Research Report

Exercise effects on activities of Na⁺,K⁺-ATPase, acetylcholinesterase and adenine nucleotides hydrolysis in ovariectomized rats

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ABSTRACT

Hormone deficiency following ovariectomy causes activation of Na⁺,K⁺-ATPase and acetylcholinesterase (AChE) that has been related to cognitive deficits in experimental animals. Considering that physical exercise presents neuroprotector effects, we decide to investigate whether exercise training would affect enzyme activation in hippocampus and cerebral cortex, as well as adenosine nucleotide hydrolysis in synaptosomes from cerebral cortex of ovariectomized rats. Female adult Wistar rats were assigned to one of the following groups: sham (submitted to surgery without removal of the ovaries), exercise, ovariectomized (Ovx) and Ovx plus exercise. Thirty days after surgery, animals were submitted to one month of exercise training, three times per week. After, rats were euthanized, blood serum was collected and hippocampus and cerebral cortex were dissected. Data demonstrated that exercise reversed the activation of Na⁺,K⁺-ATPase and AChE activities both in hippocampus and cerebral cortex of ovariectomized rats. Ovariectomy decreased AMP hydrolysis in cerebral cortex and did not alter adenine nucleotides hydrolysis in blood serum. Exercise per se decreased ADP and AMP hydrolysis in cerebral cortex. On the other hand, AMP hydrolysis in blood serum was increased by exercise in ovariectomized adult rats. Present data support that physical exercise might have beneficial effects and constitute a therapeutic alternative to hormone replacement therapy for estrogen deprivation.

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1. Introduction

Estrogen displays important roles beyond the reproductive system, such as trophic and protective role in the adult brain (Wise et al., 2001b, 2002) and it has been shown that estrogen deprivation is implicated in the pathogenesis of Alzheimer's disease and cerebral ischemia (van Duijn, 1999; Zhang et al., 1998). Studies have suggested that post-menopausal women

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are more vulnerable to such diseases and to cognitive deficits (Green and Simpkins, 2000; Wise et al., 2001a,b). Hormone replacement therapy (HRT), in the form of estrogen and progesterone or estrogen alone, has been used to treat menopause symptoms. However, due to the possible side effects of HRT, such as breast cancer and increased risk of thromboembolic accidents, there is a growing demand for alternatives for the treatment of pathological processes and symptoms associated with menopause (Miquel et al., 2006); physical exercise has been proposed as an alternative therapeutic tool.

Evidence suggests that exercise may support brain health and function; consistent to that, there are studies indicating that physical activity may reduce age-induced cognitive decline and it is recommended as a therapeutic strategy to prevent, or recover from, neurodegenerative diseases (Kramer et al., 1999; Mattson, 2000). In this context, it has been shown that exercise increases levels of brain-derived neurotrophic factor (BDNF) and other growth factors that stimulate neurogenesis, increases resistance to brain insult and was proposed to improve learning and performance (Cotman and Berchtold, 2002; van Praag et al., 2005). Studies suggest that dynamic physical exercise produces elevated regional cerebral blood flow (CBF), alterations in endogenous peptides and neurotransmitters, and increases amino acid transport through the blood brain barrier (Hollmann et al., 1994; Ide et al., 1999). Although the exact molecular mechanisms by which physical exercise affects brain function are unclear, it has been suggested that it might activate cellular and molecular pathways that contribute to neuroprotection (Cotman and Berchtold, 2002; van Praag et al., 2005).

Na$^+$,K$^+$-ATPase (E.C 3.6.1.37) is responsible for the generation of membrane potential through the active transport of sodium and potassium ions. This enzyme is necessary to maintain the ionic gradient for neuronal excitability, consuming about 40–50% of the ATP generated in brain cells (Erecinska and Silver, 1994). Na$^+$,K$^+$-ATPase activity is inhibited by free radicals and its activity is reduced in cerebral ischemia (Wyse et al., 2000) and in neurodegenerative diseases as Alzheimer's disease (Gómez-Ramos and Morán, 1997; Hattori et al., 1998). In addition, we have recently demonstrated that ovariectomy increases Na$^+$, K$^+$-ATPase activity in synaptic plasma membranes of rat hippocampus (Monteiro et al., 2005b).

ATP and acetylcholine serve as extracellular signaling substances in the nervous system and in other tissues. They can even be co-stored within synaptic vesicles and co-released from cholinergic nerves. Neither ACh nor ATP can be directly recycled. They must first be degraded to either choline or adenosine and those substances are transported back into cells. Acetylcholine is specifically hydrolyzed by acetylcholinesterase (AChE) (E.C. 3.1.1.7). This enzyme has been implicated in cholinergic and non-cholinergic actions that may play a role in neurodegenerative diseases (Cummings, 2000); it has been also shown that AChE per se activates neuronal cell death (Calder'on et al., 1998). On the other hand, it is known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Gibbs and Aggarwal, 1998; Simpkins et al., 1997). In addition, we have shown that ovariectomized female adult rats present an increase of brain AChE activity (Monteiro et al., 2005b, 2007).

ATP and the other extracellular nucleoside tri- and diphosphates can be hydrolyzed by NTPDases (nucleoside triphosphate diphosphohydrolases), which are enzymes that hydrolyze ATP and ADP, and are present in many tissue, including the vascular system (Ralevic and Burnstock, 2003) and central nervous system (SNC) of several species (Sarkis et al., 1995). The AMP produced is subsequently hydrolyzed to adenosine by an ecto-5′-nucleotidase (CD73, EC 3.1.3.5), which constitutes the rate-limiting step in this pathway (Battastini et al., 1995; Zimmermann, 1992). Extracellular ATP and its breakdown products, ADP and adenosine, have pronounced effects in a variety of biological processes (Agteresch et al., 1999). It has been suggested that steroid hormone deprivation can modulate the expression and activity of an ecto-ATPase of hippocampus and caudate nucleus (Nedeljkovic et al., 2000), and it demonstrated a down regulation in adenosine receptors in response to ovariectomy using total brain (Rose'Meyer et al., 2003).

In the present study we investigated the influence of physical exercise on the effects elicited by ovariectomy on Na$^+$, K$^+$-ATPase, AChE activities and adenine nucleotide hydrolysis in hippocampus and/or cerebral cortex of ovariectomized rats, respectively. The working hypothesis is that exercise would reverse the effects of ovariectomy over enzyme activities.

2. Results

The effect of exercise on Na$^+$,K$^+$-ATPase in female adult Wistar rats is shown in Fig. 1. Animals subjected to ovariectomy presented a significant increase (14%) of cerebral cortex Na$^+$,K$^+$-ATPase activity (Panel 1A) and exercise reversed the stimulation caused by ovariectomy [$F(3,33) = 5.17$; $p < 0.01$]. Panel 1B shows that Na$^+$,K$^+$-ATPase activity was significantly increased (11%) in hippocampus of rats subjected to ovariectomy, which was reversed by exercise [$F(3,30) = 3.49$; $p < 0.05$]. Exercise per se did not alter Na$^+$,K$^+$-ATPase activity, with exception of hippocampal Na$^+$,K$^+$-ATPase, that had a tendency to increase with exercise.

The effect of exercise on acetylcholinesterase in female adult Wistar rats is showed in Fig. 2. Fig. 2a shows that animals subjected to ovariectomy presented a significant increase (42%) of cerebral cortex AChE activity and exercise reversed such effects [$F(3,39) = 5.21$; $p < 0.01$]. Fig. 2b shows that AChE activity was significantly increased (29%) in hippocampus of rats subjected to ovariectomy, and that effect was reversed by exercise [$F(3,43) = 7.77$; $p < 0.001$]. Exercise per se did not alter AChE activity.

The effects of ovariectomy and exercise on hydrolysis of ATP, ADP and AMP in synaptosomes from cerebral cortex and blood serum of female adult Wistar rats are shown in Table 1. When compared to sham group, Ovx group did not show any significant difference in ATP hydrolysis and have a tendency to decrease ADP hydrolysis in cerebral cortex. Animals submitted to exercise per se or exercise and Ovx did not show significant changes in ATP, but decreased significantly ADP hydrolysis in this same cerebral structure [$F(3,15) = 3.67$; $p < 0.05$]. Results demonstrated a decrease in AMP hydrolysis in the cerebral cortex of exercised, ovariectomized, and ovariectomized rats submitted to exercise [$F(3,14) = 3.87$; $p < 0.05$] when compared to the sham group (Table 1).
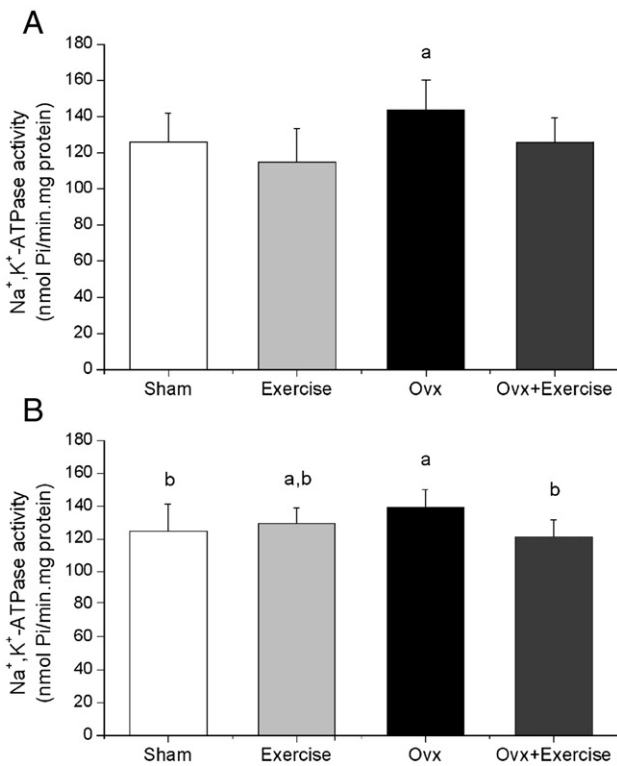
Fig. 1 – Effect of ovariectomy and exercise on Na⁺,K⁺-ATPase activity in cerebral cortex (a) and hippocampus (b) of female adults rats. Data are expressed as mean±S.D. for 8 to 10 animals in each group. $^a p < 0.05$ compared to sham group and $^b p < 0.05$ compared to Ovx group (Duncan's multiple range test). Ovx—ovariectomized.
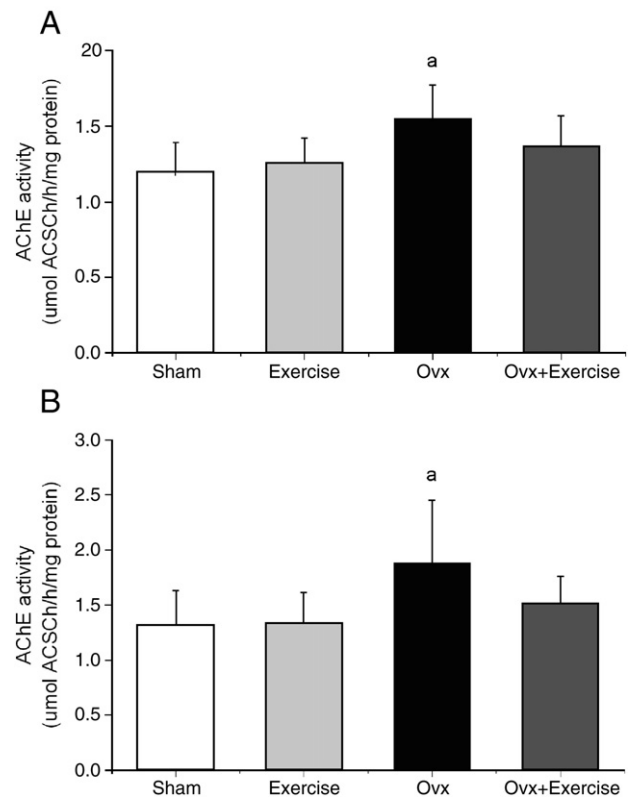
Fig. 2 – Effect of ovariectomy and exercise on acetylcholinesterase activity in cerebral cortex (a) and hippocampus (b) of female adult rats. Data are expressed as mean±S.D. for 10 to 13 animals in each group. $^a p < 0.01$ compared to sham group (Duncan's multiple range test). Ovx—ovariectomized.

In blood serum, Ovx or exercise group did not show any significant difference in ATP and ADP hydrolysis compared to sham group. However, results show an increase in AMP hydrolysis in the blood serum of ovariectomized rats submitted to exercise [$F_{(3,13)} = 4.74$; $p < 0.05$] when compared to the sham group (Table 1).

We observed that the animal weight gain was increased by ovariectomy [$F_{(3,43)} = 6.04$; $p < 0.01$] (Table 2). As can be observed in this table, exercise reversed body weight gain of ovariectomized rats.

3. Discussion

A growing number of studies indicate the brain as one of the body organs that suffers from the loss of estrogen in menopause and that damage from stroke and neurodegeneration in dementia may be retarded by estrogenic actions (McEwen, 2002). It has also been shown that post-menopausal estrogen replacement therapy reduces the risk and delay in the onset of these diseases (van Duijn, 1999; Yaffe et al., 1998). On the other hand, evidence showed that estrogen plus progestin therapy to postmenopausal women increased the risk for dementia in women aged 65 years or older and did not improve cognitive impairment in these women (Shumaker et al., 2003).

In the present study, we investigated the influence of exercise on the activation of hippocampal and cerebral cortex Na⁺,K⁺-ATPase and AChE activities caused by ovariectomy, as well as on nucleotide hydrolysis in cerebral cortex and blood serum of Ovx rats. We used this animal model of steroid hormone deprivation because ovariectomy is considered the

Table 1 – Effect of ovariectomy and exercise on nucleotide hydrolysis in synaptosomes from cerebral cortex and in serum of female adult rats.			
Groups	ATP	ADP	AMP
Cerebral cortex (nmol P/min/mg)			
Sham	86.85±7.55	40.45±9.60	19.39±2.80
Exercise	90.68±12.10	26.96±4.80*	14.38±3.34*
Ovx	88.87±13.54	36.53±8.35*#	15.42±1.49*
Ovx+exercise	76.47±16.77	27.19±5.69*	15.60±1.69*
Serum (nmol P/min/mg)			
Sham	14.45±2.75	14.46±1.70	12.26±1.38
Exercise	14.90±5.55	16.36±4.11	13.34±0.46
Ovx	14.29±1.28	12.54±2.89	11.93±1.08
Ovx+exercise	12.37±1.07	12.47±2.16	16.70±3.74*

Data are expressed as mean±S.D. for 4 to 6 animals in each group. *$p < 0.05$ compared to sham group (Duncan's multiple range test), #$p < 0.05$ compared to exercise and Ovx+exercise group.

Table 2 – Effect of ovariectomy and exercise on body weight of female adult rats.

Groups	Body weight (g)	Body weight (g)
	First day of experiment	After 30 days of exercise
Sham	187.69±12.77	222.54±20.56
Exercise	189.08±12.73	216.00±8.43
Ovx	189.17±10.44	239.83±9.70*
Ovx+exercise	189.60±10.05	227.60±14.22

Data are presented as mean±S.D. for 10 to 13 rats in each group. Ovariectomized rats were significantly different from sham groups after 30 days of training *$p < 0.01$ compared to other groups (ANOVA).

most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). The hippocampus and cerebral cortex were studied because these cerebral structures are associated with memory modulation (Daniel and Dohanich, 2001) and ovariectomized rats present memory impairments (Monteiro et al., 2005a; Singh et al., 1994).

Our results showed that ovariectomy significantly increased Na$^+$,K$^+$-ATPase and AChE activities (Figs. 1 and 2) in hippocampus and cerebral cortex of female rats submitted to ovariectomy. These results are in agreement with our previous studies showing that hippocampal Na$^+$,K$^+$-ATPase and AChE activities are increased in ovariectomized rats (Monteiro et al., 2005b, 2007). We also observed that exercise per se was unable to affect the enzyme activities. Interestingly, exercise markedly reversed the action of ovariectomy on Na$^+$,K$^+$-ATPase and AChE activities in hippocampus and cerebral cortex of ovariectomized rats (Figs. 1 and 2).

The exact mechanism of reversal of Na$^+$,K$^+$-ATPase activities by exercise is unknown. However, the activity of Na$^+$,K$^+$-ATPase can be modulated by several mechanisms. It has also been shown that the stimulation of Na$^+$,K$^+$-ATPase activity is associated with a decrease in membrane fluidity (Levin et al., 1990) and lipid peroxidation (Nanji et al., 1994). In this context, it is known that regular exercise increases resistance against reactive oxygen species (ROS) (Alessio et al., 1988) and decreases the accumulation of oxidative protein and DNA damage (Leeuwenburgh et al., 1998; Radak et al., 1999). In addition, chronic exercise also decreases the malonaldehyde (MDA) level in brain, a measure of oxidative damage to lipids, indicating a possible beneficial effect of chronic exercise on brain functioning and protection from oxidative damage (Liu et al., 2000). Also, there are data showing that diverse signal transduction pathways, leading to the formation of different mediators and the activation of a variety of kinases, regulate Na$^+$,K$^+$-ATPase activity (Bertorello and Katz, 1995). Based on this, chronic exercise also can modify signal transduction pathways that seem to be involved in the brain plasticity (Molteni et al., 2002). On the other hand, exercise may increase neurogenesis in hippocampus (van Praag et al., 1999) under estrogen-deprived conditions and that may be useful in improving brain function in climacteric women (Ji et al., 2008). Na$^+$,K$^+$-ATPase activity can also be modulated by changes in the intracellular sodium concentration (Inoue and Matsui, 1991). Another possibility could be the increase in synthesis or decreases in degradation of Na$^+$,K$^+$-ATPase.

In present study, exercise also was able to reverse the increase in AChE activity caused by ovariectomy. The reversal of AChE activity probably indicates that exercise can affect brain cholinergic mechanisms, since stimulation of AChE activity provoked an enhanced acetylcholine (ACh) hydrolysis and choline reuptake, reducing cholinergic activity in the CNS (Okuda et al., 2000). Previous study showed that soy isoflavones prevented the increase in AChE activity caused by ovariectomy (Monteiro et al., 2007) and it had been suggested that soy phytoestrogens may function as estrogen agonists in regulating choline acetyltransferase and nerve growth factor in brain of female rats (Pan et al., 1999). In this line, exercise can revert the increase in AChE activity of ovariectomized rats by increasing the number of cholinergic neurons, which also express the nerve growth factor (NGF) receptor, and exercise can also increase gene expression of NGF (Ang et al., 2003). So far we do not know the exact underlying mechanism through which AChE activity is modified in our study.

We also observed in this study that Ovx treatment significantly decreases AMP, and did not alter ADP and ATP hydrolysis in the cerebral cortex. Exercise did not prevent Ovx effect. On the other hand, exercise per se significantly decreased AMP and ADP hydrolysis. Since 5′-nucleotidase activity is involved in the hydrolysis of AMP to adenosine in the synaptic cleft (Battastini et al., 1995; Zimmermann, 1992), a decrease in this enzyme may have occurred as consequence of Ovx and/or exercise. Probably in our study the levels of adenosine, an important neuroprotective and neuromodulator agent (Bonan et al., 2001; Ribeiro et al., 2003), are lower in all tested groups when compared to sham group. In agreement with this idea, Rose'Meyer and colleagues (2003) demonstrated a down regulation in adenosine receptors in response to Ovx using total brain. However, controversial effects on the control of nucleotide levels have been promoted by Ovx or exercise. Rucker et al. (2005) showed that Ovx increases 5′-nucleotidase in synaptosomes from cerebral cortex, whereas it did not alter NTPDase activity. In addition, Dworak and colleagues (2007) did not observe any difference in brain AMP, ADP, and ATP concentrations after moderate and exhaustive treadmill exercise. Our results suggest that exercise is able to decrease the enzyme activities involved in nucleotide levels control, leading to a decrease in adenosine levels, which could suggest that the beneficial effects induced by exercise are not related to a neuroprotective action of adenosine in brain. However, it is important to consider that the different effects induced by exercise on nucleotide hydrolysis could be related with differences in exercise intensity and brain structures investigated.

Considering that the ratio nucleotides/nucleoside in the circulation could present some changes that could evoke responses in both CNS and circulatory system, in the present study we also investigated serum nucleotide hydrolysis. Results show that ATP and ADP hydrolysis were not altered in none group when compared to sham (control), suggesting that NTPDase activity in the blood serum did not change. Results also demonstrated an increase in AMP hydrolysis in the blood serum of ovariectomized rats submitted to exercise, this may result in an increase in 5′-nucleotidase activity in blood serum. Adenosine, an inhibitor of the platelet aggregation (Cristalli et al., 1995), may be increased in response to exercise in

ovariectomized rats, this can be a compensation in situations of increased platelet activity, and cardiovascular risk, such as postmenopausal conditions (Pochmann et al., 2004).

Our previous study showed that ovariectomy significantly decreases (98%) the estradiol levels in all ovariectomized rats, confirming the efficacy of the surgical procedure of ovariectomy (Monteiro et al., 2007). We also observed that the animal weight gain was increased by ovariectomy and exercise reverted weight body gain of ovariectomized rats. These findings are in agreement with Saengsirisuwan and colleagues (2009), that suggested an increased energy expenditure by exercise training, which could prevent fat accumulation and/or enhanced fat utilization of Ovx rats.

In summary, in the present study we demonstrated that exercise significantly reverses the action of ovariectomy on Na$^+$,K$^+$-ATPase and AChE activities in hippocampus and cerebral cortex of female adult rats. Ovariectomy decreased AMP hydrolysis in cerebral cortex and did not alter adenine nucleotide hydrolysis in blood serum. Exercise per se decreased the ADP and AMP hydrolysis and did not alter effects of ovariectomy in cerebral cortex, but increased the AMP hydrolysis in blood serum of ovariectomized adult rats. Based on these findings we could suggest that exercise may have a protective role against the damage brain and increased platelet activity caused by the loss of estrogen during menopause. Present data support that physical exercise might constitute a therapeutic alternative to hormone replacement therapy for estrogen deprivation.

4.   Experimental procedures

4.1.   Animals and reagents

Female adult Wistar rats (3 months, 180–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room, with free access to water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Animals were randomly assigned to one of the following groups: (n=11-15): sham (only submitted to surgery without removing of ovaries), exercise, ovariectomized (Ovx), and Ovx plus exercise. Animals were ovariectomized by the surgical removal of both ovaries under ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) intraperitoneous (i.p.) to eliminate endogenous ovarian steroids (Waynforth and Flecknell, 1992). One month after the surgery, animals were submitted to exercise.

4.2.   Exercise training

Rats were habituated to the treadmill apparatus to minimize novelty stress and randomly assigned to different experimental groups (n=11-15 in each group): non-exercised (sedentary-control group) and exercised during 20 min, 3 times a week. The exercise training consisted of running sessions on an adapted motorized rodent treadmill (INBRAMED TK 01, Porto Alegre, Brazil) at 60% of their maximal oxygen uptake (Brooks and White, 1978), a moderate exercise. Measurement of oxygen uptake (VO2) peak was carried out in all animals, indirectly before training, considering the exhaustion. Each rat ran on the treadmill at a low initial speed followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to continue running) and the time to fatigue (in min) and workload (in m/min) were taken as indexes of capacity for exercise, that was taken as VO2 max. (Arida et al., 1999; Brooks and White, 1978).

Selected animals that initially refused to run were encouraged by gently tapping their backs. Neither electric shock nor physical prodding was used in this study (Cechetti et al., 2007). The control group was transported to the experimental room and handled exactly as the experimental animals and were maintained in the turned off treadmill for 5 min without being forced to run (Scopel et al., 2006).

The animals were adapted to the treadmill by gradually increasing running speed and time, as follows: week 1, at 18 m/min for the first 3 min, 24 m/min for the next 3 min, 36 m/min for the following 6 min, 24 m/min for the following 3 min and 18 m/min for the last 3 min; week 2, at 18 m/min for the first 3 min, 36 m/min for the next 12 min, and 18 m/min for the last 3 min; weeks 3 and 4, at 18 m/min for the first 3 min, 48 m/min for the next 14 min, and 18 m/min for the last 3 min (Cechetti et al., 2007).

Approximately 12 h after the last exercise session, rats were euthanized by decapitation without anesthesia and the brain was immediately isolated, washed with saline solution and the cerebral cortex and hippocampus were dissected; and the blood was collected.
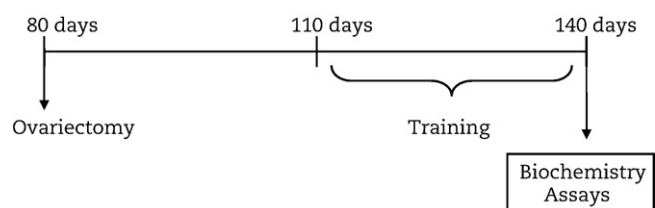
4.3.   Na$^+$,K$^+$-ATPase activity assay

For determination of Na$^+$,K$^+$-ATPase activity, the hippocampus and cerebral cortex were homogenized in 10 vol. 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4.

The reaction mixture for Na$^+$,K$^+$-ATPase activity assay contained 5.0 mM MgCl$_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na$^+$,K$^+$-ATPase activity was calculated by the difference between the two assays, as previously described (Wyse et al., 2000). Released inorganic phosphate (Pi) was

measured by the method of (Chan et al., 1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

4.4. AChE activity assay

For the AChE assay, the hippocampus and cerebral cortex were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000×g. The supernatant was used for the enzymatic AChE analyses.

Acetylcholinesterase activity was determined according to Ellman et al. (1961), with some modifications (Villescas et al., 1981). Hydrolysis rates were measured at acetylthiocholine (S) concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic Acid) (DTNB) at 25 °C. Fifty microliters of rat hippocampus and cerebral cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Specific enzyme activity was expressed as μmol ASCh per hour per milligram of protein. All samples were run in duplicate.

4.5. Adenine nucleotide hydrolysis

4.5.1. Subcellular fractionation
Cerebral cortex was removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5 and 0.1 mM EDTA) and were cut longitudinally. The cerebral cortex was gently homogenized in 10 volumes, respectively, of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer and synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000×g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

4.5.2. Isolation of blood serum fraction
Blood samples were drawn after decapitation of rats and were soon centrifuged in plastic tubes at 3000 rpm for 10 min at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments (Stefanello et al., 2003).

4.5.3. Measurement of synaptosome ATP, ADP and AMP hydrolyses
The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991). The reaction medium contained 5.0 mM KCl, 1.5 mM CaCl$_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 ml. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). The reaction medium used to assay the 5′-nucleotidase activity (AMP hydrolysis) contained 10 mM MgCl$_2$, 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 ml (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 ml 10% TCA; the released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). Controls with the addition of the enzyme preparation after addition of TCA were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein.

4.5.4. Measurement of blood serum ATP, ADP and AMP hydrolyses
ATP and ADP hydrolyses were determined using a modification of the method described by Yegutkin (1997) according to Delwing et al. (2006) The reaction mixture containing 3 mM ATP, ADP or AMP as substrate, 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 200 μl. The reaction was stopped by the addition of 200 μl of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) released was measured as described by (Chan et al., 1986). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at 5000g for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in triplicate. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein.

4.6. Protein determination

Protein was measured by the Comassie Blue method according to Bradford, using bovine serum albumin as standard (Bradford, 1976).

4.7. Statistical analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one way ANOVA followed by the Duncan multiple test when F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC-compatible computer. Values of $p < 0.05$ were considered to be significant.

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