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Research Report

Moderate exercise training and chronic caloric restriction modulate redox status in rat hippocampus

Katiane Santin[a,*], *Ricardo Fagundes da Rocha*[a,b], *Fernanda Cechetti*[a], *André Quincozes-Santos*[a], *Daniela Fraga de Souza*[a], *Patrícia Nardin*[a], *Letícia Rodrigues*[a,c], *Marina Concli Leite*[a], *José Cláudio Fonseca Moreira*[a], *Christianne Gazzana Salbego*[a], *Carlos Alberto Gonçalves*[a,c]

[a]*Department of Biochemistry, Institute of Health Basic Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Brazil*
[b]*Institute of Chemistry, Federal University of Rio Grande do Sul (UFRGS), Brazil*
[c]*Graduate Degree Program of Neurosciences, Institute of Health Basic Sciences (ICBS), Federal University of Rio Grande do Sul (UFRGS), Brazil*

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ABSTRACT

Physical activity has been related to antioxidant adaptations, which is associated with health benefits, including those to the nervous system. Additionally, available data suggest exercise and a caloric restriction regimen may reduce both the incidence and severity of neurological disorders. Therefore, our aim was to compare hippocampal redox status and glial parameters among sedentary, trained, caloric-restricted sedentary and caloric-restricted trained rats. Forty male adult rats were divided into 4 groups: ad libitum-fed sedentary (AS), ad libitum-fed exercise training (AE), calorie-restricted sedentary (RS) and calorie-restricted exercise training (RE). The caloric restriction (decrease of 30% in food intake) and exercise training (moderate in a treadmill) were carried out for 3 months. Thereafter hippocampus was surgically removed, and then redox and glial parameters were assessed. Increases in reduced glutathione (GSH) levels and total antioxidant reactivity (TAR) were observed in AE, RS and RE. The nitrite/nitrate levels decreased only in RE. We found a decrease in carbonyl content in AE, RS and RE, while no modifications were detected in thiobarbituric acid reactive substances (TBARS). Total reactive antioxidant potential (TRAP), superoxide dismutase (SOD) activity, S100B and glial fibrilary acid protein (GFAP) content did not change, but caloric restriction was able to increase glutamine synthetase (GS) activity in RS and glutamate uptake in RS and RE. Exercise training, caloric restriction and both combined can decrease oxidative damage in the hippocampus, possibly involving modulation of astroglial function, and could be used as a strategy for the prevention of neurodegenerative diseases.

1. Introduction

Much evidence suggests that aging and neurological disorders are associated with oxidative stress (Barja, 2004; Halliwell, 2001; Sinclair, 2005). The central nervous system (CNS) is prone to oxidative damage, since it presents a higher O_2 uptake (VO_2) than other organs/tissues, has lower antioxidant enzyme activity and contains large amounts of unsaturated fatty acids, which are targets for peroxidation (Dringen, 2000a; Halliwell, 2006). The main redox defense at the neural level is

* *Corresponding author at:* Rua Ramiro Barcelos, número 2600-anexo, 90035-003, Porto Alegre, RS, Brazil. Fax: +55 51 33085540.
E-mail address: katianesantin@yahoo.com.br (K. Santin).

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glutathione (GSH), and its precursors are provided mainly by astrocytes in the CNS (Dringen, 2000b).

Astrocytes are also the major glial cell responsible for glutamate removal from the synaptic cleft (Anderson and Swanson, 2000; Magistretti and Pellerin, 1999) and its conversion, through glutamine synthetase (GS) catalysis, into glutamine for replacement in the neurons (Bak et al., 2006). Several reactive oxygen/nitrogen species (RS) can decrease the uptake of glutamate by glial cells and inactivate glutamine synthetase (Aksenov et al., 1997). Moreover, oxidative stress can generate neural damage and promote excitatory amino acid release, creating a "vicious cycle" (Mailly et al., 1999). High glutamate release or failure in glutamate uptake by astrocytes, can lead to excessive and prolonged increases in intracellular free calcium (Ca^{++}) and sodium (Na^{+}), yielding excitotoxicity and often brain cell death by necrosis (Matute et al., 2006). Raised Ca^{++} levels can interfere with mitochondrial function, increasing superoxide radical ($.O_2^-$) production and activating neuronal nitric oxide synthase (nNOS) enzyme (Halliwell, 2006; Zhou and Zhu, 2009). nNOS catalyzes nitric oxide (NO.) synthesis, which then diffuses through the brain to exert its functional roles (Garthwaite, 2008). Despite the physiological functions of NO., in excessive amounts it can react with $.O_2^-$ leading to peroxynitrite ($ONOO.^-$) formation, which can damage proteins by nitration (Pacher et al., 2007).

The activity of astrocytes is commonly related to two protein markers: glial fibrilary acid protein (GFAP) and S100B. The former is the major intermediate filament protein in mature astrocytes (Rodnight et al., 1997) and its increased expression is observed in astrogliosis (O'Callaghan and Sriram, 2005). The S100B protein is a Ca^{++} binding protein, expressed and secreted by astrocytes, that has a trophic activity in neuron and glial cells with implications in neuronal survival (Tramontina et al., 2006; Van Eldik and Wainwright, 2003). Nonetheless, S100B overproduction by activated glia can lead to exacerbation of neuronal dysfunction and inflammation (Donato et al., 2009).

Caloric restriction (CR) increases the maximum and the mean life spans of laboratory rodents, suppresses a wide variety of time-related diseases, and modulates (preventing or delaying) much of the physiological changes associated with aging (Masoro, 2005; Mattson et al., 2001). Physical exercise also exerts a number of beneficial effects, including an increase in median life span (Mattson, 2000). Several reports indicate that CR and regular exercise modulate cellular antioxidant defenses and protect against free radical damage, suggesting that the beneficial adaptations could be mediated by their effects on the redox balance (Alessio and Goldfarb, 1988; Kim et al., 1996; Radak et al., 2007). Therefore, our aim was to compare hippocampal redox status and glial parameters among ad libitum-fed sedentary (AS), ad libitum-fed exercise (AE), calorie-restricted sedentary (RS) and calorie-restricted exercise (RE) rats.

2. Results

2.1. *Changes in body weight gain and serum biochemical parameters during physical training and caloric restriction*

The performances of the two rat groups (AE and RE) submitted to physical exercise during 12 weeks are shown in Fig. 1A. Their running capacity improved during the first 4 weeks of training and remained stable afterwards. No significant difference in exercise performance was observed in these groups, independently of ad libitum or restricted feeding. Body weight gain at the end of the experimental protocol is shown in Fig. 1B ($p<0,0001$). Rats submitted to caloric restriction, sedentary or not, had a decrease of 27% in body weight gain during these 12 weeks. Table 2 shows serum biochemical parameters for the four groups. No differences were observed in glycemia, total proteinemia or levels of reactive-C protein, indicating a good health state in all groups. LDL content decreased in rats submitted to caloric restriction, independent of training/sedentary protocol. No change was observed in HDL content. Total cholesterol decreased in rats submitted to caloric restriction and exercise (RE) compared to ad libitum fed rats. No significant changes were observed in triacylglycerol content among groups. Serum aspartate aminotransferase transaminase (AST) activity was lower in rats submitted to physical training (AE and RE) than in the AS and RS group. No change was detected in serum alanine aminotransferase transaminase (ALT). Urea levels decreased in rats submitted to caloric restriction, but creatinine levels were not different.

2.2. *Physical exercise and caloric restriction affect the hippocampal redox status*

The glutathione content in the hippocampus increased ($p=0.0004$) after caloric restriction and/or physical exercise (Fig. 2A). Moreover, using a chemiluminiscent assay with

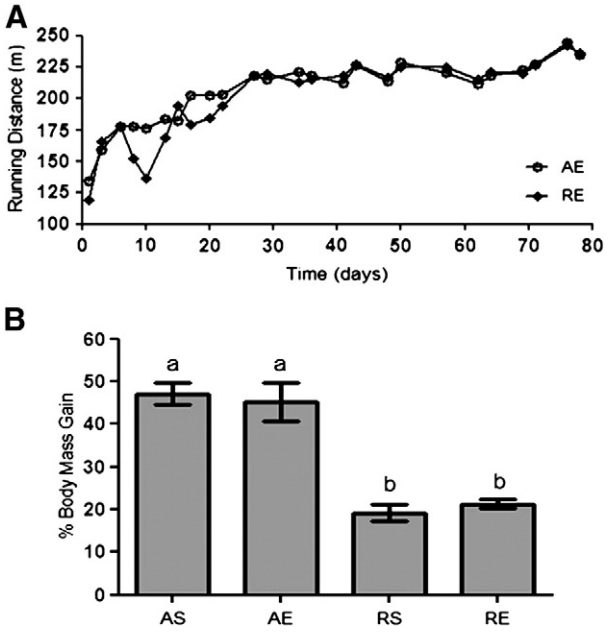

Fig. 1 – A: Rats performance throughout experiment period (n=10 for each group), evaluated only on ad libitum exercised (AE) and restricted exercised (RE) groups; Data are expressed as mean; B: Body mass gain after experiment period (n=10 for each group). Data are expressed as mean ± standard error of mean; (a) different of restricted groups (RS and RE), (b) different of ad libitum groups (AS and AE); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $\rho \leq 0.05$.

Table 1 – Laboratory chow composition.

Composition	g.Kg^{-1}
Total fat	110
Sunflower oil	5
Proteins	220
Fibers	30
Ash	60
Vitamins	20
Carbohydrates	520

Commercial nonpurified diet, Nuvilab-CRI (PR-Brazil).

luminol we found that total antioxidant reactivity (TAR) also increased ($p=0.0010$) after caloric restriction and/or physical exercise (Fig. 2B), but total reactive antioxidant potential (TRAP) was not different among groups (data not shown). It is also important to mention that hippocampal SOD activity was also not different (data not shown). Interestingly, the NO. content was reduced ($p=0.0021$) in rats submitted to the combination of caloric restriction and physical training (Fig. 2C).

Two parameters were investigated to evaluate hippocampal oxidative damage, for instance lipid peroxidation and protein carbonylation. Lipid peroxidation did not differ ($p=0.2423$) among groups (Fig. 3A), but caloric restriction and/or physical exercise decreased ($p=0.0006$)the content of protein carbonylation (Fig. 3B).

2.3. *Astroglial protein markers under the influence of caloric restriction and physical exercise*

Assuming that glutathione is predominantly astroglial, we investigated alterations in two specific markers for these cells; GFAP and S100B. No differences were observed in the hippocampal immunocontents of GFAP ($p=0.5647$) or S100B ($p=0.5216$) among the experimental groups (Figs. 4A and B, respectively).

2.4. *Astroglial activity measured by glutamate uptake and glutamine synthetase in hippocampal slices*

Two functional parameters of astroglial activity were investigated in hippocampal slices; glutamate uptake (Fig. 5A) and glutamine synthetase (Fig. 5B). Only the caloric restriction by itself (RS group) was able to induce an increase in GS activity ($p=0.0050$), while the hippocampal glutamate uptake ($p=0.0002$) was increased by caloric restriction independently of physical activity (RS and RE groups). In order to confirm the equivalent viability of ex-vivo hippocampal slices we measured MTT reduction capacity and found no differences ($p=0.5852$) among the studied groups (Fig. 5C).

3. Discussion

As life expectancy increases, the incidence of age-related neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease and stroke has increased, the last being the most prevalent (Mattson, 2000). In addition to benefiting learning and memory, extensive research demonstrates that exercise has neuroprotective effects, reducing brain injury and delaying onset of neurodegenerative diseases (Cotman et al., 2007; van Praag et al., 1999) while caloric restriction lowers the incidence of several other age-related diseases and is highly associated with increased longevity (Prolla and Mattson, 2001). Chronic exercise, through an adaptative response, is able to improve the antioxidant system, inhibiting the extension of the oxidative insult induced by acute exercise (Leeuwenburgh et al., 1997; Powers et al., 1994; Servais et al., 2003). Calorie-restricted animals display less RS-mediated damage (Barja, 2004; Merry, 2002), which may be related to a resistance to stress-induced apoptosis (Hiona and Leeuwenburgh, 2004).

Our study investigated the effects of moderate exercise training and chronic caloric restriction on hippocampus glial parameters and the CNS redox status of rats, since reactive oxygen/nitrogen species have been related to aging and neurodegenerative disease processes.

Differences in protocols results in different levels of oxidative stress, however the data are still controversial. The variability in results reported probably arises from differences in the exercise protocol (voluntary vs forced), the kind of exercise (running vs swimming) in combination with intensity (in forced exercise models) and duration of exercise exposure (acute vs chronic) (Cotman et al., 2007). Indeed, most benefits have been associated with longer-term exercise (3–12 weeks)

Table 2 – Serum biochemistry.

	AS	AE	RS	RE	P value
Glucose (mg.dL^{-1})	222±4	186±29	219±3	214±11	0.3957
Cholesterol (mg.dL^{-1})	69^{a}±4	75^{a}±5	60^{b}±4	56^{b}±2	0.0006
Triacylglicerol (mg.dL^{-1})	63±13	58±15	65±10	61±5	0.9804
HDL (mg.dL^{-1})	27±0.6	30±1.3	27±1,4	24±0.4	0.1034
LDL (mg.dL^{-1})	32^{a}±2	34^{a}±4	20^{b}±1	19^{b}±2	0.0009
Creatinine(mg.dL^{-1})	0.58±0.05	0.60±0.04	0.56±0.02	0.51±0.04	0.4919
Urea (mg.dL^{-1})	62^{a}±2.3	57^{a}±2.6	45^{b}±2.4	46^{b}±26	0.0003
Total Proteins (g.dL^{-1})	6.2±0.3	6.2±0.2	6.1±0.1	6.1±0.2	0.9394
C-Reactve Protein (mg.dL^{-1})	3.6±0.1	3.3±0.2	3.5±0.1	3.5±0.1	0.4353
AST (U.L^{-1})	112^{c}±5	86^{d}±1	95±2	87±1	0.0153
ALT (U.L^{-1})	55±2	50±1	51±4	49±4	0.5410

Data are expressed as mean±standard error of mean (n=5 for each group). (a) different of restricted groups (RS and RE), (b) different of ad libitum groups (AS and AE), (c) different of ad libitum exercised (AE), (d) different of ad libitum sedentary (AS). Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $p\leq 0{,}05$.

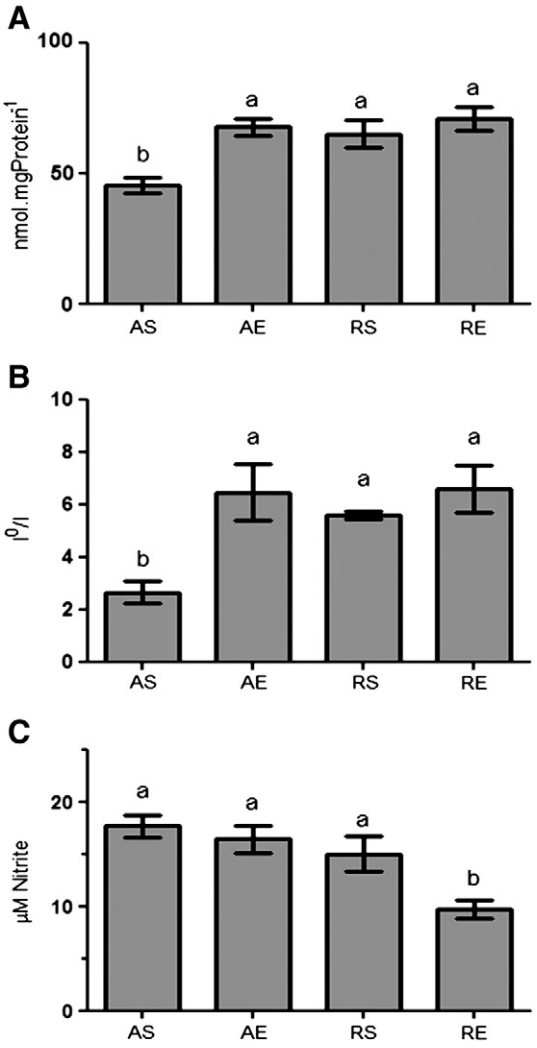

Fig. 2 – A: Reduced glutathione (GSH) content; (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); B: Total antioxidant reactivity (TAR); (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); C: Nitrite (NO2–) and nitrate (NO3–) levels. (a) different of restricted exercised (RE), (b) different of ad libitum sedentary (AS), of ad libitum exercised (AE) and of restricted sedentary (RS); Data are expressed as mean ± standard error of mean (n = 5 for each group); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $\rho \leq 0.05$.

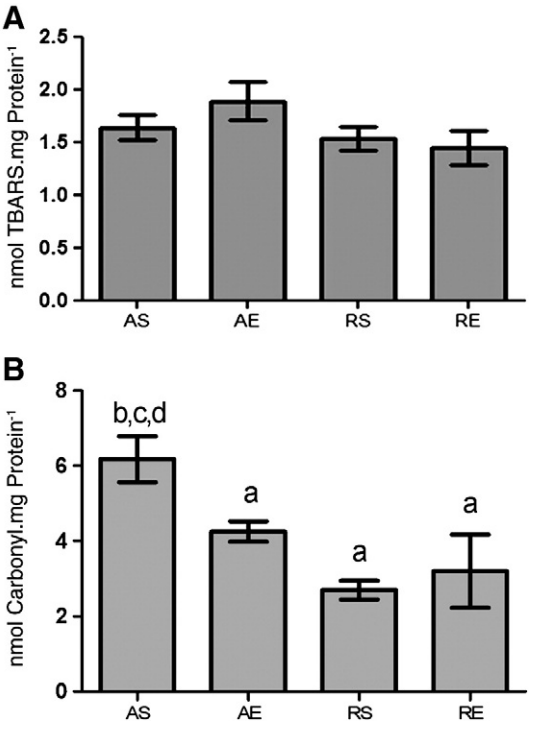

Fig. 3 – A: Thiobarbituric acid reactive species (TBARS) content; B: Protein carbonyl content; Data are expressed as mean ± standard error of mean (n = 5 for each group); (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), of restricted sedentary (RS) and of restricted exercised (RE); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $\rho \leq 0.05$.

(O'Callaghan et al., 2007; Schweitzer et al., 2006; van Praag et al., 2005). It has been demonstrated that performance of moderate intensity exercise (treadmill training protocol) with a frequency of three times a week, for 12-weeks, reduces damage in hippocampal slices from Wistar rats that were submitted to an in vitro ischemia protocol, suggesting exercise-induced neuroprotection (Cechetti et al., 2007).

The CR increases longevity, independently of whether protein intake is also reduced or not, providing evidence that energy intake could play a greater role in life extension than protein intake reduction (Masoro, 2005). A 30% reduction in calorie intake extends the life spans of rats and mice by 30–40%, and maintenance of this regimen for 2–4 months results in lower levels of oxidative stress in the hippocampus, compared to mice fed ad libitum, indicating that suppression of oxidative stress may be one mechanism underlying the neuroprotective effect of caloric restriction (Mattson, 2000).

As an indirect measure of training status, we observed that the rats' performance improved during the 3 months of running training, in agreement with other studies (Radak et al., 2005; Radak et al., 2006). Moreover, the CR-fed rats (RS and RE) presented a lower body mass gain than ad libitum-fed rats (AS and AE), in accordance with other reports (Horska et al., 1999; Wanagat et al., 1999). To ensure that the animals were not undernourished or physically exhausted, we performed biochemical serum analysis. The CR-fed rats, RS and RE, showed benefits in health, demonstrating lower levels of total cholesterol and LDL cholesterol than ad libitum-fed rats. Additionally, the exercised rats, AE and RE, displayed lower AST activities than the sedentary, AS and RS group, suggesting that the exercise training program used in this experiment results in lower muscular tissue damage (Nelson and Cox, 2005). Although creatinine levels were not different, we found decreased urea serum levels in CR-fed rats, indicating that renal glutaminase activity may be altered in these animals, as observed in a prior study (Ribeiro et al., 2009). Since no

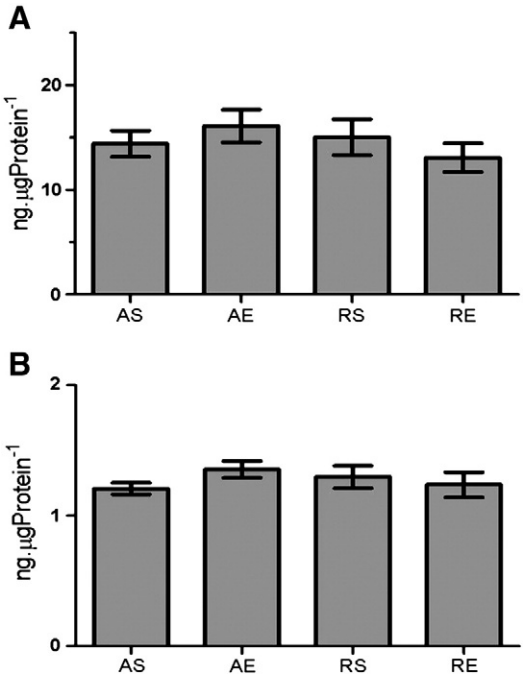

Fig. 4 – A: Glial fibrilary acid protein (GFAP) content; B: S100B content; Data are expressed as mean ± standard error of mean (n = 5 for each group); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $\rho \leq 0.05$.

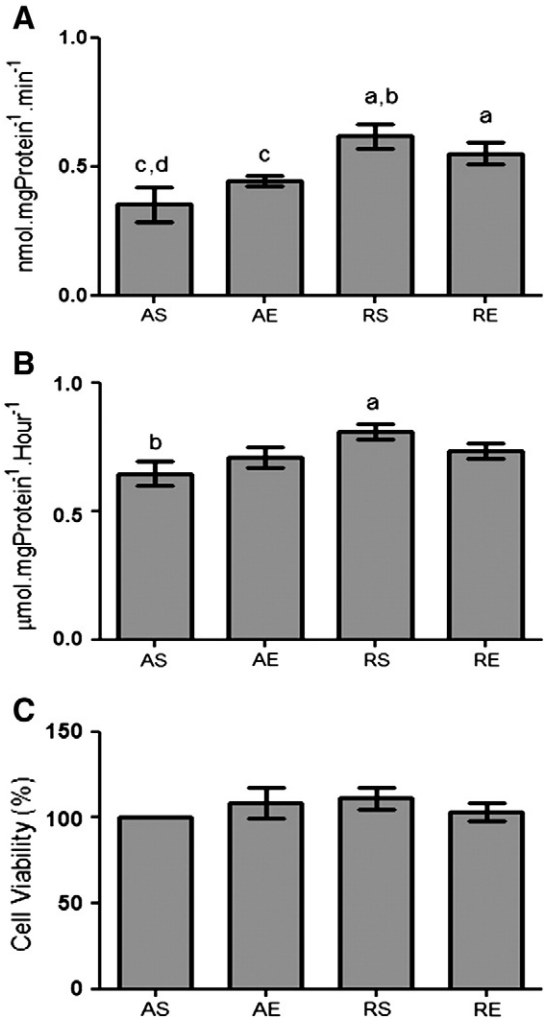

Fig. 5 – A: Glutamate uptake; (a) different of ad libitum sedentary (AS), (b) different of ad libitum exercised (AE), (c) different of restricted sedentary (RS) and (d) different of restricted exercised (RE) B: Glutamine synthetase activity; (a) different of ad libitum sedentary (AS), (b) different of restricted sedentary (RS), C: Cell viability; Data are expressed as mean ± standard error of mean (n = 5 for each group); Differences were determined with Anova one way followed by Newman Keul's pos hoc and the accepted significance level was $\rho \leq 0.05$.

differences in other parameters were observed, rats were deemed to be healthy.

GSH is an important redox sensor for most cell types, and plays a crucial role as a redox buffer in the CNS (Aoyama et al., 2008). Previous studies have shown that GSH declines with aging (Maher, 2005) and that GSH deficiency is involved in neurodegenerative diseases (Christen, 2000; Sian et al., 1994). The three interventions studied were each able to increase GSH levels and, probably as a consequence, the TAR. TRAP and the TAR are parameters of total non-enzymatic capacity, although TAR is more related to antioxidant quality while TRAP is more related to antioxidant amount (Lissi et al., 1995).

Our study demonstrated that non-enzymatic adaptation may be responsible for the reduced damage, as opposed to enzymatic adaptation, since, in the present study, no modifications were found in SOD activity. Somani and coworkers also found no changes in hippocampal SOD activity after 7.5-weeks of exercise (Somani et al., 1995). In contrast, Devi and Kiran found increased SOD activity in the hippocampus after 4 months of swimming exercise, compared to controls, while Asku and coworkers demonstrated a decreased SOD activity when regular treadmill exercise was performed at different strengths (Aksu et al., 2009; Devi and Kiran, 2004). Different results in these studies may be due to the differences in type, duration and intensity of the exercise.

Protein carbonyl content, a measure of protein oxidation, increases with age most rapidly in the hippocampus and striatum (Dubey et al., 1996), regions associated with significant losses in function due to the aging process. To evaluate oxidative damage in the hippocampus, we measured the lipid peroxidation by TBARS and protein oxidation by carbonyl content. Although no differences were seen for TBARS, the exercise training and caloric restriction, and both combined, decreased carbonyl contents, indicating an attenuation of aging that is in agreement with other caloric restriction studies (Dubey et al., 1996; Forster et al., 2000). Other authors found similar results for TBARS following chronic exercise (Aksu et al., 2009; Coskun et al., 2005).

NO. plays important roles in central nervous system, participating in neurogenesis, neuron differentiation and development, memory and neuroprotection (Garthwaite, 2008). In addition, NO. can react very fast with O_2^- to form peroxynitrite ($ONOO^-$), which can directly oxidize and nitrate proteins, lipids

and DNA (Alvarez and Radi, 2003). NO_2^-/NO_3^-levels are a good indication of NO. production, since these molecules are the metabolism end products of NO. (Halliwell and Whiteman, 2004; Levine, 2002). The decreased NO_2^-/NO_3^-levels observed following the combination of the two approaches could indicate that only regular exercise associated with chronic caloric restriction are effective in reducing NO.. Interestingly, Asku and coworkers did not find any change in brain (prefrontal cortex, striatum and hippocampus) nitrate–nitrite levels after chronic treadmill exercise by itself (Aksu et al., 2009).

Astrocytes are closely associated with neurons in glutamatergic transmission and, consequently, with synaptic plasticity and neuroprotection (Chen and Swanson, 2003; Tramontina et al., 2006). In the present study, we investigated important astroglial functions such as glutamate uptake (which avoids excitotoxicity damage that could lead to neuronal death) (Danbolt, 2001), and the ability to convert glutamate into glutamine via glutamine synthetase activity. Although only caloric restriction by itself (RS group) was able to increase GS activity, this intervention, associated or not to exercise, showed an increase in glutamate uptake from extracellular media due to modulation in activity and/or the amount of glutamate transport, enhancing one of the most important functions of astrocytes. This is in agreement with prior work (Ribeiro et al., 2009).

After injury of the CNS, either as a result of trauma, disease, genetic disorders or chemical insult, astrocytes become reactive, termed astrogliosis, and this activation is characterized by an increase in GFAP (Eng et al., 2000). Increasing evidence indicates that S100B exerts functional roles by acting as an intracellular regulator and an extracellular signal (Donato et al., 2009). S100B is secreted by an unknown mechanism and has dual effects: at nanomolar levels, S100B stimulates neurite growth and promotes neuronal survival, and at micromolar levels this protein produces undesirable events such as neuronal apoptosis (Donato et al., 2009; Van Eldik and Wainwright, 2003). High levels of brain tissue S100B have been found in neurodegenerative disorders, including Alzheimer's disease (Griffin et al., 1998). In the present study, GFAP and S100B contents were not influenced by caloric restriction and/or exercise, indicating absence of astrogliosis and non predisposition to apoptosis (O'Callaghan and Sriram, 2005).

4. Experimental procedures

4.1. Chemicals

N-methyl-D-glucamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and all other reagents were purchased from Sigma Chemical CO (St. Louis, MO); L-[3H]glutamate (specific activity 30 Ci.mmol^{-1}) was purchased from Amersham International, UK.

4.2. Animals

All experiments were approved by the Local Animal Care Committee. Experiments were carried out with 40 male 60-day-old Wistar rats obtained from our breeding colony. The animals were kept under standard laboratory conditions (12 h light/dark, 22±2 °C) with water ad libitum. Animals were weight matched and divided into four experimental groups (n=10 to each): ad libitum-fed sedentary (AS); ad libitum-fed exercise (AE); calorie-restricted sedentary (RS) and calorie-restricted exercise (RE).

4.3. Training protocol

Rats were habituated with the treadmill apparatus to minimize novelty stress. Moderate exercise training was conducted as previously described (Cechetti et al., 2007). Briefly, running sessions consisted of 20 min (min), three times a week for twelve weeks, on an adapted motorized rodent treadmill (INBRAMED TK01®, Brazil) at 60% of their maximal oxygen uptake (VO_2max) (Brooks and White, 1978). The oxygen uptake peak (VO_2peak) was measured in all animals, indirectly before training. All rats ran on a treadmill at a low initial speed followed by increases in 5 m.min^{-1} speed every 3 min until the exhaustion point. The time to fatigue (in min) and workload (in m.min^{-1}) were recorded as indexes of capacity for exercise, which was taken as VO_2max (Cechetti et al., 2007). Neither electric shock nor physical prodding was used in this study.

4.4. Caloric restriction diet

All the animals received a regular laboratory chow (Nuvilab-CR1® from Nuvital, Brazil), as presented in Table 1. The caloric restriction diet was conducted as previously described (Ribeiro et al., 2009). Briefly, the treatment was progressive, being initiated at 10% restriction in the first week, changing to 20% at the second week and to 30% at the third week, maintained until the end of the experiment (at week twelve). Hence, the rats on the RS and RE were provided with an amount of food equivalent to 70% of that consumed by rats in the AS and AE groups, respectively. The food intake was monitored daily, and the animals were weighed weekly (Chang et al., 2007; Horska et al., 1999).

4.5. Biochemical analysis

48 h after the last training session, animals were overnight-starved (12 h fasting) and anesthetized with an intramuscular injection of ketamine and xylazine (75 and 10 mg.Kg^{-1}, respectively). Blood samples were obtained from intracardiac punction, and the animals were killed by decapitation. The blood samples were kept at room temperature (25 °C) for 30 min and then centrifuged at 1000 *g* for 5 min. The serum was collected and the biochemical analyses were carried out on the same day, in a Multi-test Analyzer (Labmax 240® from Labtest, Brazil), using specific kits supplied by Labtest: total protein, C-reactive protein (CRP), aspartate aminotransferase transaminase (AST), alanine aminotransferase transaminase (ALT), creatinine, urea, glucose, triacylglycerol, total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL).

4.6. Hippocampal dissection

The brains were removed and placed in cold saline medium with the following composition (in mM): NaCl 120, KCl 2, $CaCl_2$ 1, $MgSO_4$ 1, HEPES 1, KH_2PO_4 1 and glucose 10 adjusted to pH 7.4 and previously aerated with oxygen (O_2). The hippocampi were quickly dissected out and one hemisphere of each animal was

transformed in transverse sections (300 μm) using a Mcllwain tissue chopper, while the other one was stored at −70 °C for subsequent analysis.

4.7. Glutathione content

Reduced GSH content was determined as previously described (Browne and Armstrong, 1998). Briefly, slices were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 0.005 M EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdeyde (1 mg.mL^{-1} of methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM). GSH concentrations were calculated as nmol.mg protein^{-1}.

4.8. TRAP and TAR

The non-enzymatic antioxidant potential of the reproductive tract structures was estimated by the total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) (Lissi et al., 1995). The reaction was initiated by adding luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, 4 mM) – an external probe for monitoring radical production – and AAPH (2,2′-Azobis-2-methyl-propianamidine-dihydrochloride, 10 mM) – a free radical source that produces peroxyl radical at a constant rate – in glycine buffer (0.1 M, pH 8.6) at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read with a liquid scintillation counter (Wallace 1409®) as counts per minutes. Sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (100 μg of protein) in a TRAP protocol, and the area under the curve (AUC) was quantified (Dresch et al., 2009). The TAR was calculated and expressed as a ratio of the luminescense without sample for the first luminescence after sample addition.

4.9. Superoxide dismutase activity

Superoxide dismutase (E.C.1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich, 1972). Results were expressed as Units SOD.mg protein^{-1}.

4.10. Nitrite-nitrate levels

The NO. metabolites, nitrites (NO_2^-) and nitrates (NO_3^-) were determined as previously described (Hevel and Marletta, 1994). Five hundred microliters of supernatant were deproteinized with 20 μL 25% (w/v) trichloroacetic acid and centrifuged at 1800×*g*. The supernatant was immediately neutralized with 35 μL 2 M potassium bicarbonate and used for the quantification of $NO_2^-$and NO_3^-. $NO_3^-$was reduced to $NO_2^-$by nitrate reductase. The total NO_2^-in the incubation was measured by a colorimetric assay at 540 nm based on the Griess reaction. A standard curve was performed in the same way using sodium nitrate (0–80 μM). Results were expressed as μM NO_2^-.mg protein^{-1}.

4.11. Measurement of protein carbonyls

The oxidative damage to proteins was measure by the quantification of carbonyl groups, based on a reaction with dinitrophenylhidrazine, as previously described (Levine et al., 1990). Proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhidrazine and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl.mg protein^{-1}.

4.12. TBARS

As an index of lipid peroxidation, the formation of thiobarbituric acid reactive species (TBARS) was monitored during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 mL of 10% trichloroacetic acid and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. Thiobarbituric acid reactive species were determined by the absorbance in a spetrophotometer at 532 nm. Results were expressed as thiobarbituric acid reactive species.mg protein^{-1}.

4.13. GFAP measurement

Enzyme-linked immunossorbent assay was carried out for measurement of GFAP content, as previously described (Tramontina et al., 2007). Briefly, the microtiter plate was coated with 100 μL samples containing 500 ng of protein for 24 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase or 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 493 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng.mL^{-1}.

4.14. S100B measurement

An enzyme-linked immunossorbent assay was carried out for measurement of intracellular S100B content, as previously described (Leite et al., 2008). Briefly, 50 μL of sample (5–10 ng.μL^{-1} of total protein) plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate previously coated with anti-S100B monoclonal antibody (SH-B1, from Sigma). Anti-S100 polyclonal antibody (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng.mL^{-1}.

4.15. Glutamate uptake assay

Hippocampal slices were transferred immediately to 24-well culture plates, each well containing 0.3 mL of physiological medium and only one slice. Glutamate uptake was measured as previously described (Thomazi et al., 2004) with some modifications. Medium was replaced by Hank's balanced salt solution (HBSS) containing NaCl 137 mM; Na2HPO4 0.63 mM, NaHCO3 4.17 mM, KH2PO4 0.44 mM, KCl 5.36 mM, CaCl2 1.26 mM, MgSO4

0.41 mM, MgCl2 0.41 mM and glucose 5.55 mM, in pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.66 Ci.mL^{-1} L-[2,3- 3H] glutamate. Incubation was stopped after 10 min by removal of the medium and rinsing the slices twice with ice-cold HBSS. Slices were then lysed in a solution containing 0.5 M NaOH. Final glutamate uptake was obtained by discounting non-specific uptake from specific uptake in assays carried out in sodium-free medium, prepared by replacing NaCl with choline chloride in the HBSS. Radioactivity was measured with a scintillation counter and the results were expressed as nmol.mg protein^{-1}.min^{-1}.

4.16. *Glutamine synthetase activity*

The enzymatic assay was performed, as previously described (dos Santos et al., 2006). Briefly, homogenate (0.1 mL) was added to 0.1 mL of reaction mixture containing (in mM): 10 MgCl2; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 150 ATP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the supernatant's absorbance was measured at 530 nm and compared to the absorbance generated by standard quantities of -glutamylhydroxamate, treated with ferric chloride reagent.

4.17. *Mitochondrial viability assay*

Mitochondrial activity was evaluated by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, after a recovery period, slices were incubated in a medium, containing 45 μg.mL^{-1} MTT, for 45 min at 37 °C. Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of the soluble yellow MTT dye to the insoluble purple formazan, which was extracted in dimethyl sulfoxide (DMSO) (Mosmann, 1983). The optical density was measured at 570 and 630 nm, and the net A570–A630 was taken as an index of cell viability (Siqueira et al., 2004).

4.18. *Protein content*

The total protein content was determined by the modified method of Lowry (Peterson, 1977), using bovine serum albumin (BSA) as standard.

4.19. *Statistical analysis*

Data are expressed as mean ± standard error of mean (SE). The values were considered significant when $\rho \leq 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Bonferroni's test when indicated.

5. Conclusion

The present study demonstrated an improvement in antioxidant system following exercise training, caloric restriction and both in combination, leading to a significant modulation of astroglial functions. Moreover, caloric restriction improved glutamate uptake and glutamine synthetase activity, which could be related to a lower risk of excitotoxicity. These findings provide new insights into how caloric restriction, allied with regular physical activity, could be a strategy for the prevention of neurodegenerative diseases. We believe that these results combined show for the first time that these approaches avoid oxidative stress and are highly involved on glia function. Mattson and others has evaluated the effect of caloric restriction on neural function, but in the present work we evaluated the glial function, what is a novelty, mainly when regarding exercise training together.

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