Mol Cell Biochem (2012) 371:147–156
DOI 10.1007/s11010-012-1431-7

Exercise training prevents ecto-nucleotidases alterations in platelets of hypertensive rats

Andréia Machado Cardoso · Margarete Dulce Bagatini · Caroline Curry Martins · Fátima Hussein Abdalla · Daniela Zanini · Roberta Schmatz · Jessié Gutierres · Victor Camera Pimentel · Gustavo Thomé · Claudio Alberto Martins Leal · Juliano Marchi Vieira · Naiara Stefanello · Fernando da Silva Fiorin · Jucimara Baldissareli · Luiz Fernando Freire Royes · Adriane Bello Klein · Vera Maria Morsch · Maria Rosa Chitolina Schetinger

Received: 25 April 2012 / Accepted: 3 August 2012 / Published online: 23 August 2012

Abstract In this study, we investigated the effect of 6 weeks of swimming training on the ecto-nucleotidase activities and platelet aggregation from rats that developed hypertension in response to oral administration of L-NAME. The rats were divided into four groups: control ($n = 10$), exercise ($n = 10$), L-NAME ($n = 10$), and exercise L-NAME ($n = 10$). The animals were trained five times per week in an adapted swimming system for 60 min with a gradual increase of the workload up to 5 % of animal's body weight. The results showed an increase in ATP, ADP, AMP, and adenosine hydrolysis, indicating an augment in NTPDase (from 35.3 ± 8.1 to 53.0 ± 15.1 nmol Pi/min/mg protein for ATP; and from 21.7 ± 7.0 to 46.4 ± 15.6 nmol Pi/min/mg protein for ADP as substrate), ecto-5′-nucleotidase (from 8.0 ± 5.7 to 28.1 ± 6.9 nmol Pi/min/mg protein), and ADA (from 0.8 ± 0.5 to 3.9 ± 0.8 U/L) activities in platelets from L-NAME-treated rats when compared to other groups ($p < 0.05$). A significant augment on platelet aggregation in L-NAME group was also observed. Exercise training was efficient in preventing these alterations in the exercise L-NAME group, besides showing a significant hypotensive effect. In conclusion, our results clearly indicated a protector action of moderate intensity exercise on nucleotides and nucleoside hydrolysis and on platelet aggregation, which highlights the exercise training effect to avoid hypertension complications related to ecto-nucleotidase activities.

Keywords Hypertension · L-NAME · Ecto-nucleotidases · Platelet aggregation · Exercise training

A. M. Cardoso · C. C. Martins · F. H. Abdalla · D. Zanini · R. Schmatz · J. Gutierres · V. C. Pimentel · G. Thomé · C. A. M. Leal · J. M. Vieira · N. Stefanello · F. da Silva Fiorin · J. Baldissareli · L. F. F. Royes · V. M. Morsch · M. R. C. Schetinger
Post-Graduation Program in Toxicological Biochemistry, Department of Chemistry, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil

A. M. Cardoso · M. D. Bagatini · C. C. Martins · F. H. Abdalla · D. Zanini · R. Schmatz · J. Gutierres · V. C. Pimentel · G. Thomé · C. A. M. Leal · J. M. Vieira · N. Stefanello · F. da Silva Fiorin · J. Baldissareli · L. F. F. Royes · A. B. Klein · V. M. Morsch · M. R. C. Schetinger
Health Basic Sciences Institute, Department of Physiology of Federal University of Rio Grande do Sul, RS, Brazil

M. D. Bagatini
Collegiate of Nursing, University of Southern Frontier, Chapecó Campus, Chapecó, SC, Brazil

M. D. Bagatini (✉)
Curso de Enfermagem, Universidade Federal da Fronteira Sul, Chapecó, SC 89812-000, Brazil
e-mail: margaretebagatini@yahoo.com.br

M. R. C. Schetinger (✉)
Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Av. Roraima, Santa Maria, RS 97105-900, Brazil
e-mail: mariachitolina@gmail.com

Introduction

Currently, hypertension affects more than 1 billion adults worldwide and 90–95 % of these patients have essential hypertension [1]. This disease is considered an independent risk factor for stroke, myocardial infarction, heart failure, and arterial aneurysm, besides being the leading cause of chronic renal failure [2].

It is well established that nitric oxide (NO) produced in the vascular endothelial cells shows a potent vasodilator

effect [3] and plays an important role in the local regulation of platelet–vessel wall interactions as well as in vascular resistance and growth [4]. On the basis of these effects, NO has been proposed to have antihypertensive, antithrombotic, and anti-atherosclerotic properties [3].

The chronic inhibition of NO produces volume-dependent elevation of blood pressure and its physiological and pathological characteristics resemble essential hypertension [5]. Several studies have administered in vivo an inhibitor of nitric oxide biosynthesis, the *Nω*-nitro-L-arginine methyl ester hydrochloride (L-NAME), which is an L-arginine analog, to induce hypertension in rats [6–8].

Extracellular nucleotides and nucleosides released by platelets in vasculature have an important participation in the regulation of thrombus formation, being some of the most important blood molecules involved in the regulation of blood flow [9, 10]. Platelet aggregation can be described as having a central role in hypertension complications and ADP released by platelets is a molecule that exhibits a potent induction of this phenomenon, even in micromolar concentrations. On the other hand, adenosine, which is the final product of ATP hydrolysis, plays an important role in the purinergic metabolism, inhibiting platelet aggregation and exerting a reduction of the vascular injury [9]. ATP seems to have a dual role in the induction and/or inhibition of platelet activation, i.e., at low concentrations, ATP can induces platelet aggregation, whereas at high concentrations, it can inhibits such situation [11, 12].

Recent studies [7, 8] and works developed by our group [13, 14] have correlated alterations in extracellular nucleotides and nucleosides and hypertension, suggesting that the purinergic system is altered in this pathology. Moreover, it is important to underline that these extracellular molecules are important signaling that mediate diverse biological and pathological processes in several tissues as already demonstrated by our research group [15–18]. This way, the levels of these molecules must be carefully controlled.

The importance of adenine nucleotides in homeostasis and thrombosis is greatly correlated with the essential role of an enzymatic system that provides an adequate control of these signaling molecules in the vascular system. This complex is composed by the enzymes NTPDase (nucleoside triphosphate diphosphohydrolase), that hydrolyzes ATP and ADP to AMP [20]; E-NPP (nucleotide pyrophosphatase/phosphodiesterase) that converts 5′-phosphodiester bonds in nucleotides and their derivatives producing AMP [21]; ecto-5′-nucleotidase, that hydrolyzing AMP produces adenosine [10, 19]; and, ecto-adenosine deaminase (ADA) that converts adenosine to inosine and is described as the last enzyme in the purinergic cascade, having an important role in the regulation of platelet activation [22].

People exercise daily to maintain good cardiovascular health. Many people with cardiovascular diseases are engaged in organized group exercise rehabilitation programs or pursue individual exercise with or without medication [23]. However, the effects of physical training on ecto-nucleotidase activities in platelets are still unknown, although it has been recently evidenced that when red blood cells are exposed to a low oxygen tension environment, as in an exercise session, they release ATP and its metabolites, and there is the involvement of nucleotidases in this process as a way to control blood flow [24]. Indeed, the information available about exercises, mainly their chronic effects, and ecto-nucleotidases is very scarce, highlighting the importance of this study.

According to what was described above, the enzymes E-NTPDase, E-NPP, ecto-5′-nucleotidase, and ADA are present on the surface of intact platelets [7, 19] and their activities are altered in hypertension [7]. On the other hand, physical exercise is recognized by having hypotensive effects and it has been hypothesized that it could contribute to the ecto-nucleotidase modulation in favor of maintaining good cardiovascular health. This way, in this study we investigated the ecto-nucleotidase activities in the platelets of rats that developed hypertension in response to the oral administration of L-NAME and the effect of physical exercise on ecto-nucleotidase activities of these hypertensive rats comparing with normotensive control animals and normotensive exercised animals. Moreover, we investigated changes in the ecto-nucleotidase activities after an acute single bout of swimming in order to explain exercise chronic alterations.

Materials and methods

Chemicals

The substrates ATP, ADP, AMP, *p*-nitrophenyl thymidine 5′-monophosphate (*p*-Nph-5′-TMP), adenosine, as well as Trizma base, sodium azide HEPES, L-NAME, and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, K_2HPO_4, from Reagen (Colombo, PR, Brazil). All the other chemicals used in this experiment were of the highest purity.

Animals

Adult male Wistar rats (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 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria

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

Week	Monday	Tuesday	Wednesday	Thursday	Friday
1st	20 min.	30 min.	40 min.	50 min	60 min.
	wo	wo	wo	wo	wo
2nd	40 min.	50 min.	60 min.	60 min.	60 min.
	2 % bw	2 % bw	2 % bw	2 % bw	2 % bw
3rd	40 min.	50 min.	60 min.	60 min.	60 min.
	5 % bw	5 % bw	5 % bw	5 % bw	5 % bw
4th, 5th, 6th	60 min.	60 min.	60 min.	60 min.	60 min.
	5 % bw	5 % bw	5 % bw	5 % bw	5 % bw

wo without overload, *bw* body weight ($n = 10$ to each group)

(protocol under number: 029/2011). All protocols are in accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council) and all efforts were made to minimize the number of animals used in this study and their suffering.

Experimental protocol

Rats were randomly divided into four groups, normotensive (Control), normotensive plus exercise (Exercise), hypertensive (L-NAME), and hypertensive plus exercise (Exercise L-NAME). In the hypertensive groups, hypertension was induced by the oral administration of the nitric oxide synthase (NOS) inhibitor L-NAME (30 mg/kg bodyweight/day) [7] by gavage during all experiment (L-NAME-treated groups). In the normotensive groups, the animals received water by gavage throughout the entire experiment to be submitted to the same stress (control groups). These rats were euthanized 24 h after the last exercise session [25] and blood was collected by cardiac puncture.

Exercise protocol

Swimming was the exercise chosen for this study. The protocol used was according to the protocols adapted from Medeiros et al. [26] and Gobatto et al. [27] as follows.

Swimming protocol

All rats were adapted to water before training beginning. The adaptation was to keep the animals in shallow water at 31 ± 1 °C [26, 27] for 5 days, with duration of 1 h. This procedure was performed in the same time of the day. The adjustment reduces stress, without, however, promoting adaptations to the training.

The animals were trained five times per week in an adapted swimming system with water heated to 31 ± 1 °C for 6 weeks with duration of 60 min. The workload (weight on the back) was gradually increased up to 5 % of the animal's body weight (Table 1).

According to Medeiros et al. [26], this protocol is regarded as training of moderate intensity and long duration, and it is effective in promoting cardiovascular adaptations and increased muscle oxidative capacity.

The sedentary animals were placed in shallow water, heated to 31 ± 1 °C, 30 min, 5 days a week to be subjected to the same stress, however, without suffering the effects of physical training.

Acute exercise protocol

With the purpose to complement the work and explain chronic changes in ecto-nucleotidase activities, we aimed to verify changes in these enzymes due to an acute bout of exercise. A number of ten healthy normotensive rats were randomly divided into two groups: a group which should remain at rest ($n = 5$) and a group which was submitted to an adaptation training week before the swimming test. In the swimming session test rats performed 60 min of swimming with 5 % animal's body weight workload (weight on the back). Rats were killed immediately after the acute swimming test, blood was collected and platelets were separated to further analysis.

Hemodynamic parameter determination

In all rats, systolic blood pressure (SBP) and heart rate (HR) were measured in awake animals, by tail-cuff plethysmography (Kent Scientific; RTBP1001 Rat Tail Blood Pressure System for rats and mice, Litchfield, USA). Rats were conditioned with the apparatus before measurements were taken. SBP was recorded at the end of experiment (last treatment week). The heart rate values were derived from the pulsations detected by SBP.

Platelet preparation

Platelet-rich plasma (PRP) was prepared by the method of Lunkes et al. [28] with the following minor modifications.

Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160×*g* during 15 min. After this, one part of PRP was used for determine platelet aggregation. The rest of PRP was centrifuged at 1,400×*g* for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM NaCl, 2.5 mM KCl, and 5.5 mM glucose. The platelet pellets were resuspended in HEPES buffer and used to determine enzymatic activities.

NTPDase and ecto-5′-nucleotidase activity determination

As described by Lunkes et al. [28], the NTPDase measure was performed in a medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose, and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 μL. The ecto-5′-nucleotidase activity was carried out as previously described by Lunkes et al. [28] to measure AMP hydrolysis. However, the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$ to perform the assay. The enzyme activities were expressed as nmol Pi released/min/mg of protein. In brief, 20 μL of the enzyme preparation were added to the reaction mixture and the pre-incubation proceeded for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and AMP at a concentration final of 2 mM. The time of incubation was 60 min. Both enzyme assays were stopped by the addition of 200 μL of 10 % trichloroacetic acid (TCA) to provide a final concentration of 5 %. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. [29] using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition.

E-NPP activity determination: measurement of *p*-Nph-5′-TMP hydrolysis in platelets

As previously described by Fürstenau et al. [19], the Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) determination was measured using *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) as substrate and was expressed as nmol of *p*-nitrophenol released per minute per milligram of protein (nmol *p*-nitrophenol released/min/mg protein). The reaction medium containing 50 mM Tris–HCl buffer, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, and 5.0 mM $CaCl_2$, pH 8.9, was preincubated with approximately 20 mg per tube of platelet protein for 10 min at 37 °C in a final volume of 200 mL. The enzyme reaction was started by the addition of *p*-Nph-5′-TMP at a final concentration of 0.5 mM. After 80 min of incubation, 200 mL NaOH 0.2 N was added to the medium to stop the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8 × 10^{-3}/M/cm.

Adenosine deaminase activity determination (ADA)

ADA activity determination was performed as described by Guisti and Galanti [22] which is based on the direct measurement of the formation of ammonia, produced when adenosine deaminase acts in excess of adenosine. In brief, 50 μL of platelets reacted with 21 mmol/L of adenosine, pH 6.5, and was incubated at 37 °C for 60 min. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Platelet aggregation test

Sample was prepared according to Yun-Choi et al. [30]. The preparation of platelet-rich plasma (PRP) was obtained as previously described. After, the remained was once more centrifuged for 15 min at 2,000×*g*, to obtain the platelet poor plasma (PPP). A platelet count was performed in the PRP and it was adjusted to 300 × 10^3 platelets/mL, by dilution with PPP, obtaining the platelet equalized plasma (PEP).

For ex vivo platelet aggregation, the Born and Cross [31] technique was performed by turbidimetric measurement with a Chrono-log optical aggregometer, with AGGRO/LINK® Model 810-CA software for Windows version 5.1. After calibration of the aggregometer, the rats data concerning the assays and reagents were entered on a computer coupled to the equipment, and the test was then performed. Aggregation was measured at 37 °C and expressed as the maximal percent change in light transmittance from baseline at 5 min after the addition of the agonist adenosine diphosphate (ADP) 10 and 5 μM, with platelet poor plasma as a reference.

Protein determination

Protein content was measured by the Coomassie blue method according to Bradford [32], using bovine serum albumin as the standard. This assay is based on the binding of the dye Coomassie blue G-250 to protein, and this binding is accompanied by measuring the absorbance maximum of the solution at 595 nm.

Statistical analysis

Data are presented as mean ± SD and were analyzed statistically by two-way ANOVA, followed by Duncan's multiple range test. For comparison of the two groups of acute protocol, was used student *t* test. Differences between groups were considered to be significant when $p < 0.05$.

Results

In this study, the oral administration of L-NAME by gavage was associated with a significant rise in SBP when compared with the control groups, validating the hypertensive model. On the other hand, we could observe that exercise clearly possesses hypotensive effect, reducing significantly SBP in the exercise L-NAME group (Fig. 1).

No difference was observed in the food and water consumption after the administration of L-NAME among the experimental groups (data not shown). Moreover, the heart rate, expressed as cycles per minute (cpm), remained unchanged in the L-NAME-treated group and in the exercised groups compared with the respective control group. However, although not statistically significant, we could observe that in the exercised groups, heart rate values were lower than the control and L-NAME-treated groups, which was an expected result (Table 2). Regarding body weight (BW), no difference was observed among the experimental groups (data not shown).

Results obtained for NTPDase and ecto-5′-nucleotidase activities in platelets are shown in Fig. 2. As can be observed, ATP, ADP, and AMP hydrolysis were significantly increased in the L-NAME-treated group when compared with the control groups and exercised L-NAME group ($p < 0.05$) (Fig. 2a–c). However, it is interesting to note that exercise per se had the ability to increase ADP hydrolysis in the exercise group and decrease ADP hydrolysis in the exercise L-NAME group (Fig. 2b). Regarding acute effect of a single bout exercise (Fig. 2d), we observed a significant rise in ATP, ADP, and AMP hydrolysis ($p < 0.05$).

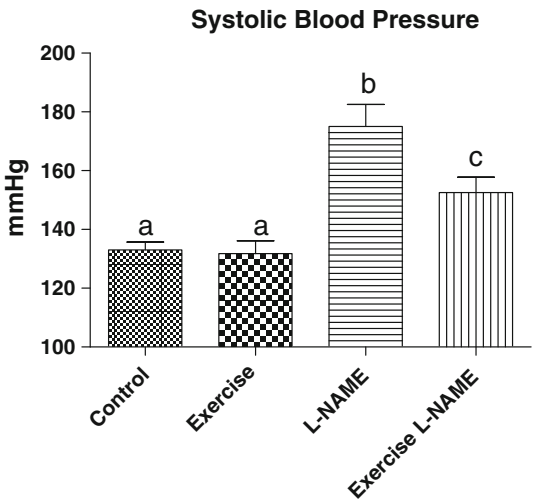

Fig. 1 Final systolic blood pressure (SBP) measurements of control group, exercise group, L-NAME group, and exercise L-NAME group. SBP was followed as described in materials. Data are presented as mean ± SD. Groups with *different letters* are statistically different ($p < 0.05$, $n = 10$ to each group)

Table 2 Heart rate values of control group, exercise control group, L-NAME group, and exercise L-NAME group

	Control	Exercise	L-NAME	Exercise L-NAME
Heart rate (cpm)	469 ± 57	435 ± 47	481 ± 56	452 ± 51

Results are presented as mean ± SD ($n = 10$ to each group)

Figure 3 shows ADA activity on chronic treatment (Fig. 3a) and acute exercise session (Fig. 3b). An increase in the ADA activity was observed in L-NAME-treated group when compared with other groups ($p < 0.05$) and swimming training was efficient in keeping the activity of this enzyme in levels close to normotensive groups (Fig. 3a). An acute bout of exercise clearly results in a significant rise in the ADA activity (Fig. 3b, $p < 0.05$).

Regarding E-NPP activity, no significant changes could be observed. However, there is an indicative that the E-NPP activity had an increase in the L-NAME-treated group when compared with other groups and that the exercise could prevent this, although these values did not reach significant levels (Fig. 4a). As a result of an acute bout of swimming, we also observed just an indicative of a rise in the E-NPP activity, but without statistic relevance (Fig. 4b).

Results obtained for platelet aggregation are shown in Fig. 5. As can be observed, there is a significant augment in platelet aggregation related to L-NAME-treated group at the two different concentrations of ADP (Fig. 5a, b), which was prevented by exercise in exercise L-NAME group.

Discussion

Several studies have shown the beneficial effects of regular physical activity in reducing the elevated blood pressure in both human [1, 33] and animal hypertension models. Regarding experimental models of hypertension, physical training was efficient in reducing blood pressure in spontaneously hypertensive rats [34], Dahl salt-sensitive and salt-resistant rats [35], deoxycorticosterone acetate (DOCA)-induced hypertension [36], hypertension due to manipulation of kidney arteries [37], and hypertension induced by L-NAME administration [6, 38, 39].

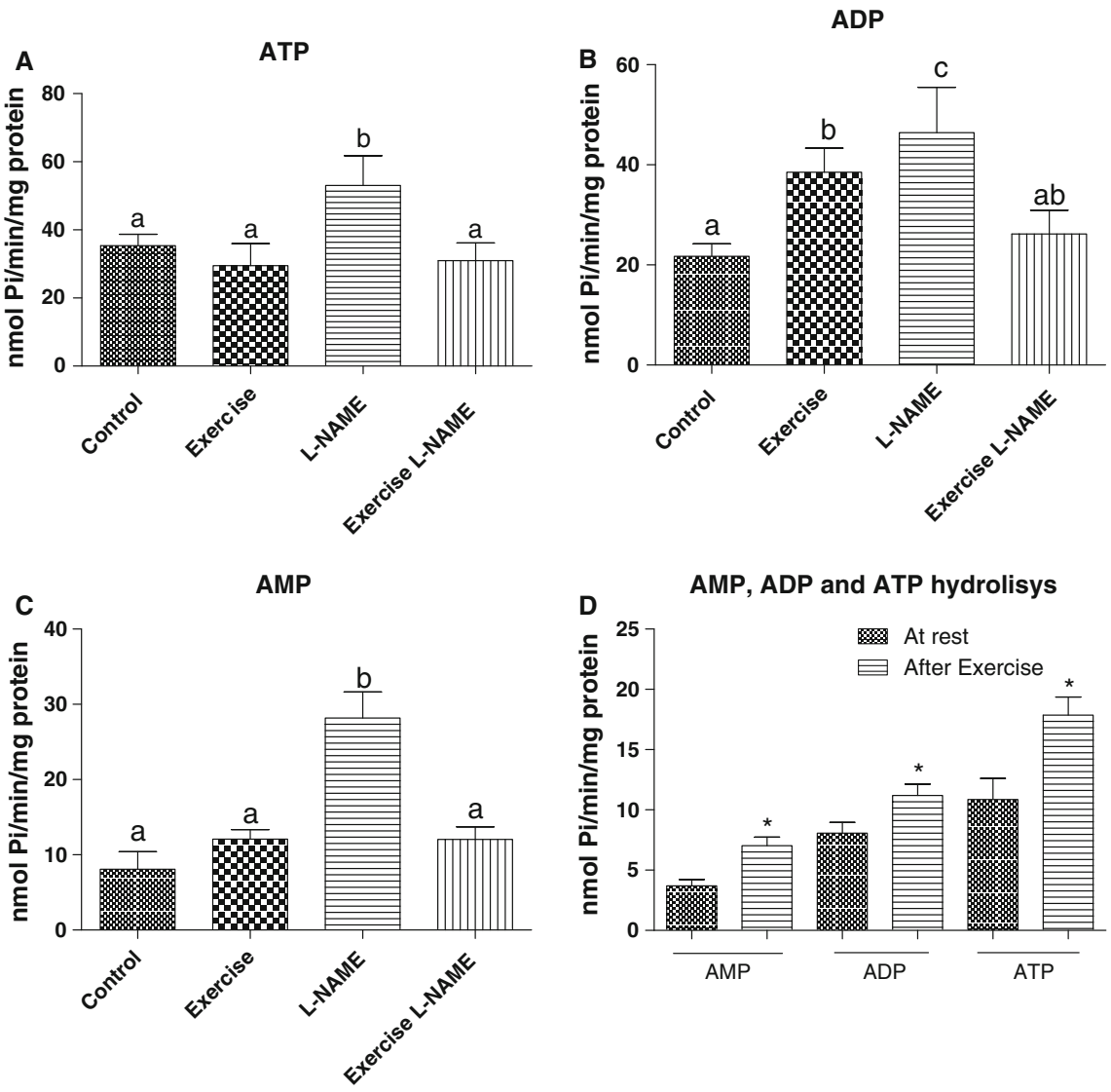

Fig. 2 **a** NTPDase (ATP), **b** NTPDase (ADP) and **c** ecto-5′-nucleotidase (AMP) activities in platelets of control group, exercise group, L-NAME group, and exercise L-NAME group. Data are presented as mean ± SD. Groups with *different letters* are statistically different ($p < 0.05$, $n = 10$ to each group). **d** Ecto-5′-nucleotidase (AMP), NTPDases (ATP and ADP) activities in platelets of rats at rest and after an acute bout of exercise. Data are presented as mean ± SD. *Mean difference between each nucleotide hydrolysis ($p < 0.05$, $n = 5$ to each group)

Encouraging regular physical exercise has been recommended by health professionals to maintain good cardiovascular fitness and prevent or treat hypertension. For the evaluation of training-related effects in hypertension models, several kinds of exercise protocols have been applied. Regularly performed aerobic exercises significantly reduce the high blood pressure in rats with spontaneous hypertension [36, 40] and in rats with hypertension induced by L-NAME administration [6, 38, 39] corroborating our results obtained with rats performing 6 weeks of swimming protocol, which corresponds to a moderate aerobic exercise.

The beneficial effects of physical exercises on hypertension have been explained by several pathways, including, for example, an increase in the nitric oxide production, which has a vasodilatation effect [41]. However, until now there are no studies proposing to assess ecto-nucleotidase activities, even noting that these enzymes are known as having cardioprotector effects [10]. This way, this is the first study that aimed to evaluate the ecto-nucleotidase activities related to exercise benefits in hypertension.

Ecto-nucleotidases are enzymes that have an interesting role in thromboregulation mechanisms. Alterations in their activities have been verified in various studies from our

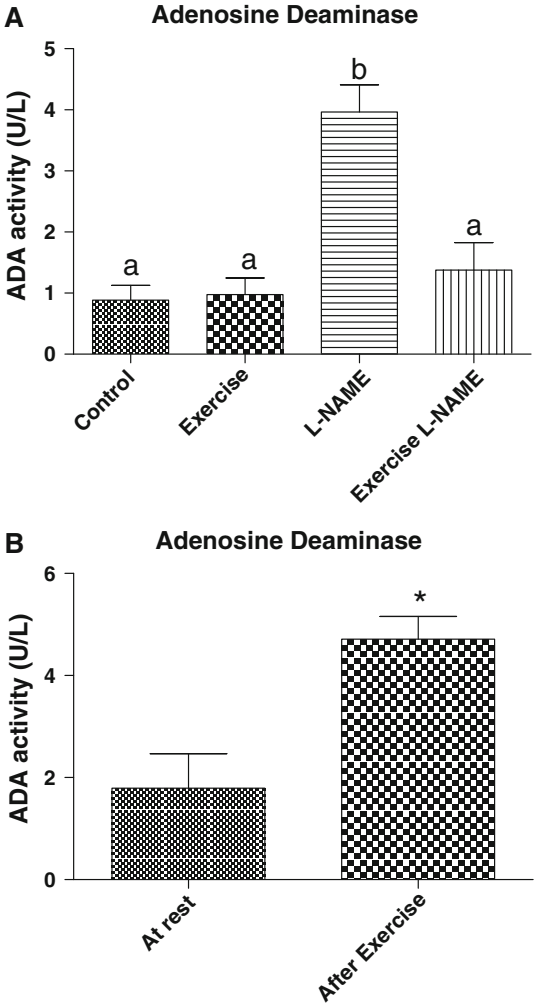

Fig. 3 **a** Adenosine deaminase (ADA) activity in platelets of control group, exercise group, L-NAME group, and exercise L-NAME group. Data are presented as mean ± SD. Groups with *different letters* are statistically different ($p < 0.05$, $n = 10$ to each group). **b** Adenosine deaminase (ADA) activity in platelets of rats at rest and after an acute bout of exercise. Data are presented as mean ± SD. *Mean difference between nucleoside hydrolysis ($p < 0.05$, $n = 5$ to each group)

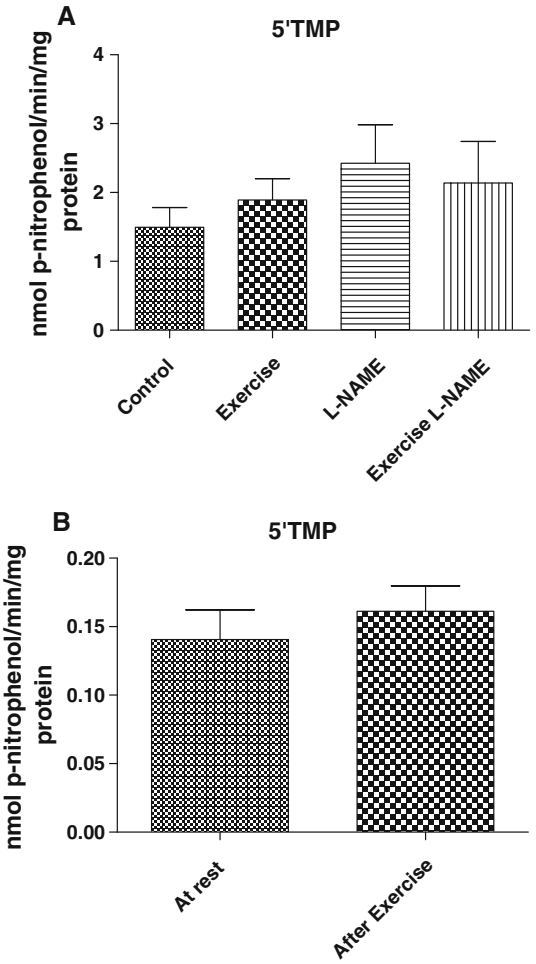

Fig. 4 **a** E-NPP activity in platelets of control group, exercise group, L-NAME group, and exercise L-NAME group ($n = 10$ to each group). **b** E-NPP activity in platelets of rats at rest and after an acute bout of exercise ($n = 10$ to each group). Data are presented as mean ± SD

group [34], suggesting that they could be important physiological and pathological parameters of several pathologies, including hypertension [14].

Regarding L-NAME-induced hypertension in rats, it has recently been demonstrated that ecto-nucleotidase activities are altered and a decrease was found in these enzyme activities [7] after 15 days of L-NAME administration. In contrast, our results showed an increase in the NTPDase and ecto-5′-nucleotidase activities in L-NAME treated rats after 60 days treatment in ATP, ADP, and AMP hydrolysis. These differences between studies could be related to the duration of L-NAME administration, since our study had a long-term duration when compared to Fürstenau et al. [7] study. Furthermore, is important to highlight that in Fürstenau et al. [7] study, they found a decreased in purine levels and suggested a compensatory mechanism of endothelial NTPDase 1 that could be activated in order to justify the depletion of ADP and AMP levels. This information can help us to clarify why in our study platelet ecto-nucleotidases are activated. With the prolonged time of L-NAME administration, these platelet enzymes, in L-NAME-treated rats of our study, can be doing the same role as endothelial NTPDase 1.

In this line of thought, we suggest that a compensatory mechanism of NTPDase (ATP and ADP) and ecto-5′-nucleotidase in platelets could be acting concerning the development of hypertension in the L-NAME group. Since ATP and ADP hydrolysis favors adenosine production, a rapid hydrolysis of these nucleotides is beneficial to hypertension control because of the vasodilatation and the inhibition of platelet aggregation

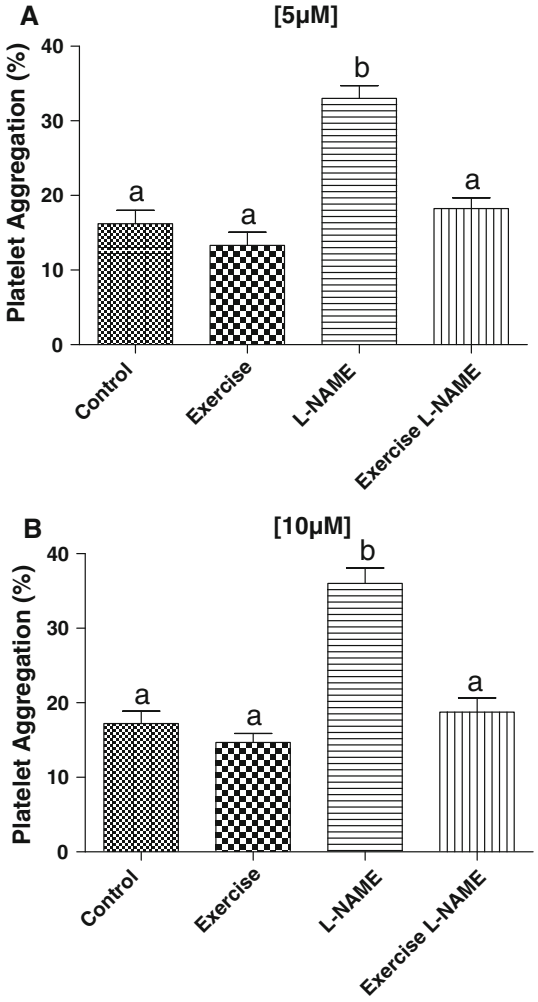

Fig. 5 Platelet aggregation profile of control group, exercise group, L-NAME group, and exercise L-NAME group. Platelet aggregation was evaluated by using ADP at concentrations of 5 and 10 μM as agonist (graphs **a** and **b**, respectively). The results are expressed as percentage of aggregation. Groups with *different letters* are statistically different ($p < 0.05$, $n = 10$ to each group)

properties of adenosine. Consequently, the organism could be avoiding thrombotic processes by compensatory ADP depletion and adenosine production.

Other point that has to be highlighted is that platelet aggregation also can be regulated by the NTPDase 1 present in vasculature. This enzyme has a great importance in controlling vascular tone and hydrolyzing circulating nucleotides as it faces the blood circulation [42]. According to Sévigny et al. [42], vascular NTPDase 1 abrogates platelet aggregation by depleting ADP and, this way, precludes the activation of specific ADP receptors in platelets, such as P2Y1 and P2Y12. Thus, NTPDase 1 present in vasculature and in platelets can be acting together to avoid hypertension complications. As such, further studies are necessary in order to clarify the induction raised here.

E-NPPs are another group of enzymes that participate in the cascade of nucleotide hydrolysis resulting in the production of nucleotide monophosphate. It is made up of three members (NPP1–3) responsible for the conversion of cyclic AMP to AMP, ATP to AMP, and ADP to AMP and responsible for hydrolyzing 5′-phosphodiester bonds in nucleotides and their derivatives, having both purines and pyrimidines as substrates [19, 21].

In our study, no significant changes were found regarding the E-NPP activity, even though a little tendency could be observed in favor of its activation in L-NAME-treated rats. From this trend, we could speculate that the same compensatory mechanism of NTPDase (ATP and AMP) could be acting, and the E-NPP activity could be statistically activated if rats stayed more time in this pathological condition. An increase in the E-NPP activity may lead to an increase in AMP levels, which can be hydrolyzed by ecto-5′-nucleotidase action into adenosine [12].

However, the ADA activity in L-NAME-treated rats is augmented, i.e., the same adenosine formed as a compensatory mechanism to avoid hypertension complications is being hydrolyzed and will probably not exert its effects. This could be understood because in hypertension there is a predisposition to thrombus formation [14]. There is an intrinsic cell–cell interaction between platelets, neutrophils, erythrocytes, and endothelial cells in this kind of complication [43]. This way, it would not be fair to say that the enhancement in platelet NTPDase activity could totally avoid thrombus formation, since the augment on ATP and ADP hydrolysis could not be sufficient to prevent this action in the microenvironment of such pathological condition.

Adenosine is involved in thromboregulation, which is a process or group of processes by which circulating blood cells and cells of the vessel wall interact to regulate or inhibit thrombus formation [43]. As we see through the great increase in the ADA activity and, consequently, the decrease in adenosine levels, thromboregulation did not occur efficiently in L-NAME-treated rats, which can be a great factor of hypertension development observed in this study.

Reinforcing this line of reasoning, we investigated platelet aggregation. We observed an increase in the platelet aggregation in hypertensive rats at different concentrations of ADP as agonist. Since adenosine plays an important role in the purinergic metabolism inhibiting platelet aggregation and exerting a reduction of vascular injury [9], this increase in platelet aggregation observed in L-NAME-treated rats can be explained by the probable absence of adenosine levels in consequence of ADA activation. Even adenosine levels were not measured in this study, the strong activation of ADA allows us to infer that

the amount of adenosine is diminished in L-NAME-treated rats, this way, contributing to the platelet activation.

Regarding exercise effects in L-NAME-treated rats, a protector action of moderate intensity exercise (swimming) in ecto-nucleotidase activities could be clearly observed, since in the L-NAME-exercised group, NTPDase (ATP and ADP), ecto-5′-nucleotidase, E-NPP, and ADA activities kept similar to control group. We have to underline the effect of exercise on the adenosine hydrolysis, leaving a larger amount of extracellular adenosine available in the middle, which can explain the prevention of platelet aggregation in the L-NAME-exercised group. These findings confirm the great protector of exercise training effects to avoid hypertension complications.

With the purpose of complementing the work and explaining chronic changes in ecto-nucleotidase activities, we verified changes in these enzymes due to an acute bout of exercise (Figs. 2d, 3b, 4b). Some studies [24, 44, 45] have shown that during exercise, red blood cells may control coronary blood flow by releasing ATP in areas of low oxygen tension caused by increased myocardial oxygen extraction. This increase in ATP and its metabolite (ADP, AMP, and adenosine) concentrations in the bloodstream, although being released by erythrocytes, may explain the rise in ecto-nucleotidase activities as a result of a single bout exercise found in our work, since these enzymes are present on the surface of platelets and probably are in contact with nucleotides released from red cells.

A study developed by Yegutkin et al. [46] is the unique that have assessed changes in platelet ecto-nucleotidase activities in response to an acute exercise. They showed that strenuous exercise significantly augments platelet activity via transient ADP release, producing acute prothrombotic responses. In contrast, our study indicated an augment in the whole nucleotide hydrolysis, including ADP. These differences on findings probably are related to the intensity of exercises, suggesting that ecto-nucleotidases, regarding moderate exercise, respond differently from ecto-nucleotidases regarding strenuous exercise.

As reported before, chronic exercise in hypertensive rats prevented ecto-nucleotidase alterations, platelet aggregation and reduced blood pressure in the L-NAME-exercised group. It is reasonable to assume that the augment of the ecto-nucleotidase activities as a result of an acute exercise can produce an adaptation by the organism, i.e., with several exercise sessions ecto-nucleotidases became prepared to receive an exercise stimulus. This way, they keep with low activity when the organism is at rest as showed by our results, probably because low ATP is being released from red cells or other sources [24] in an organism already adapted to training as in our study. However, this possible mechanism is encouraged to be more investigated in further studies.

It is interesting to note that exercise per se was able to increase NTPDase (ADP) activity on normotensive rats. Since NTPDase (ADP) activity is augmented, it means that more ADP is being hydrolyzed [10] and it could explain one of the training mechanisms on tromboregulation. Since less concentration of ADP is in the microenvironment and ADP is the most important molecule that pursues pro-aggregant action, the platelet aggregation occurs in low intensity [10, 13]. This training effect could be understood as one of the main protector actions of swimming in L-NAME-treated rats.

In conclusion, our study suggests that moderate exercise training prevents ecto-nucleotidase alterations in platelets of rats that developed hypertension in response to oral administration of L-NAME, probably for the adaptation of these enzymes to stimuli caused by the exercise sessions that can modulate ecto-nucleotidase activities and inhibit platelet activation, highlighting the great protector exercise training effects to avoid hypertension complications.

This modulation can be involved in the hypotensive effect of exercise and the mechanisms of this link are encouraged to be investigated in further studies.

Acknowledgments The authors wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Nacional de Ciência e Tecnologia (INCT) and Rede Instituto Brasileiro de Neurociência (IBN-Net). Furthermore, the authors would like to thank to the members of BioEx (Laboratório de Bioquímica do Exercício—UFSM) and to professor Cristina Ribas Fürstenau for the help during the experiment.

References

1. Rogers VL, Go AS, Lloyd-Jones DM et al. on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2011) Heart disease and stroke statistics-2011 update: a report from the American Heart Association. Circulation 123:180–209
2. Lloyd-Jones D, Adams R, Carnethon M et al (2009) Heart disease and stroke statistics-2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119(3):480–486
3. Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–142
4. Radomski MW, Palmer RMJ, Moncada S (1990) Characterization of the L-arginine–nitric oxide pathway in human platelets. Br J Pharmacol 101:325–328
5. Lerman LO, Chade AR, Sica V, Napoli C (2005) Animal models of hypertension: an overview. J Lab Clin Med 146:160–173
6. Husain K (2002) Exercise conditioning attenuates the hypertensive effects of nitric oxide synthase inhibitor in rat. Mol Cel Biochem 231:129–137
7. Fürstenau CR, Trentin DS, Gossenheimer AN et al (2008) Ectonucleotidase activities are altered in serum and platelets of L-NAME-treated rats. Blood Cells Mol Dis 41:223–229

8. Fürstenau CR, Ramos DB, Vuaden FC et al (2010) L-NAME-treatment alters ectonucleotidase activities in kidney membranes of rats. Life Sci 87:325–332
9. Bakker WW, Poelstra A, Barradas K, Mikhailidis MA (1994) Platelets and ectonucleotidases. Platelets 5:121–129
10. Atkinson B, Dwyer K, Enjyoji K, Robson SC (2006) Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. Blood Cells Mol Dis 36:217–222
11. Soslau G, Youngprapakorn D (1997) A possible dual physiological role of extracellular ATP in modulation of platelet aggregation. Biochim Biophys Acta 1355:131–140
12. Colgan SP, Eltzschig HK, Eckle T, Thompson LF (2006) Physiological roles for ecto-5′-nucleotidase (CD73). Purinergic Signal 2:351–360
13. Schetinger MR, Morsch VM, Bonan C, Wyse A (2007) NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. BioFactors 31:77–98
14. Lunkes GL, Lunkes D, Stefanello F et al (2003) Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thromb Res 109:189–194
15. Spanevello RM, Mazzanti CM, Bagatini M, Correa M, Schmatz R, Stefanello N, Thome G, Morsch VM, Becker L, Belle L, Oliveira L, Schetinger MRC (2010) Activities of the enzymes that hydrolyze adenine nucleotides in platelets from multiple sclerosis patients. J Neurol 257:24–30
16. Schmatz R, Schetinger MRC, Spanevello RM, Mazzanti CM, Stefanello N, Maldonado PA, Gutierres J, Corrêa MC, Girotto E, Moretto MB, Morsch VM (2009) Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. Life Sci 84:345–350
17. Bagatini MD, Martins CC, Gasparetto D, Spanevello RM, Becker LV, Rosa CS, Battisti V, Bellé L, Gonçalves JF, Schetinger MRC, Santos RB, Oliveira LZ, Morsch VM (2011) Enzymes that hydrolyze adenine nucleotides in patients with ischemic heart disease. Clin Chim Acta 412:159–164
18. Bagatini MD, Martins CC, Spanevello RM, Rosa CS, Gonçalves JF, Schetinger MRC, Santos RB, Morsch VM (2008) Hydrolysis of adenine nucleotides in platelets from patients with acute myocardial infarction. Clin Biochem 41:1181–1185
19. Fürstenau CR, Trentin DS, Barreto-Chaves MLM, Sarkis JJF (2006) Ecto-nucleotide pyrophosphatase/phosphodiesterase as part of a multiple system for nucleotide hydrolysis by platelets from rats: kinetic characterization and biochemical properties. Platelets 17:84–91
20. Robson SC, Sevigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430
21. Stefan C, Jansen S, Bollen M (2005) NPP-type ectophosphodiesterases: unity in diversity. Trends Biochem Sci 30:542–550
22. Guisti G, Galanti B (1984) Colorimetric method. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinheim, pp 315–323
23. Dunbar CC (1992) The antihypertensive effects of exercise training. NY State J Med 92(6):250–255
24. Gorman MW, Feigl EO (2011) Control of coronary blood flow during exercise. Exerc Sport Sci Rev 40(1):37–42. doi: 10.1097/JES.0b013e3182348cdd
25. Kuru O, Sentürk ÜK, Koçer G (2009) Effect of exercise training on resistance arteries in rats with chronic NOS inhibition. J Appl Physiol 107:896–902
26. Medeiros A, Gianolla RM, Kalil LMP et al (2000) Efeito do treinamento físico de natação sobre o sistema cardiovascular de ratos normotensos. Rev Paul Ed Física 14:7–15
27. Gobatto CA, Mello MAR, Sibuya CY et al (2001) Maximal lactate steady state in rats submitted to swimming exercise. Comp Biochem Physiol A 130:21–27
28. Lunkes G, Lunkes D, Morsch V et al (2004) NTPDase e 5′-nucleotidase activities in rats alloxan -induced diabetes. Diabetes Res Clin Pract 65:1–6
29. Chan K, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal Biochem 157:375–380
30. Yun-Choi HS, Park KM, Pyo MK (2000) Epinephrine induced platelet aggregation in rat platelet-rich plasma. Thromb Res 100(6):511–518
31. Born GVR, Cross MJ (1963) The aggregation of blood platelets. J Physiol 951:68–78
32. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:218–254
33. Tsai J, Liu J, Kao C et al (2002) Beneficial effects on blood pressure and lipid profile of programmed exercise training in subjects with white coat hypertension. AJH 15:571–576
34. Veras-Silva AS, Mattos KC, Gava NS et al (1997) Low-intensity exercise training decreases cardiac output and hypertension in spontaneously hypertensive rats. Am J Physiol 273:2627–2631
35. Shepherd RE, Kuehne ML, Kenno KA et al (1982) Attenuation of blood pressure increases in Dahl salt-sensitive rats by exercise. J Appl Physiol 52(6):1608–1613
36. Fregly MJ (1984) Effect of an exercise regimen on development of hypertension in rats. J Appl Physiol 56(2):381–387
37. Marcus KD, Tipton CM (1985) Exercise training and its effects with renal hypertensive rats. J Appl Physiol 59(5):1410–1415
38. Kuru O, Senturk UK, Demir N et al (2002) Effect of exercise on blood pressure in rats with chronic NOS inhibition. Eur J Appl Physiol 87(2):134–140
39. Kuru O, Gulkesen H, Demir N, Gunduz F (2005) Physical training increases renal injury in rats with chronic NOS inhibition. Ren Fail 27:459–463
40. Tipton CM, Sebastian LA, Overton JM et al (1991) Chronic exercise and its hemodynamic influences on resting blood pressure of hypertensive rats. J Appl Physiol 71(6):2206–2210
41. Maeda S, Miyauchi T, Kakiyama T (2001) Effects of exercise training of 8 weeks and detraining on plasma levels of endothelium-derived factors, endothelin-1 and nitric oxide, in healthy young humans. Life Sci 69:1005–1016
42. Sévigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, Imai M, Zimmermann H, Robson CS (2002) Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1(NTPDase1) and NTPDase 2 have implications for thromboregulation. Blood 99:2801–2809
43. Kawashima Y, Nagasawa T, Ninomiya H (2000) Contribution of ecto-5′ nucleotidase to the inhibition of platelet aggregation by human endothelial cells. Blood 96:2157–2162
44. Farias M, Gorman MW, Savage MV, Feigl EO (2005) Plasma ATP during exercise: possible role in regulation of coronary blood flow. Am J Physiol Heart Circ Physiol 288:1586–1590
45. Gorman MW, Rooke GA, Savage MV, Jayasekara MPS, Jacobson KA, Feigl EO (2010) Adenine nucleotide control of coronary blood flow during exercise. Am J Physiol Heart Circ Physiol 299:1981–1989
46. Yegutkin GG, Samburski SS, Mortensen SP, Jalkanen S, Gonzalez-Alonso J (2007) Intravascular ADP and soluble nucleotidases contribute to acute prothrombotic state during vigorous exercise in humans. J Physiol 579:553–564