Swimming Training Prevents Alterations in Acetylcholinesterase and Butyrylcholinesterase Activities in Hypertensive Rats

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BACKGROUND
Cholinergic enzyme activities are altered in hypertension, reflecting a low-grade inflammation. Regular physical exercise exerts anti-inflammatory effects and has been described as a coadjutant in the treatment of hypertension. In this study, we investigated the effect of 6 weeks of swimming training on cholinergic enzyme activities (acetylcholinesterase and butyrylcholinesterase) in Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)–induced hypertensive rats.

METHODS
The rats were divided into 4 groups: control (n = 10), exercise (n = 10), L-NAME (n = 10), and exercise L-NAME (n = 10). The animals were trained 5 times per week in an adapted swimming system for 60 minutes with a gradual increase of the workload up to 5% of animal’s body weight. Enzyme activities were measured spectrophotometrically in lymphocytes, whole blood, and serum.

RESULTS
A significant rise in acetylcholinesterase activity was observed in lymphocytes and whole blood as well as in serum butyrylcholinesterase activity in the L-NAME group when compared with the other groups (P < 0.05), and the increase in cholinesterase activities was positively correlated with the rise in blood pressure (r = 0.5721, r = 0.6121, and r = 0.5811, respectively). Swimming training was efficient in preventing these alterations in the exercise L-NAME group, which displayed values similar to those of the control group. Exercise training demonstrated a significant hypotensive effect in hypertensive rats.

CONCLUSIONS
Exercise training was shown to prevent increased cholinesterase related to inflammatory processes in hypertensive rats, providing a new insight about protective exercise mechanisms to avoid hypertension-related inflammation.

Keywords: acetylcholinesterase; blood pressure; butyrylcholinesterase; hypertension; inflammation; swimming training.

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Hypertension represents a major risk factor for stroke, myocardial infarction, heart failure, aneurysms of the arteries, and chronic kidney disease, making it an important public health challenge. It is well established that a chronic low-grade inflammation accompanies hypertension1,2 and the release of proinflammatory cytokines modifies the normal state of vasodilatation mainly because of a low availability of nitric oxide (NO).3 The chronic administration of Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), which is an L-arginine analogue, has been widely used to induce hypertension in rats because it produces a volume-dependent elevation of blood pressure (BP) through the inhibition of NO production.5–12

Several studies have highlighted the relationship between the cholinergic system, inflammatory processes, and the development of hypertension. Lymphocytes express most of the cholinergic components, and the neurotransmitter acetylcholine (ACh) has been described as possessing

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anti-inflammatory properties.\textsuperscript{2,13} ACh can act by inhibiting the production of tumor necrosis factor, interleukin 1, macrophage migration inhibitory factor, and high mobility group box 1. Also, it suppresses the activation of nuclear factor-kappa B expression.\textsuperscript{2} The enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are also responsible for ACh hydrolysis and thus can modulate immune (AChE) and butyrylcholinesterase (BuChE) are also responsible for ACh hydrolysis and thus can modulate immune response.\textsuperscript{2,13} However, although studies have reported the relationship between hypertension and alterations in plasma cholinesterase activities,\textsuperscript{3} the relationship between hypertension and the alteration in AChE and BuChE activities in lymphocytes remains unknown.

Concerns about increased stroke, myocardial infarction, and heart failure frequency in patients with hypertension have led many health-care professionals to adopt protective measures. In this context, a large number of studies have shown that lifestyle changes can improve BP control and decrease the risk of associated health complications.\textsuperscript{3,5,14–16} Physical training has been prescribed as a coadjuvant treatment for hypertension, mainly because of its anti-inflammatory effects.\textsuperscript{17} A recent review addressing this issue has focused on 3 possible mechanisms: reduction in visceral fat mass, increased production and release of anti-inflammatory cytokines, and reduced expression of Toll-like receptors on monocytes and macrophages.\textsuperscript{17}

However, the effects of physical training on cholinesterase activities related to the inflammatory process are still unknown. Furthermore, there is no information about exercise (mainly its chronic effects) and hypertension-induced changes in cholinesterase activities. There are few studies elucidating the influence of exercise on these enzymes in other systems and/or contexts.\textsuperscript{15,18,19} This study investigated the effect of chronic swimming training on AChE activity in lymphocytes and whole blood as well as in serum BuChE activity in rats who developed hypertension in response to the oral administration of L-NAME. Moreover, we investigated changes in cholinergic enzyme activities after an acute single bout of swimming to explain chronic exercise alterations.

METHODS

Chemicals

L-NAME, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), Triton X-100, and ethopropazine hydrochloride were obtained from Sigma Chemical (St. Louis, MO). Ficoll-Histopaque (Lymphoprep) was purchased from Nycomed Pharma (Oslo, Norway). All other reagents used in the experiments were of analytical grade and the highest purity.

Animals

Adult male Wistar rats (aged 70–90 days; 220–300 g) from the Central Animal House of the Federal University of Santa Maria were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12-hour light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee of the Federal University of Santa Maria (protocol number: 029/2011).

Experimental protocol

Rats were randomly divided into 4 groups, normotensive (Control, n = 10), normotensive plus exercise (Exercise, n = 10), hypertensive (L-NAME, n = 10) and hypertensive plus exercise (Exercise L-NAME, n = 10). In the hypertensive groups, hypertension was induced by the oral administration of NO synthase inhibitor (L-NAME). Although most studies induce hypertension through drinking water, Ribeiro et al.\textsuperscript{10} observed that average dairy water intake per box was 26% lower in L-NAME–treated rats than in controls. Thus, gavage administration was used to be certain about the dose ingested by rats and to be certain that all rats received the same dose. In addition, despite hypertension induction, high doses\textsuperscript{2,10} or long-term administration\textsuperscript{3} of L-NAME may induce severe proteinuria and renal injury. Thus, a moderate dose of L-NAME, as used by Furstenau et al. (30 mg/kg/day),\textsuperscript{8} was given for a moderate length of time under the assumption that our rats would become hypertensive and that renal injury would occur but it would not be overly deleterious. Gavages with L-NAME started 1 week before physical training. After 1 week\textsuperscript{9} (day 8), BP was measured in all groups. L-NAME–treated animals presented elevated BP (data not shown). After the certification that the animals were hypertensive, the physical training program started. In the normotensive groups, the animals received water by gavage throughout the entire experiment to undergo the same level of stress. These rats were anesthetized and killed 24 hours after the last exercise session.\textsuperscript{11,12} Blood was collected by cardiac puncture.

Exercise protocol

Swimming was the exercise chosen for this study. The use of swimming rats as a model of exercise presents advantages over treadmill running because swimming is a natural ability of rats and it circumvents the need to select animals, which is necessary in experimental protocols using treadmill running.\textsuperscript{20} Furthermore, the animals are not likely to suffer from foot injuries and physical trauma. An additional advantage is that swimming provides a uniform type of physical activity with the use of ankle and flexor muscles.\textsuperscript{16}

Swimming protocol

All rats were adapted to water before training began. The adaptation consisted of keeping the animals in shallow water at 31 ± 1 °C\textsuperscript{20} for 1 hour during 5 days. This procedure was performed between 10:00 AM and 12:00 PM. The adjustment reduces stress without promoting adaptation to the training.

Animals were trained for 60 minutes 5 times per week for 6 weeks in an adapted swimming system with water heated to 31 ± 1 °C. The training occurred between 10:00 AM and 12:00 PM. The training tank used for this study was 80 cm in length, 50 cm in width, and 90 cm in depth.\textsuperscript{20} The workload (weight on the back) was gradually increased to up to 5% of the animal's body weight (Table 1).

Sedentary animals were placed in shallow water (5 cm in depth) heated to 31 ± 1 °C, for 60 minutes 5 times per week without the work load to undergo the same level of stress without being submitted to the effects of physical training.

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Table 1. Swimming protocol, with training time from week 1 to week 6, held from Monday to Friday

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There were n = 10 rats per group.
Abbreviations: BW, body weight; WO, without overload.

Acute exercise protocol

As a complement to the study and to explain chronic changes in cholinesterase activities, we aimed to verify changes in these enzymes due to an acute bout of exercise. For this purpose, a subset of healthy normotensive rats were randomly divided into 2 groups: a group that would remain at rest (n = 5) and a group that was submitted to an adaptation training week before the swimming test (n = 5). In the swimming session test, rats performed 60 minutes of swimming with a workload (weight on the back) of 5% of the animal’s body weight. Rats were anesthetized and killed immediately after the acute swimming test, blood was collected, and lymphocytes and serum were separated for further analysis. The same acute protocol was performed with hypertensive rats, but because there was no difference between hypertensive and normotensive groups, we omitted these results.

Hemodynamic parameter determination

In all rats, systolic BP was measured in awake animals by tail-cuff plethysmography (RTBP1001 Rat Tail Blood Pressure System for rats and mice; Kent Scientific, Torrington, CT). Rats were conditioned with the apparatus before measurements were taken. BP was recorded at the end of experiment (last treatment week).

Blood collection

Twenty-four hours after the last swimming session, the animals were anesthetized with halothane and killed. Halothane was administered by the closed technique in a dose of 0.5%, according to Halothane bull (Tanohalo 1:1 ml; CRISTÁLIA Produtos Químicos Farmacêuticos) adapted to rats. The animals were kept in a closed chamber, which became an environment saturated with anesthetic, for approximately 2 minutes. Blood was collected by cardiac puncture in tubes with ethylenediaminetetraacetic acid as anticoagulant for lymphocytes separation. Tubes without anticoagulant were used to obtain serum.

Isolation of mononuclear cells

Mononuclear leukocytes were isolated from blood collected with ethylenediaminetetraacetic acid and separated on Ficoll-Histopaque density gradients as described by Böyum. Despite the methodology described above to be used for separating mononuclear cells, the work done by Jaques et al. demonstrated that there is a high incidence of lymphocytes in separate samples and the amount of monocytes is almost insignificant. For this reason, we will consider the samples as containing only lymphocytes.

AChE activity determination

Whole blood AChE activity was determined by the method of Elmann et al. modified by Worek et al. To achieve temperature equilibration and complete reaction of sample matrix sulfhydryl groups with DTNB, the mixture was incubated for 10 minutes before addition of substrate. Enzyme activity was corrected for spontaneous hydrolysis of the substrate and DTNB degradation. The BuChE was inhibited by ethopropazine. AChE activity was measured at 436 nm and 37°C using polystyrol cuvetts.

AChE activity in lymphocytes was determined according to the method described by Ellman et al. modified by Fitzgerald and Costa. Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. Then 0.2 ml of intact cells were added to a solution containing 1.0 mM acetylthiocholine (ATC), 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 minutes at 27°C, the absorbance was read on a spectrophotometer at 412 nm. The results are expressed as μmol/h/mg of protein.

BuChE activity determination

The BuChE enzymatic assay was determined in serum by a modification of the spectrophotometric method of Ellman et al. The reaction mixture (2 ml final volume) contained 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM DTNB. The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid nitrobenzoic, measured by absorbance at 412 nm during 2 minutes of incubation at 25°C. Enzyme activity was expressed in μmol BuSCh/h/mg of protein.

Protein determination

Protein was measured by the Coomassie blue method according to Bradford using serum albumin as standard.

Statistical analysis

Data were analyzed statistically using 2-way analysis of variance using SSPS 18.0 for Windows (SPSS, Chicago, IL), followed by Tukey’s multiple range tests. Some data were analyzed using Student t test and others by Pearson correlation. Differences were considered significant when P < 0.05, and variables are presented as mean ± SD.

RESULTS

Oral administration of L-NAME induced a significant increase in BP when compared with the other groups,
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Corroborating previous studies that have demonstrated the same effect,\textsuperscript{5,6-10} on the other hand, exercise clearly affords a hypotensive effect, significantly reducing BP in the Exercise L-NAME group (interaction between factors $F(3,28) = 7.63$; $P = 0.01$) (Figure 1). No difference was observed in the body weight and food and water consumption after the administration of L-NAME between the experimental groups (data not shown).

Results obtained for the AChE activity in lymphocytes are shown in Figure 2. Statistical analysis showed a significant hypertension vs. exercise interaction ($F(3,36) = 4.80$; $P = 0.049$). Post hoc comparison revealed that the hypertension development was associated with a significant rise in AChE activity in the L-NAME group when compared with the other groups ($P < 0.05$). Also, swimming training protected against the L-Name–induced BP increase in the Exercise L-NAME group ($P < 0.05$) (Figure 2a). Regarding the acute effect of a single bout of exercise (Figure 2b), we observed a significant increase in the AChE activity after exercise ($P = 0.01$). Figure 2c shows a positive Pearson correlation between AChE activity and systolic BP ($r = 0.5721$; $P = 0.01$), indicating that AChE activity rises as blood pressure rises.

Figure 3 shows results obtained for AChE activity in whole blood. Statistical analysis showed a significant hypertension vs. exercise interaction ($F(3,36) = 6.49$; $P = 0.01$). Hypertension development was associated with a significant increase in AChE activity in the L-NAME group when compared with the other groups ($P < 0.05$), and swimming training was efficient in preventing this alteration in the Exercise L-NAME group. Exercise per se decreased AChE activity in the Exercise group when compared with the other

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**Figure 1.** Final systolic blood pressure measurements of Control group, Exercise group, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) group, and Exercise L-NAME group. Systolic blood pressure was followed as described in the Methods section. Data are presented as means ± SD. Groups (n = 10 per group) with different letters are statistically different (2-way analysis of variance, $P < 0.05$).

**Figure 2.** Acetylcholinesterase (AChE) activity in lymphocytes. (a) AChE activity in lymphocytes of Control group, Exercise group, Nu-Nitro-L-arginine methyl ester hydrochloride (L-NAME) group, and Exercise L-NAME group. Data are presented as means ± minimums and maximums. Groups (n = 10 per group) with different letters are statistically different (2-way analysis of variance, $P < 0.05$). (b) AChE activity in lymphocytes of rats at rest and after an acute bout of exercise. Data are presented as means ± SD. * indicates statistical difference (Student t test, $P < 0.05$) (n = 5 per group). (c) Pearson correlation between AChE activity and systolic blood pressure ($r = 0.5721$; $P < 0.05$).

**Figure 3.**
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Regarding the acute effect of a single bout of exercise (Figure 3b), we observed a significant increase in AChE activity immediately after exercise ($P = 0.01$). Figure 3c shows a positive Pearson correlation between AChE activity and systolic BP ($r = 0.6121; P = 0.02$), indicating that AChE activity rises as blood pressure rises.

BuChE activity, measured in serum, presented the same behavior as lymphocytic AChE activity, as shown in Figure 4 (interaction between factors $F(3,36) = 9.16; P = 0.005$). Hypertension development was associated with a significant rise in BuChE activity in the L-NAME group when compared with the other groups ($P < 0.05$), and swimming training was efficient in preventing this alteration in the Exercise L-NAME group (Figure 4a). Figure 4b shows that after a single bout of exercise, BuChE activity was significantly increased ($P < 0.01$). Figure 4c shows a positive Pearson correlation between BuChE activity and systolic BP ($r = 0.5811; P = 0.03$), indicating that AChE activity increases as blood pressure increases.

**DISCUSSION**

Hypertension is accompanied by vascular inflammation, which is characterized by infiltration of immune cells. In addition to vascular growth and proliferation of vascular smooth muscle cells, inflammation plays a key role in the vascular remodeling, which participates in the mechanisms leading to BP elevation. On the other hand, regular physical activity has been shown to have a central role in hypertension prevention or treatment and its anti-inflammatory effect is one of the main mechanisms involved.

Several studies have shown the beneficial effects of regular physical activity in reducing elevated BP. Thus, regular physical activity has been recommended by health professionals to maintain good cardiovascular fitness and prevent or treat hypertension. It has been widely recognized that lymphocytic cholinergic activity reflects well the changes in immune system function. In addition, plasma alteration in AChE and BuChE activities serves as a marker of low-grade systemic inflammation. Using this line of reasoning, we have demonstrated that the development of hypertension was associated with a significant increase in AChE activity in lymphocytes and whole blood as well as in BuChE activity measured in serum in the L-NAME group when compared with the other groups. In addition, the rise in AChE and BuChE activities positively correlated with the increase in BP. These experimental data agree with several studies that have already shown the significant increase of these enzymes in the plasma and tissue of patients with Alzheimer’s disease, diabetes mellitus, hypertension, insulin resistance, hyperlipidemia, and leukemia. Ofek et al. showed that subjects expressing high levels of plasma cholinesterases may produce higher levels of proinflammatory cytokines under infectious insults. This

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**Figure 3.** Acetylcholinesterase (AChE) activity in whole blood. (a) AChE activity in whole blood of Control group, Exercise group, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) group, and Exercise L-NAME group. Data are presented as means ± SD. Groups (n = 10 per group) with different letters are statistically different (2-way analysis of variance, $P < 0.05$). (b) AChE activity in whole blood of rats at rest and after an acute bout of exercise. Data are presented as means ± SD. * indicates statistical difference (Student t test, $P < 0.05$) (n = 5 per group). (c) Pearson correlation between AChE activity and systolic blood pressure ($r = 0.6121; P < 0.05$).
suggests that the increase in cholinesterase activities is a way to combat infections through the release of proinflammatory cytokines (positive response). However, this alternative is less plausible for our study because acute infection is not comparable with the low-grade inflammation verified in hypertension. Instead, our study corroborates Ofecket et al., clearly showing the positive correlation between inflammation and rise in cholinesterase activities. In addition, the hypertensive rats probably presented augmented levels of proinflammatory cytokines when compared with the normotensive rats, although this has not been measured. Our findings are also corroborated by Ben-Assayang et al., who demonstrated the power of circulation cholinesterase measurements as useful early diagnostic tools for the occurrence of stroke.

As a result of increased activities of AChE and BuChE, the levels of ACh probably decreased in L-NAME–treated rats. In this context, the cholinergic anti-inflammatory pathway, mediated by ACh, which acts by inhibiting the production of tumor necrosis factor, interleukin 1, macrophage migration inhibitory factor, and high mobility group box 1 and suppresses the activation of nuclear factor kappa B expression, becomes impaired. Thus, it is plausible to propose that both AChE and BuChE enhance inflammation in hypertensive rats by inactivating ACh, and this situation may represent an augmented risk to the development of hypertension-associated complications. In the same context, it is important to point out that, although we have not identified the lymphocytes T and B, the main effects are probably related to the T cells because these cells show enhanced synthesis and release of ACh as well as increased AChE expression when compared with B cells.

Of great interest to our work, we tested the effect of 6 weeks of swimming training on AChE and BuChE activities in hypertensive rats and demonstrated that this training was efficient in preventing the alterations in these enzyme activities in the Exercise L-NAME group. The maintenance of these enzyme activities by physical training indicates that the levels of ACh are probably preserved in the vascular extracellular medium and thus help to avoid inflammatory processes induced by the development of hypertension. Other studies have assessed the effects of chronic physical training on the rat brain. However, this is the first study aiming to verify the effects of exercise on the cholinergic system and in relation to hypertensive inflammation. Ben et al. found that physical training prevented an increase in AChE activity in the brain of ovariectomized rats, but exercise per se presented no effect. Ben-Ari et al. verified that exercise increased the number of AChE-positive fibers in the molecular layer, reduced cerebellar cytokine levels, and suppressed serum AChE activity, suggesting anti-inflammatory protection by enhanced cholinergic signaling. In our study, swimming training presented a similar prevention of AChE activity alterations, despite the differences in the research focus. It is plausible to infer that one of the mechanisms by which swimming training prevented alterations in cholinesterase activities is related to the involvement of microRNA 132 in potentiating cholinergic anti-inflammatory signaling by targeting AChE.

To understand chronic alterations displayed by swimming training in the cholinergic system, we verified the acute
effect of a single bout of exercise. We observed a significant increase in AChE and BuChE activities immediately after exercise. Similar findings were reported by Schulps et al.\textsuperscript{15} in erythrocyte AChE activity in players immediately after a game. Moreover, Kaufer et al.\textsuperscript{17} also verified an increase in brain AChE gene expression after 10 minutes of acute swimming. On the contrary, Tsakiriset et al.\textsuperscript{18} found a decrease in AChE activity in response to acute exercise in rat brain. The differences between findings are probably related to AChE localization and the experimental protocol because effects on ACh modulation differ depending on the cell surface and stimuli.

As stated by Schulps et al.,\textsuperscript{15} AChE stimulation in response to acute exercise may be related to the additional release of ACh as well as of catecholamines, serotonin, and/or cortisol in the blood. The large ACh release may be related to its endothelium-dependent vasodilatation function,\textsuperscript{38} as well as to an anti-inflammatory microenvironment caused by the acute exercise bout. Furthermore, it may be related to the discharge of anti-inflammatory cytokines and the modulation of immune cells already described in a review by Gleeson et al.\textsuperscript{17} because ACh has the above-mentioned anti-inflammatory properties.\textsuperscript{2} In the same context, Gilboa-Geffen et al.\textsuperscript{39} reported that stress-induced increases in ACh act to mitigate inflammatory response and restore homeostasis.

As reported before, chronic exercise in hypertensive rats prevented cholinesterase stimulation and reduced BP in the Exercise L-NAME group. It is reasonable to assume that the increase of cholinesterase activities as a result of acute exercise may produce an adaptation by the organism. That is, with several exercise sessions cholinesterase becomes prepared to receive an exercise stimulus and a major ACh release. In this sense, when the organism is in a resting condition, it maintains low enzyme activity, as shown by our results, probably because low ACh is required in blood circulation in an organism already adapted to training, as in our study. Another possible explanation for the low activity of AChE and BuChE due to chronic exercise effects found in our study is that even if a large amount of circulating ACh is being released in an organism at rest and already adapted to physical training, the enzyme activities are kept low to allow ACh action. The second inference seems to be more plausible mainly because of the ACh action in endothelial cells that mediates vasorelaxation and thus may contribute to the hypotensive effects of exercise. In endothelial cells, ACh acts through muscarinic (M3 or M5) receptors. This results in an activated endothelial NO synthase, which leads to the production of NO stimulating soluble guanylyl cyclase to produce cyclic guanosine monophosphate. Protein kinase G activated by cyclic guanosine monophosphate promotes relaxation.\textsuperscript{38} However, further studies are necessary to elucidate these possible mechanisms.

In conclusion, our study suggests that moderate exercise training prevents cholinesterase alterations related to inflammatory processes in rats that developed hypertension in response to oral administration of L-NAME. This was probably because of the adaptation of these enzymes to stimuli caused by the exercise sessions, which can modulate cholinesterase activities. This modulation can be understood as another anti-inflammatory mechanism generated by exercise that contributes to the control of BP, highlighting the great protective effect of exercise training to avoid hypertension-related inflammation.

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**DISCLOSURE**

The authors declared no conflict of interest.

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