented the age-related decrease in the mitochondrial GSH/GSSG ratio in brain (see Figure 2). GSH levels were 22 ± 7 nmols/g ($n = 3$) and GSSG levels were 0.44 ± 0.12 nmols/g ($n = 3$) in brain mitochondria of old mice treated with GSH.

Effect of Aging and Sulphur-containing Antioxidants on Oxidative Damage to Mitochondrial DNA in Brain

The levels of the oxidized base 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodG) are indicators of oxidative damage to DNA.[9] 8oxodG levels in mitochondrial DNA (mtDNA) from brain were higher ($P < 0.01$) in old mice than in young mice (see Figure 3). Administration of GSH or AMTC prevented this age-related increase in oxidative damage to brain mtDNA (see Figure 3).

Effect of Age and of Antioxidants on the Motor Co-ordination Test in Mice

In order to find out whether administration of sulphur-containing antioxidants was able to prevent the decline in physiological performance that occurs upon aging, mice were subjected to the motor co-ordination test described in Ref. [16].

Mice were subjected to the test once per month from 12 months old to 18 months old, when they were sacrificed. Figure 4 shows that administration of GSH or AMTCA was able to improve the physiological performance in the motor co-ordination test. Figure 5 shows an inverse linear relationship between the motor co-ordination performance of old mice and the oxidative damage to their brain mitochondrial DNA.

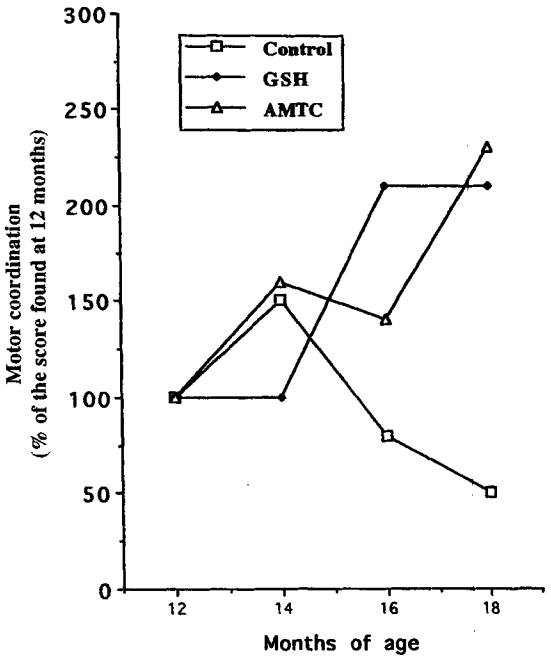

FIGURE 4 Effect of aging and sulphur-containing antioxidants on motor co-ordination in mice. Values are the average of each group for $n=4$–6. The vertical scale indicates the percentage of the score for motor co-ordination in old mice (14–18 months old) vs. the score for 12 months old mice. The score for motor co-ordination is the transported biomass, which is calculated as indicated in Methods.

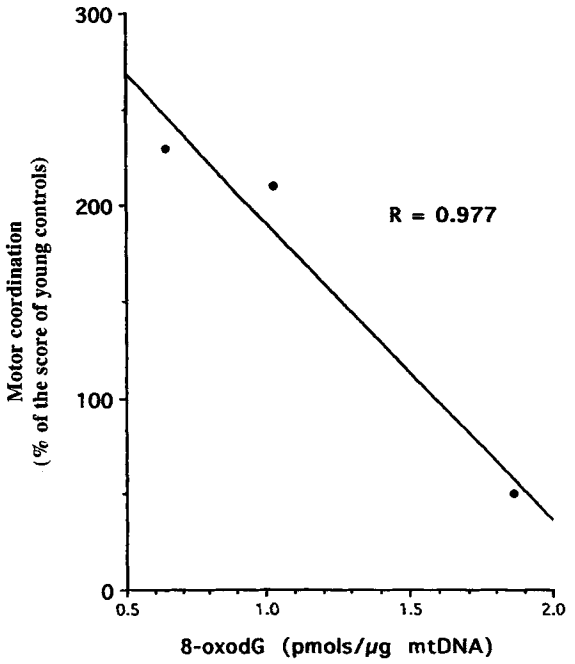

FIGURE 5 Inverse relationship between motor co-ordination and oxidative damage to brain mitochondrial DNA in mice. Points are average for $n=3$–4 values. The vertical scale indicates the percentage of the score for motor co-ordination in old mice vs. the score for 12 months old mice. The score for motor co-ordination is the transported biomass, which is calculated as indicated in Methods.

DISCUSSION

The experimental evidences obtained so far in favour of the free radical theory of aging have given support for studies on prevention of those impairments associated with aging by antioxidant administration.[17,18] Miquel *et al.*[7] showed that administration of thiazolidine carboxylate increases the vitality and life span of mice. We found that oral administration of glutathione is effective to prevent GSH depletion by various agents.[12] This effect is due to the hydrolysis of glutathione in the gut, so that glutathione is a source of its constituent amino acids, which are absorbed and then eventually are substrates for synthesis *de novo* of glutathione in tissue cells. On the other hand, Furukawa *et al.*[19] reported that oral glutathione protects against the age-associated decline in immune response. In addition, administration of some sulphur-containing antioxidants, such as thiazolidine carboxylate derivatives, partially protect against glutathione oxidation in tissues of rodents and in Drosophila.[13,18,20]

In the present paper, we show that administration of sulphur-containing antioxidants, such as GSH or a thiazolidine derivative (AMTC), prevents not only the mitochondrial oxidative stress but also the physiological impairment associated with aging. The effects are much less marked when glutathione is determined in whole brain (see Figures 1 and 2). GSH/GSSG ratio is higher in the mitochondria than in the whole cell in young animals. There is a remarkable decrease (85%) of this ratio in mitochondria upon aging. Administration of GSH or AMTC prevented glutathione oxidation in brain mitochondria, however, these treatments did not affect significantly the

age-related oxidative stress in the whole tissue. Administration of GSH or AMTC also prevented the oxidative damage to mitochondrial DNA that occurs upon aging. The effects of AMTC or GSH treatment on the age-related oxidative stress were accompanied by an increase in motor co-ordination of mice. These results suggest that oxidative stress in mitochondria, and not in the whole cell, may be a key factor in the physiological impairment associated with aging.

We showed in the past, that oral administration of glutathione is effective in increasing glutathione levels in tissues. We showed that this is not due to a direct glutathione absorption and uptake by tissues. Rather, glutathione is hydrolysed to its constituent amino acids which are absorbed and serve as precursors for glutathione synthesis in tissues.[12]

The possibility of starting late in life is critical when studying dietary modifications related to aging. For instance Weindruch and Walford showed[21] that late onset dietary restriction could prolong life span in mice. In this paper we show that late onset administration of antioxidants prevents damage to mitochondrial DNA and loss of motor co-ordination in mice.

It has been proposed that oxidative stress is involved in the aging process in the brain that lead to dysfunction.[22–24] Dietary restriction[25] or administration of the spin-trapping agent N-tert-butyl-a-phenylnitrone[26] decreased the protein oxidative damage in the brain of rodents with a concurrent improvement in age-associated behavioural deficits. Sohal and co-workers have reported that age-related declines of cognitive function and motor skills involve oxidative damage to proteins within different regions of the brain.[11] We show here that age-related losses in motor co-ordination are associated with oxidative damage to mitochondrial DNA. Recently, Perrig *et al.*[27] have found a correlation between performance on tests of memory and plasma ascorbic acid and β-carotene levels in humans. These results, together with ours, support the role of oxidative stress in brain aging and the beneficial effect of antioxidant nutrients in the protection against this process. However, further studies on dietary supplementation with antioxidants need to be done to establish adequately their beneficial action.

The fact that oxidative stress is associated with diseases such as Alzheimer's[28] or Parkinson's[22,23,29] and that antioxidant administration can protect against oxidative stress shows that antioxidant administration may also be effective to protect against neurodegenerative diseases associated with the old age as appears to be the case in the case of Alzheimer's disease.[30]

In summary, we have found that oxidative stress occurs in whole brain and particularly in brain mitochondria from mice upon aging. Late onset administration of sulphur-containing antioxidants prevented the age-associated oxidative stress in mitochondria as well as the age-associated impairment in the neuromuscular co-ordination in mice. The age-associated loss of motor coordination is associated with an increase in damage to mitochondrial DNA.

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