Protective Effect of L-Cysteine and Glutathione on Rat Brain Na^+,K^+-ATPase Inhibition Induced by Free Radicals

Stylianos Tsakiris[a,*], Panagoula Angelogianni[a], Kleopatra H. Schulpis[b] and Panagiotis Behrakis[a]

[a] Department of Experimental Physiology, University of Athens, Medical School, P. O.Box 65257, GR-154 01 Athens, Greece. Fax: 0030-1-7775295. E-mail: stsakir@cc.uoa.gr
[b] Inborn Errors of Metabolism Department, Institut of Child Health, GR-115 27 Athens, Greece

* Author for correspondence and reprint requests

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The aim of this study was to investigate whether the preincubation of brain homogenates with L-phenylalanine (Phe), L-cysteine (Cys) or reduced glutathione (GSH) could reverse the free radical effects on Na^+,K^+-ATPase activity. Two well established systems were used for the production of free radicals: 1) $FeSO_4$ (84 μM) plus ascorbic acid (400 μM) and 2) $FeSO_4$, ascorbic acid and H_2O_2 (1 mM) for 10 min at 37 °C in homogenates of adult rat whole brain. Changes in brain Na^+,K^+-ATPase activity and total antioxidant status (TAS) were studied in the presence of each system separately, with or without Phe, Cys or GSH. TAS value reflects the amount of free radicals and the capacity of the antioxidant enzymes to limit the free radicals in the homogenate. Na^+,K^+-ATPase was inhibited by 35–50% and TAS value was decreased by 50–60% by both systems of free radical production. The enzymatic inhibition was completely reversed and TAS value increased by 150–180% when brain homogenates were preincubated with 0.83 mM Cys or GSH. However, this Na^+,K^+-ATPase inhibition was not affected by 1.80 mM Phe, which produced a 45–50% increase in TAS value. It is suggested that the antioxidant action of Cys and GSH may be due to the binding of free radicals to sulfhydryl groups of the molecule, so that free radicals cannot induce Na^+,K^+-ATPase inhibition. Moreover, Cys and GSH could regulate towards normal values the neural excitability and metabolic energy production, which may be disturbed by free radical action on Na^+,K^+-ATPase.

Introduction

Free radicals in brain could represent one of the main causes of cellular dysfunction occurring during aging (Harman, 1983; Floyd *et al.*, 1984; Hall and Braughler, 1989). Some basic evidence has suggested the above hypothesis: (a) brain contains high amounts of polyunsaturated fatty acids; (b) compared with other tissues, brain utilizes one-fifth of the total oxygen demand of the body, and (c) brain is not particularly enriched in any of the antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase). Furthermore, it has been demonstrated that a general reduction of the antioxidant protection mechanisms occurs during aging. From among the enzymatic systems, an age-dependent decrease in superoxide dismutase activity has been reported (Reiss and Gershon, 1976; Mizumo and Ohta, 1986); with regard to nonenzymatic antioxidants, a decrease in brain ascorbate (Adlard *et al.*, 1973), glutathione (Hazelton and Lang, 1980; Benzi *et al.*, 1989) and α-tocopherol levels (Meydani *et al.*, 1986) has been shown to occur during aging.

Na^+,K^+-ATPase (EC 3.6.1.3) is the enzymatic basis of univalent cation transport (Sweadner and Goldin, 1980). It is implicated in neural excitability (Sastry and Phillis, 1977), activity-dependent metabolism of energy (Mata *et al.*, 1980) and Na^+-dependent tryptophan uptake system (Herrero *et al.*, 1983). Na^+,K^+-ATPase activity can be regulated by noradrenergic (Swann, 1984) and/or serotoninergic mechanisms (Hernàndez, 1987). Brain Na^+,K^+-ATPase activity may be decreased in Alzheimer's disease due to the decreased concentrations of noradrenaline (Palmer *et al.*, 1987). In addition, it was shown to be stimulated in aged rats, possibly due to the rise in the number of glial cells

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with age (Tsakiris *et al.*, 1996). Moreover, in Alzheimer's disease and aging, free radical action was shown to rise in the brain (Götz *et al.*, 1994; Williams, 1995).

In this study, Na^+,K^+-ATPase activity was estimated in homogenized whole brain, pituitary and distinct brain regions in adult (4 months old) and aged rats (22 months old). The observed inhibition of Na^+,K^+-ATPase activity in some brain regions of aged rats was similar to that noticed in adult brain, in which enzyme inhibition was induced for 7–10 min at 37 °C by using two separate systems of free radical production: 1) $FeSO_4$ and ascorbic acid (Viani *et al.*, 1991; Ghosh *et al.*, 1993) and 2) $FeSO_4$, ascorbic acid and H_2O_2 (Xu *et al.*, 1997). In the presence of iron, a Fenton reaction will occur between Fe^{2+} and H_2O_2 giving rise to the reactive hydroxyl radical (.OH). Proteins (and possibly Na^+,K^+-ATPase) are susceptible to free radical attack, especially by the .OH radical. Furthermore, it was investigated by evaluating the total antioxidant status (TAS) value whether the preincubation of brain homogenates with L-phenylalanine (Phe), L-cysteine (Cys) or reduced glutathione (GSH) could reverse the free radical effects on Na^+,K^+-ATPase activity.

Materials and Methods

Animals

Albino Wistar rats of both sexes (Saint Savvas Hospital, Athens, Greece) were used in all experiments. Body weight was 225±10 g (mean±SD) for adult (4 mo) and 326±34 g for aged (22 mo) rats. Adult or aged rats were housed four in a cage, at a constant room temperature (22±1 °C) under a 12hL:12hD (light 08.00–20.00 h) cycle and acclimated 1 week before use. Food and water were provided ad lib. Animals were cared for in accordance with the principles of the "*Guide to the Care and Use of Experimental Animals*".

Tissue preparation

Rats were sacrificed by decapitation. Rat whole brain or the pituitary, hypothalamus, frontal cortex and hippocampus were rapidly removed, weighed and thoroughly washed with isotonic saline. Pools of three pituitaries or individual tissues were homogenized in 10 vol ice-cold (0–4 °C) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4 and 300 mM sucrose using an ice-chilled glass homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1000×*g* for 10 min to remove nuclei and debris. In the resulting supernatant, the protein content was determined according to Lowry *et al.* (1951) and then the enzyme activities were measured. The enzyme incubation mixture was kept at 37 °C.

Free radical production and PHE/CYS/GSH-preincubation

Two well established systems were used to produce free radicals: 1) FeSO4 (84 μM) and ascorbic acid (400 μM) (Viani *et al.*, 1991; Ghosh *et al.*, 1993) and 2) FeSO4, ascorbic acid and H2O2 (1 mM) (Xu *et al.*, 1997) at 37 °C in homogenates of adult rat whole brain. In the absence of homogenized tissue TAS was 0.017±0.003 μmol/ml x min representing the radical production activity of a Fenton-type reaction. In the presence of homogenate TAS increased to 0.049±0.008 μmol/mlxmin using the above systems due to the existence of enzymatic and nonenzymatic antioxidants. Changes were studied in brain Na^+,K^+-ATPase activity in the presence of the above mentioned systems. Whole brain homogenates were preincubated with Phe at 30 mg/dl (1.80 mM), Cys at 10 mg/dl (0.83 mM) and 30 mg/dl (2.48 mM) or GSH at 25.5 mg/dl (0.83 mM) for 1 h, in order to investigate the possible protective role of these compounds on Na^+,K^+-ATPase against free radical action. During the preincubation period, these compounds were stable at 37 °C. A Phe concentration of 1.80 mM is usually found in the plasma of phenylketonuric patients (Missiou-Tsagaraki *et al.*, 1988), whereas a Cys concentration of 0.83 mM may be found in the plasma of humans taking Cys (500 mg/day per os) as an antioxidant compound (Murakami and Webb, 1981).

Biochemical determinations

Na^+,K^+-ATPase activity was calculated as the difference between total ATPase activity (Na^+,K^+,Mg^{2+}-dependent) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl,

4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP, and 80–100 μg protein of the homogenate, in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of the Mg^{2+}-dependent ATPase. The values of Mg^{2+}-dependent ATPase were similar in the presence of ouabain in the reaction mixture and in its absence and without NaCl and KCl. The reaction was started by adding ATP and was stopped after a 20 min incubation period by the addition of 2 ml of a mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 (Bowler and Tirri, 1974). The yellow colour which developed was read at 390 nm.

Total antioxidant status

The total antioxidant capacity was measured spectrophotometrically by a commercial kit (Randox Laboratories Ltd, Cat No NX 2332) as previously reported (Gaal *et al.*, 1995). ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{\cdot+}$. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this colour production to a degree which is proportional to their concentration. Measurements were done in fresh brain homogenates in the presence of iron/ascorbate/H_2O_2 systems with or without Phe, Cys or GSH.

Statistical analysis

The data were analyzed by two-tailed Student's *t*-test. A p value of <0.05 was considered statistically significant.

Results and Discussion

The effect of aging on Na^+,K^+-ATPase activity determined in homogenized whole brain, pituitary and distinct brain regions is presented in Table I. Whole brain Na^+,K^+-ATPase stimulation in 22 months old rats has been reported earlier (Tsakiris *et al.*, 1996). This stimulation may be due to the rise in the number of glial cells with age, in which Na^+,K^+-ATPase activity is higher in comparison to that in neuronal perikarya (Henn *et al.*, 1972; Grisar *et al.*, 1979). The enzyme activity was unaltered in hypothalamus, whereas it was decreased in pituitary (by 38%), in frontal cortex (by 24%) and in hippocampus (by 22%). Brain Na^+,K^+-ATPase activity may decrease in Alzheimer's disease (Palmer *et al.*, 1987). On the contrary, increased free radical action has been shown in the brain of aged rats as well as in Alzheimer's brain (Götz *et al.*, 1994; Williams, 1995).

Table I. Effect of aging on Na^+,K^+-ATPase activity determined in homogenized whole brain, pituitary and specific brain areas.

	Na^+,K^+-ATPase activity (μmol Pi/h x mg protein)	
Tissue	4 mo old rats	22 mo old rats
Whole brain	1.91±0.07	3.10±0.12***
Pituitary	2.28±0.22	1.42±0.13***
Hypothalamus	7.46±0.69	7.52±0.92
Frontal cortex	6.95±0.69	5.28±0.49*
Hippocampus	7.62±0.78	5.92±0.41*

Values represent means±SD of five independent experiments (five pools of three animals each) for adult or aged rats for the pituitary, and eight independent experiments (eight rats) for 4-month-old rats and six experiments (six rats) for 22-month-old rats for the whole brain as well as the specific brain areas. The average value of each experiment came from three determinations. *:$p<0.05$; ***:$p<0.001$; compared to 4-month-old rats.

Figure 1 presents time dependence of free radical production on Na^+,K^+-ATPase activity in adult brain homogenates by using the two well established systems $FeSO_4$/ascorbate with or without H_2O_2 (Viani *et al.*, 1991; Ghosh *et al.*, 1993; Xu *et al.*, 1997). The incubation system containing $FeSO_4$ (84 μM), ascorbic acid (400 μM) and H_2O_2 (1 mM) inhibited Na^+,K^+-ATPase activity to a higher degree compared to that of $FeSO_4$ plus ascorbic acid. A 10 min incubation of brain homogenates with $FeSO_4$ (84 μM), ascorbic acid (400 μM) or H_2O_2 (1 mM) separately did not induce Na^+,K^+-ATPase inhibition ($p>0.05$). Therefore, Fenton reaction of free radicals may induce an enzymatic inhibition of up to 50% which was observed during a 10 min incubation. This enzyme inhibition may be due to free radicals directly attacking the ATP binding site of the brain Na^+,K^+-ATPase (Xu *et al.*, 1997).

The effect of 1 h preincubation of brain homogenates with Phe (1.80 mM), Cys (0.83 mM or

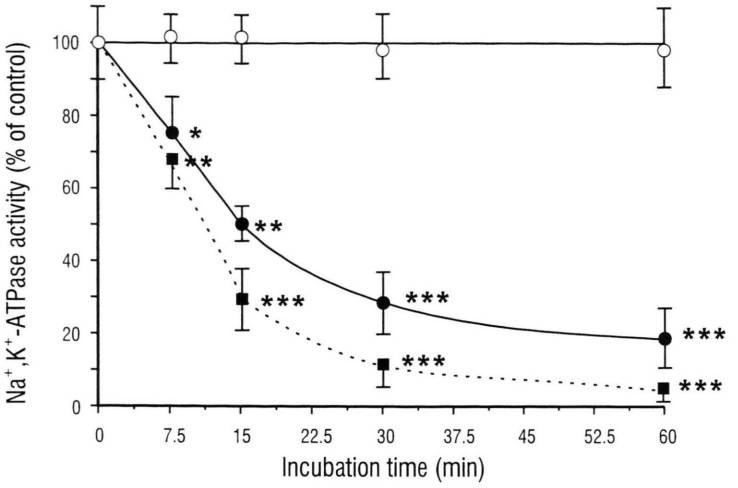

Fig. 1. Time-dependent effect of free radical production by the systems of $FeSO_4$/ascorbic acid/H_2O_2 on Na^+,K^+-ATPase activity determined in homogenized brain of 4 mo old rats. Points and vertical bars represent mean values±SD for the control (○),the system of $FeSO_4$ (84 μM) plus ascorbic acid (400 μM) (●) and that of $FeSO_4$, ascorbic acid, H_2O_2 (1 mM) (■). Values represent means ± SD of three independent experiments. The average values of each experiment arise from three determinations. Control value of Na^+,K^+-ATPase activity was 2.50±0.25 μmol Pi/hxmg protein. *:p<0.05; **:p<0.01; ***:p<0.001; compared to control.

2.48 mM) or GSH (0.83 mM) on Na^+,K^+-ATPase inhibition induced by free radicals is presented in Table II. Na^+,K^+-ATPase inhibition (32–50%, $p<0.01$) induced by the two systems of free radicals was not affected by Phe ($p>0.05$). It has been reported that ·OH radicals are especially bound to Phe (Sun *et al.*, 1993; Van der Vliet *et al.*, 1994). Therefore, the Na^+,K^+-ATPase inhibition may not be due to ·OH radicals but to other kind(s) of free radicals. Moreover, it is well known that Tris-HCl buffer and sucrose at a very high concentration are hydroxyl radical scavengers in the incubation mixture. However, 1 h preincubation of brain homogenates with Phe (1.80 mM) was able to induce a complete protection of brain acetylcholinesterase inhibition by the two systems of free radicals using a similar enzymatic incubation mixture (S. Tsakiris et al, unpublished results). Therefore, the ability of Phe to bind ·OH radicals is not affected by the preexistence of Tris and sucrose in the mixture but is dependent on the behaviour of the enzyme. However, Phe alone stimulated Na^+,K^+-ATPase of brain homogenate (Tsakiris *et al.*, 1998) to a lower degree (3.37 μmol Pi/h x mg protein, 35% increase) as compared to that of Cys alone (6.20 μmol Pi/h x mg protein, 150% increase) or GSH alone (5.53 μmol Pi/h x mg protein, 120% increase). A direct as well as an indirect effect of Phe on Na^+,K^+-ATPase have been suggested by us in a previous study (Tsakiris *et al.*, 1998). The indirect effect of Phe could be the result of brain noradrenaline enhancement, the synthesis of which in non-phenylketonuric rats can be increased by high Phe concentrations (Tsakiris *et al.*, 1998). In addi-

Table II. Effect of iron/ascorbate/H_2O_2 free radical production and L-phenylalanine, L-cysteine or glutathione preincubation on Na^+,K^+-ATPase activity determined in homogenized adult whole brain.

Treatment	Na^+,K^+-ATPase activity (μmol Pi/h x mg protein)
Control	2.50±0.25
Control+Phe	3.37±0.30**
Control+Cys	6.20±0.80***
Control+GSH	5.53±0.28***
$FeSO_4$+ascorbic acid	1.70±0.10**
Phe+($FeSO_4$+ascorbic acid)	1.65±0.13**
Cys+($FeSO_4$+ascorbic acid)	2.55±0.20
GSH+($FeSO_4$+ascorbic acid)	2.45±0.23
$FeSO_4$+ascorbic acid+H_2O_2	1.12±0.08**
Phe+($FeSO_4$+ascorbic acid+H_2O_2)	1.20±0.10**
Cys+($FeSO_4$+ascorbic acid+H_2O_2)	2.35±0.27
GSH+($FeSO_4$+ascorbic acid+H_2O_2)	2.33±0.24

$FeSO_4$ (84 μM), ascorbic acid (400 μM) or $FeSO_4$, ascorbic acid and H_2O_2 (1 mM) were incubated for 10 min at 37 °C with the 1 ml cuvette mixture containing 0.1 mg protein from homogenized adult brain (4 month old). The cuvette mixture and assay conditions of enzyme activity are described in detail in materials and methods. L-phenylalanine (Phe) (1.80 mM), L-cysteine (Cys) (0.83 mM or 2.48 mM) or glutathione reduced (GSH) (0.83 mM) was preincubated for 1 h. Values represent means±SD of three independent experiments. The average value of each experiment came from three determinations. **:p<0.01; ***: p<0.001; compared to control.

tion, a direct effect of Cys or GSH on this enzyme may occur, as well as an indirect one through free radical binding on Cys or GSH. Contrary to Phe plus free radical production, Na^+,K^+-ATPase inhibition was completely reversed by Cys or GSH preincubation ($p>0.05$; compared to control). Therefore, Cys and GSH could regulate towards normal values the neural excitability (Sastry and Phillis, 1977) and metabolic energy production (Mata *et al.*, 1980), which may be disturbed by free radical action on Na^+,K^+-ATPase.

Figure 2 presents the effect of 1 h preincubation of brain homogenates with Phe (1.80 mM) alone (B) and prior to free radical production (F, K), Cys (0.83 mM or 2.48 mM) alone (C) and prior to free radical production (G, L) or GSH (0.83 mM) alone (D) and prior to free radical production (H, M) on TAS changes in the homogenate. In this figure, TAS changes, induced only by $FeSO_4$ + ascorbic acid and $FeSO_4$+ascorbic acid+H_2O_2, are represented by E and I columns, respectively. The above TAS values are compared to a control value (A). We found that the production of free radicals by the two systems reduced TAS value by 50–60% (E, I). When Phe alone was added (B), TAS remained unchanged ($p>0.05$). However, when Phe was incubated prior to free radical production (F, K), TAS was increased by 45–50%. Contrary to Phe, Cys or GSH produced a 150–180% increase in TAS value when added either alone (C, D) or prior to free radical production (G, H, L, M). TAS value reflects the amount of free radicals and the capacity of the antioxidant enzymes to limit the free radicals in the homogenate. TAS was found to be reduced (E, I) because the amount of free radicals increased. Phe, which binds to ·OH radicals only (Sun *et al.*, 1993; Van der Vliet *et al.*, 1994), stimulated TAS value by 45–50% (F, K). However, Cys and GSH stimulated TAS more than Phe (G, H, L, M), as Cys and GSH may bind to free radicals and activate antioxidant enzymes to limit free radicals more extensively.

Free radicals may bind to Cys or GSH, which contain sulfhydryl groups, so they are not able to induce brain Na^+,K^+-ATPase inhibition any more. The above results suggest that Cys protection against the inhibitory action of free radicals on brain Na^+,K^+-ATPase activity, could explain partly the mechanism of its action as an antioxidant drug. Na^+,K^+-ATPase inhibition observed in some aged brain areas (Table I) could be due to the decreased GSH concentration in the brain (Hazelton and

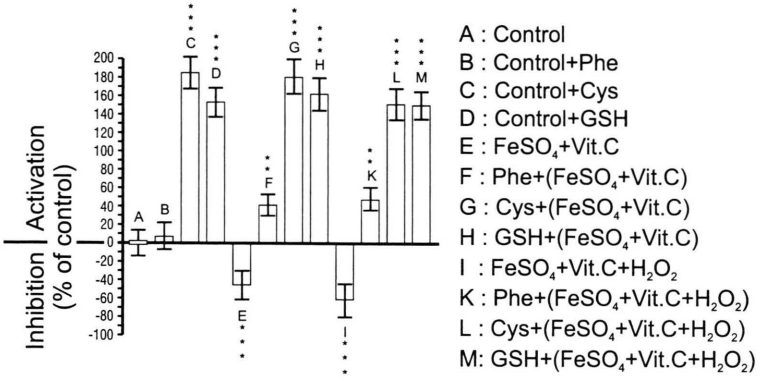

Fig. 2. Effect of $FeSO_4$/ascorbic acid/H_2O_2 free radical production and L-phenylalanine (Phe), L-cysteine (Cys) or reduced glutathione (GSH) preincubation on total antioxidant status (TAS) value determined in fresh homogenized adult whole brain. $FeSO_4$ (84 μM), ascorbic acid (400 μM) or $FeSO_4$, ascorbic acid and H_2O_2 (1 mM) were incubated with 50 mM Tris-HCl, pH 7.4, 240 mM sucrose, 0.1 mg protein from the homogenate in the presence or absence of Phe (1.80 mM), Cys (0.83 mM or 2.48 mM) or GSH (0.83 mM) for 10 min at 37 °C in a volume of 1 ml. Phe, Cys or GSH were preincubated separately for 1 h. A volume of 0.020 ml from the 1 ml incubation mixture was used for TAS value determination as described in Materials and Methods. TAS value of brain homogenate was 0.15±0.02 μmol/g fresh brain weight and that of the control (in the absence of $FeSO_4$/ascorbic acid/H_2O_2 systems plus Phe, Cys and GSH) was 0.57±0.08 mmol/l. Values are expressed as percentage of the control. Bars represent means±SD of seven experiments. **:$p<0.01$; ***:$p<0.001$; compared to control.

Lang, 1980; Benzi *et al.*, 1989) and/or to the rise of free radical action during aging.

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