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Effects induced by exercise on lymphocyte β-adrenergic receptors and plasma catecholamine levels in performance horses

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ABSTRACT

The effect of dynamic exercise on complete blood cell count, lymphocyte β-adrenergic receptor and plasma catecholamine (adrenaline and noradrenaline) levels in horses performing different disciplines were investigated during rest and after exercise. Blood samples were collected from jumping horses (n = 6), Arabian Endurance horses (n = 6) and Standardbred trotters (n = 6) before and immediately after competition. Dynamic exercise caused a significant increase in red blood cell count (Standardbred trotters: P = 0.0012), haemoglobin concentration (jumping horses: P = 0.001; Standardbred trotters: P = 0.01), haematocrit percentage (Standardbred trotters: P = 0.005), neutrophil percentage (jumping horses: P = 0.0003), lymphocyte percentage (jumping horses: P = 0.0003), monocyte percentage (Standardbred trotters: P = 0.0008), lymphocyte β-AR numbers (jumping horses: P = 0.01; Arabian Endurance horses: P = 0.016; Standardbred trotters: P = 0.05), plasma adrenaline concentration (Standardbred trotters: P = 0.0001) and plasma noradrenaline levels (Standardbred trotters: P = 0.003). It is concluded that acute increases in plasma catecholamine concentrations depended on the exercise performed and may induce up-regulation of β-AR in equine lymphocytes. However, the exact mechanism of β-AR up-regulation still remains unclear.

Keywords: Horse, β-Adrenergic receptors, Catecholamines, Lymphocytes, Exercise

INTRODUCTION

The sympathetic nervous system generally plays an important role in the mediation of the response to exercise, modifying cardiovascular system function, pulmonary function, thermoregulation, muscle tone and metabolic function. The influence of the sympathetic nervous system during exercise is mediated by the release of adrenaline and noradrenaline, which exert their effects by binding to α- and β-adrenergic receptors (β-AR) in target tissues (McKeever, 1993). In basal conditions, the β-AR signal transduction pathway in human lymphocytes is significantly correlated with that in other body tissues. Particularly, the number and the responsiveness of β-AR to an agonist in human lymphocytes are closely related to those in the myocardium (Brodde et al., 1986). The correlation between the number of β-AR in lymphocytes and myocardial cell membranes was also demonstrated in healthy dogs by Re et al. (1999). Moreover, a significant correlation between β-AR downregulation in circulating lymphocytes and in the myocardium in dogs affected by dilated cardiomyopathy has been observed, thus suggesting that it may be possible to measure the density of β-AR in circulating lymphocytes in order to indirectly monitor β-AR changes during pathological conditions (Re et al., 1999). However, differences between β-AR subtype distribution in circulating lymphocytes in human and in animals must be taken into account for the interpretation of results from different studies. In fact, it has been demonstrated that human lymphocytes contain a homogeneous population of β-AR represented by β_2-adrenergic receptors (β_2-AR) (Williams et al., 1976), whereas canine and equine lymphocytes express a non-homogeneous population of β-AR. In particular, dogs possess about 35% b1-adrenergic receptors and about 65% β_2-AR (Re et al., 1999). On the other hand, horses are more similar to humans, showing a substantial amount of β_2-AR (>90%) (Abraham et al., 2001). Dynamic exercise induces a significant increase in the number of β-AR in human lymphocytes (Fujii et al., 1996) and in rat myocardium (Izawa et al., 1989). For this reason, lymphocytes derived from peripheral blood are often utilized as a model for determining the effects of acute dynamic exercise on the β-AR system. The ‘‘fight or flight’’ response caused by the adrenergic system activation is mediated by neurotransmitters such as noradrenaline, dopamine and adrenaline (Lefkowitz et al., 2001). This response involves the local release of noradrenaline from the sympathetic nerve ending and a systemic release of adrenaline from the adrenal medulla. Exercise has been shown to increase plasma catecholamine concentrations both in human beings (Mastorakos and Pavlatou, 2005) and horses (Hada et al., 2003). Catecholamine release plays an important role in the activation and modulation of many physiological and biochemical responses to

exercise, such as the maintenance of cardiovascular and respiratory functions, activation of metabolic pathways, resulting in elevated blood concentration of glucose and free fatty acids, which facilitate the delivery of substrate to tissues. The release of these hormones reflects the increased sympathetic nervous system activity. Since to date no data exist on the modification in lymphocyte β- AR number induced by exercise in horses, the aim of the present study was to investigate the concentration of β-AR in lymphocytes and of plasma catecholamine levels before and after exercise in horses performing different disciplines. According to the fact that equine lymphocytes show a substantial amount of β_2-AR, as previously described, we measured total β-AR concentrations in equine lymphocytes.

MATERIALS AND METHODS

Horses

Eighteen intact or castrated healthy male and female horses were divided into three groups according to their performance attitude:

- group I: six jumping horses, 6.3 ± 0.8 (mean ± SEM) year old, weighing 562 ± 17.5 kg.
- group II: six Arabian Endurance horses, 9.8 ± 0.9 year old, weighing 485 ± 23.5 kg.
- group III: six Standardbred trotters, 4 ± 0.4 year old, weighing 555.6 ± 16.7 kg.

All the horses had not received any medications during the last 15 days, except for routine vaccination. Animals were fed daily with a standard diet including hay and protein supplementation and automatic vessels provided water ad libitum. Horses were trained for an average of 5 days per week and competed once a week. In particular, group I horses were trained with standard exercise 5 days every week walking, trotting, cantering and jumping; they had show jumping 1 day weekly in a jumping race composed of 15 jumps heightening at most 1.40 m. Group II horses were trained with standard exercise 3 days per week walking, trotting and cantering in the countryside and raced once per week having endurance competitions for 30 km. Group III horses were trained with standard exercise 6 days weekly trotting in a racetrack and competed 1 day weekly trotting on a distance of 1600 m.

Blood samples collection

Forty millilitre of whole blood (1.6 mg EDTA/ml blood) were collected by jugular venipuncture before and immediately after competitions in order to determine complete blood cell (CBC) count, lymphocyte β-AR and plasma catecholamine concentrations. Aliquots of 30 ml of blood were used to separate lymphocytes and aliquots of 5 ml of blood were immediately centrifuged and stored (-80 °C) until plasma catecholamine levels were measured. The remained aliquots of blood (5 ml) were used to perform the CBC count by the use of a blood analyser (SEAC hemat 8, Calenzano, Florence, Italy).

Isolation of lymphocytes

Lymphocytes were isolated by a density gradient centrifugation method (Böyum, 1968) introducing some minor modifications. Briefly, 30 ml of fresh whole blood were diluted with an equal volume of phosphate-buffered saline (PBS) (25 mM Na2HPO4; 1.8 mM KH2PO4; 170 mM NaCl; 3.35 mM KCl; pH 7.4). About 20 ml of the diluted blood was carefully layered on 20 ml sodium diatrizoate (Histopaque-1077, Sigma Diagnostic, St. Louis, MO, USA) in 50 ml tubes and centrifuged at 1000g for 20 min at room temperature. The plasma/platelet layer was carefully removed and the mononuclear cell layer, consisting exclusively of lymphocytes, was transferred to 50 ml centrifuge tubes and washed twice with 40 ml of PBS by centrifugation at 3200g for 5 min at 4 °C. The resulting pellets were gently re-suspended in 1 ml of incubation buffer (50 mM Tris–HCl; 250 mM sucrose; 1 mM EDTA; pH 7.4). Lymphocytes were then counted with an automatic cell counter (SEAC hemat 8, Calenzano, Florence, Italy) and the suspension was adjusted by incubation buffer to a concentration of 1.4 x 10^6 cells/100 µl.

β-Adrenergic receptor binding assays

β -AR measurement was performed using the method described by Re et al. (1999). Aliquots of lymphocyte suspensions (100 µl) were incubated for 60 min at 37 °C with increasing concentrations of the non-selective β -AR antagonist (-)[3H]CGP 12177 (NEN Du Pont De Nemours Italiana, Cologno Monzese, Milan, Italy) (0.125–4 nM) in a total volume of 200 µl. Non-specific binding was calculated in the presence of 100 µM (-) isoproterenol and subtracted from the total (-)[3H]CGP 12177 binding to obtain specific binding at each concentration used. Incubation was stopped by adding 2 ml of ice-cold

buffered saline solution (50 mM Tris–HCl; 154 mM NaCl; pH 7.4), and the incubation mixtures were immediately filtered under vacuum over pre-soaked glass microfiber filters (Whatman GF/C, Whatman International Ltd., Maidstone, England). The filters were then rapidly washed with 4 ml of the buffered saline solution for three times and solubilized with 4 ml of scintillation fluid (Filter Count, Canberra Packard, Groningen, The Netherlands). The radioactivity retained on the wet filters was measured by the use of a beta counter (Tri-Carb 1600 TR, Canberra Packard, Groningen, The Netherlands) at an efficiency of 60%. The maximum number of binding sites (B_{max}) and the equilibrium dissociation constants (K_d) for (-)[3H]CGP 12177, expressed as femtomoles per mg of protein and nanomolar (nM) values, respectively, were calculated from plots according to Scatchard (1949) analysis. The number of binding sites per cell was calculated using the equation $n = B \times N/c$, where n is the number of binding sites, B is the number of ligand moles bound, N is the Avogadro number and c is the number of cells per tube.

Plasma catecholamine levels

Plasma adrenaline and noradrenaline concentrations were determined by radioimmunoassay using a commercial kit validated for the horse (Rietmann et al., 2004) (TriCat TM, IBL Hamburg, Germany).

Statistical analysis

The data measured among the different groups were statistically processed by the Kolmogorov–Smirnov test to assess normal distribution. This test showed a P value larger than 0.01 for all data measured among different groups of this study, suggesting that the frequency distribution did not differ significantly from normal distribution. Statistical analysis of complete blood cell counts, lymphocyte β -AR concentrations and plasma catecholamine levels was performed using a repeated measures ANOVA (SPSS, Chicago, Illinois, USA) with Tukey post test. Significance was taken for $P < 0.05$. All measurement was performed in triplicate and data are expressed as means ± SEM.

RESULTS

Complete blood cell count

Exercise caused an increase in red blood cell number, haemoglobin concentration, haematocrit percentage, neutrophil and monocyte count, whereas white blood cells and circulating lymphocytes decreased (Table 1). When horses were divided in groups according to their performance attitude, a different basal value in haemoglobin concentration (group I vs. group II: $P < 0.001$; group I vs. group III: $P < 0.001$), haematocrit percentage (group I vs. group III: $P < 0.05$), neutrophils (group I vs. group II: $P < 0.001$; group II vs. group III: $P < 0.05$), lymphocytes (group I vs. group II: $P < 0.01$) and eosinophils (group II vs. group III: $P < 0.05$) was observed. At the same time, red blood cell (group I vs. group III: $P < 0.001$; group II vs. group III: $P < 0.001$), platelets (group I vs. group II: $P < 0.05$) and white blood cell number (group I vs. group II: $P < 0.01$; group I vs. group III: $P < 0.01$) showed significant differences among the three experimental groups after exercise.

Lymphocyte b-AR concentrations

The linearity of the Scatchard plots was demonstrated by the values of the correlation coefficient ($r > 0.90$). Dissociation constant values for total lymphocyte β -AR before and after competition were not significantly different in group I (0.12 ± 0.04 nM vs.0.22 ± 0.08 nM, $P = 0.79$), group II (0.12 ± 0.03 nM vs. 0.43 ± 0.14 nM, $P = 0.39$) and group III (0.19 ± 0.06 nM vs. 0.43 ± 0.14 nM, $P = 0.09$). Total lymphocyte β -AR concentrations increased after exercise from 3011 ± 293.8 to 13,765 ± 2765 sites/cell in group I ($P = 0.01$), from 3943 ± 375.3 to 13,334 ± 2624 sites/cell in group II ($P = 0.016$) and from 4904 ± 504.1 to 10,754 ± 1154 sites/cell in group III ($P = 0.05$) (Fig. 1). No differences were detected between groups ($P > 0.05$).

Plasma catecholamine concentrations

Plasma adrenaline levels increased after exercise from 0.046 ± 0.002 to 0.055 ± 0.007 ng/ml in group I ($P > 0.05$), from 0.044 ± 0.003 to 0.055 ± 0.009 ng/ml in group II ($P > 0.05$) and from 0.042 ± 0.003 to 0.110 ± 0.004 ng/ml in group III ($P = 0.0001$) (Fig. 2). An increase in plasma noradrenaline levels after exercise was also observed. In particular,

noradrenaline increased from 0.13 ± 0.011 to 0.14 ± 0.004 ng/ml in group I ($P > 0.05$), from 0.17 ± 0.013 to 0.22 ± 0.021 ng/ml in group II ($P > 0.05$) and from 0.13 ± 0.013 to 0.28 ± 0.033 ng/ml in group III ($P = 0.003$) (Fig. 3). Additionally, post exercise plasma adrenaline was significantly higher in group III compared to group I ($P = 0.01$) and group II ($P = 0.001$) and post exercise plasma noradrenaline was significantly higher in group II and group III compared to group I ($P = 0.021$ and $P = 0.007$, respectively) (Fig. 3). No significant difference in basal values for adrenaline or noradrenaline was observed among groups.

DISCUSSION

Responses of blood leukocytes and their subpopulations to acute exercise are well known. White blood cells and neutrophils increase during and after exercise, whereas lymphocyte concentrations increase during exercise and fall below basal values after long-duration physical works (Pedersen and Hoffman-Goetz, 2000). However, in our study we did not observe a significant decrease in WBC after prolonged exercise. A significant neutrophilia in group I ($P = 0.0003$), a significant lymphopenia in group I ($P = 0.0003$) and a significant monocytosis in group III ($P = 0.0008$) were found. The exact mechanism of leukopenia is unclear; the other findings are in agreement with those reported by Robson et al. (2003). During exercise, alterations to leukocyte kinetics have been explained by rises in hormone concentrations, such as cortisol (Shinkai et al., 1996), adrenaline (Tønnesen et al., 1987) and growth hormone (Kappel et al., 1993). In addition, the shear stress caused by an increased cardiac output may increase mobilization of leukocytes from marginated pools (Tønnesen et al., 1987). Previous studies have demonstrated an inverse relationship between plasma cortisol concentration and blood lymphocyte count (Fukuda et al., 1994; Shinkai et al., 1996). Moreover, cortisol also stimulates the release of neutrophils from the bone marrow into the circulation (Yang and Hill, 1991). These mechanisms may fully explain the post-race lymphopenia and neutrophilia observed in the current study. Another physiological response to exercise is the increase in the total number of circulating red blood cells, haematocrit percentage and haemoglobin concentration (Böning et al., 2010), agreeing with the observations in the current study. In fact, it is well recognized that in horses and dogs, as well as in several other species, the spleen acts as a reservoir for erythrocytes, storing a large volume of blood, approximately up to the 40–50% of the entire circulation (Stewart and McKenzie, 2002). The study conducted by McKeever et al. (1993)

demonstrated that splenectomised horses displayed a significant reduction in haematocrit percentage after exercise in comparison with non-splenectomised subjects. The release of stored erythrocytes from the spleen into the systemic circulation is under the influence of the sympathetic nervous system and circulating catecholamines, which cause the contraction of smooth muscle within the splenic capsule. However, exercise also causes some reduction in plasma volume, which is attributed to a fluid shift from the intravascular to the extravascular compartment as a result of fluid loss through sweating. This haemoconcentration results in an increase in haematocrit and haemoglobin levels (Stewart and McKenzie, 2002). The increase in haematocrit percentage is a function of exercise intensity; in fact a linear relationship between haematocrit and speed exists up to a haematocrit of 60–70% (Erickson and Poole, 2004). Since erythrocytes have an oxygen-carrying capacity, this sort of auto-transfusion has an important role in improving oxygen transport and aerobic performance during exercise (Stewart and McKenzie, 2002). In the present study, the basal and post exercise value of total lymphocyte β -AR in athlete horses belonging to different disciplines were measured for the first time. Dynamic exercise significantly increased lymphocyte β -AR concentrations in jumping horses ($P = 0.01$), Arabian Endurance horses ($P = 0.016$) and in Standardbred trotters ($P = 0.05$). The lymphocyte β -AR up-regulation observed during our study in performance horses is in agreement with data obtained in previous studies performed in human beings (Fujii et al., 1996). However, this regulatory mechanism is not well understood, but different hypotheses have been formulated. Human studies demonstrated that exercise induces the increase of circulating T helper, T suppressor/cytotoxic and natural killer (NK) cells from the spleen (Goebel and Mills, 2000). T Helper cells express low β -AR concentrations, whereas higher concentrations of β -AR are present on T suppressor/cytotoxic and NK cell membranes. Moreover, NK cells are more responsive to exercise and other stressors than any other lymphocyte subpopulations (Pedersen and Hoffman-Goetz, 2000). Therefore, a correlation seems to exist between the density of β -AR expressed by lymphocyte subpopulations and their responsiveness to exercise. According to Benschop et al. (1993), adrenaline strongly contributes to the recruitment of NK cells from the marginated pools in blood vessels, lymph nodes and intestine. However, this redistribution of circulating lymphocyte subpopulations can only account for an increase in β -AR concentration of about 20% (Maisel et al., 1990). In our study, exercise induced approximately a 3.4-fold increase in lymphocyte β-AR concentration, therefore it seems that redistribution of circulating lymphocyte subsets, with different β -AR densities, is not sufficient to explain

the β -AR up-regulation observed after the performance. Van Tits et al. (1990) observed that men exposed to isoproterenol and adrenaline infusion showed an increase in β -AR density in lymphocyte subsets, especially as far as NK cells were concerned. They suggested that this phenomenon may be caused by a release of lymphocyte subsets relatively rich in β -AR concentration from the spleen into the circulation and/or by a redistribution of lymphocyte subsets between spleen and circulation, whereby freshly released splenic lymphocytes appeared to express higher β -AR concentrations than those found in the circulation. In fact, in their report, the increase in lymphocyte β -AR number was attenuated by 40% in splenectomised patients compared with normal subjects. Fujii et al. (1996) reported that dynamic exercise induced the translocation of β -AR from the inside to the outside of the lymphocyte plasma membrane, resulting in a higher receptor number and increased functionality, measured by isoproterenol-stimulated cAMP production. In another study, Fujii et al. (1997) demonstrated that the exercise-induced increase in β -AR density in human lymphocytes was associated with the increase of its mRNA expression. The exposure to β -adrenergic agonists seemed to control this process; in fact, it has been demonstrated that short-term (30 min) exposure of DDT1MF-2 hamster smooth muscle cells to 100 nM adrenaline activates the rate of β_2-AR gene transcription, resulting in an increase in β_2-AR mRNA level. However, de novo protein synthesis takes more time (hours) to occur, thus synthesis of new receptors seems to not account for the β -AR up-regulation measured in our study. In equine medicine, González et al. (1998) performed a study on the effects induced by exercise on erythrocyte β -AR concentrations. In this case, they demonstrated a down-regulation of β -AR, that was probably caused by the translocation from intracellular space to the plasmatic membrane of β-adrenergic receptor kinase (β -ARK). Such translocation would cause receptor phosphorylation and then G-protein uncoupling with consequent receptor sequestration. This mechanism could be an important response to exercise, thus preventing red blood cell aggregation. In fact, during a race, the faster flow of erythrocyte through capillary vessels could be impaired by the rise in plasma catecholamine levels, since these hormones decrease red cell plasticity. On this basis, exercisedinduced modulation of the adrenergic system may be different in the various blood cell populations and may play distinct roles in the adaptation to or in the manifestation of adverse effects during exercise. As far as plasma catecholamine levels are concerned, our study demonstrated that adrenaline and noradrenaline concentrations increased after exercise, reaching significant differences ($P = 0.0001$ and $P = 0.003$, respectively) only in Standardbred trotters. These data agree with other studies performed

in human beings (Mastorakos and Pavlatou, 2005) and in horses (Hada et al., 2003), and it has been suggested that the increase in plasma concentrations of adrenaline and noradrenaline may depend on the duration and intensity of dynamic exercise (Pedersen and Hoffman-Goetz, 2000). Circulating catecholamine initiates many of the metabolic events required for the maintenance of dynamic exercise: adrenaline stimulates the conversion of muscle glycogen to glucose phosphate and ultimately pyruvate, activates tissue lipases, inhibits insulin release, increases heart rate and cardiac output, is involved in the redistribution of blood flow during exercise, facilitates neuromuscular transmission in skeletal muscle and increases respiratory rate (Erickson and Poole, 2004). In conclusion, the activation of the sympathoadrenergic system in the horse caused by physical exercise may contribute to ameliorate the performance during a stressful situation. Moreover, it may be important with respect to adjustment of drug doses for trained animals, according to their discipline, compared to a untrained animals.

Acknowledgments

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FIGURES AND TABLES

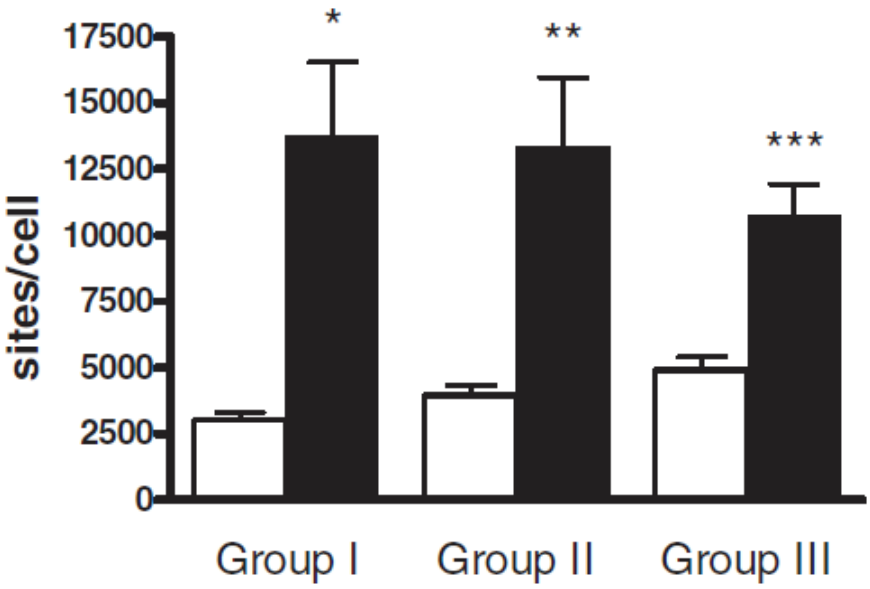

Fig. 1. Mean ± SEM values (n = 6) of lymphocyte b-AR before (white bar) and after (dark bar) exercise. Repeated measures ANOVA before and after exercise in group I (*P = 0.001), group II (**P = 0.016) and group III (***P = 0.05).

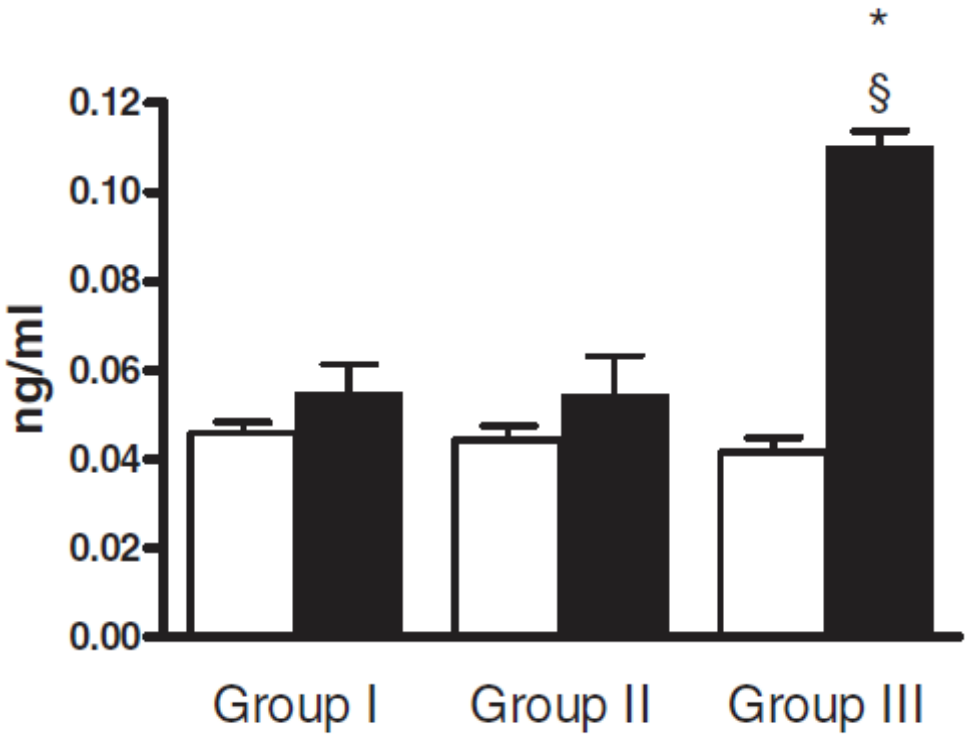

Fig. 2. Mean ± SEM values (n = 6) of plasma adrenaline concentrations before (white bar) and after (dark bar) exercise. Repeated measures ANOVA before and after exercise in group III (*P = 0.0001) and in group III vs. group I (§P = 0.001) and in group III vs. group II (§P = 0.0001).

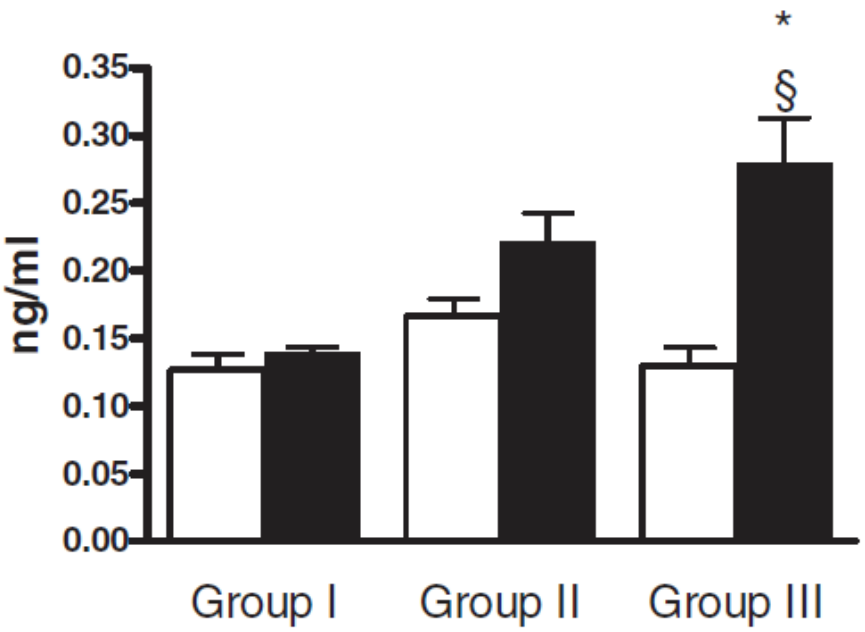

Fig. 3. Mean ± SEM values (n = 6) of plasma noradrenaline concentrations before (white bar) and after (dark bar) exercise. Repeated measures ANOVA before and after exercise in group III (*P = 0.003) and in group I vs. group II (§P = 0.021) and in group I vs. group III (§P = 0.007).

	Group I			Group II			Group III		
	Before exercise	After exercise	*P*	Before exercise	After exercise	*P*	Before exercise	After exercise	*P*
RBC (10^{12} cells/L)	7.3 ± 0.5	8.1 ± 0.1	N.S.	7.6 ± 0.3	8.0 ± 0.3	N.S.	8.5 ± 0.2	9.5 ± 0.1	0.0012
Hb (g/L)	10.0 ± 0.6	12.8 ± 0.1	0.001	12.8 ± 0.2	13.4 ± 0.4	N.S.	13.8 ± 0.3	14.8 ± 0.1	0.01
PCV (L/L)	32.3 ± 1.9	35.7 ± 0.1	N.S.	36.0 ± 1.1	37.5 ± 1.2	N.S.	38.6 ± 0.9	4.2 ± 0.3	0.005
PTL (10^{9} cells/L)	146.3 ± 4.7	147.0 ± 8.6	N.S.	134.2 ± 14.5	106.9 ± 11.5	N.S.	113.0 ± 11.0	123.0 ± 3.7	N.S.
WBC (10^{9} cells/L)	6.8 ± 0.4	6.4 ± 0.5	N.S.	9.6 ± 1.7	9.1 ± 0.6	N.S.	9.0 ± 0.3	8.8 ± 0.1	N.S.
N (%)	54.4 ± 2.1	67.0 ± 0.9	0.0003	73.0 ± 3.5	75.2 ± 2.7	N.S.	63.0 ± 2.9	66.5 ± 2.2	N.S.
E (%)	2.3 ± 0.9	0.5 ± 0.3	N.S.	0.5 ± 0.3	2.3 ± 0.8	N.S.	3.7 ± 0.9	1.7 ± 0.6	N.S.
B (%)	1.1 ± 0.5	1.0 ± 0.4	N.S.	0.5 ± 0.3	0.7 ± 0.5	N.S.	0.2 ± 0.2	1.0 ± 0.6	N.S.
L (%)	39.3 ± 2.1	27.0 ± 0.9	0.0003	21.2 ± 2.7	17.7 ± 3.2	N.S.	30.0 ± 3.5	26.7 ± 3.1	N.S.
M (%)	2.7 ± 0.5	4.0 ± 0.6	N.S.	1.2 ± 0.5	3.3 ± 1.0	N.S.	2.5 ± 0.3	4.5 ± 0.3	0.0008

Table 1

Complete blood cell count in groups I, II and III, before and after exercise and significant differences. RBC, red blood cells; Hb, haemoglobin; PCV, packed cell volume; PTL, platelets; WBC, white blood cells; N, neutrophil; E, eosinophil; B, basophil; L, lymphocyte; M, monocyte. Mean ± SEM, n = 6, Repeated measures ANOVA.