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Peripheral serotoninergic response to physical exercise in athletic horses

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The purpose of this study was to evaluate the influence of exercise on plasma tryptophan (TRP) and free serotonin (f5-HT), whole blood-5-HT (WB-5-HT) and f5-HT/WB-5-HT ratio in Italian Saddle horses. Six clinically healthy Italian Saddle horses were subjected to a 450 meters obstacles course. Blood samples were collected from each horse by jugular venipuncture using vacutainer tubes with K_3-EDTA at rest, immediately after exercise, and after 30 min. TRP, f5-HT and WB-5-HT were analyzed by HPLC. Immediately after exercise, statistically significant increases of f5-HT ($p < 0.001$) and WB-5-HT ($p < 0.001$) were observed. After 30 min, f5-HT and WB-5-HT decreased compared to immediately after exercise, but were still significantly higher than rest values ($p < 0.01$ and $p < 0.05$, respectively). A significant linear regression between f5-HT and WB-5-HT was observed during experimental conditions. f5-HT and WB-5-HT modifications after exercise suggest an important role of peripheral serotoninergic markers in response to physical activity. The possible source of extra serotonin detected after show jumping should be clarified by further investigation.

Keywords: athletic horses, exercise, plasma, serotonin, tryptophan

Introduction

The monoamine neurotransmitter serotonin (5-HT) plays an important role in regulating various physiological functions, such as the regulation of sleep or wakefulness, appetite, nociception, mood, stress and maternal or sexual behaviour. Peripheral 5-HT is synthesised from tryptophan (TRP) by tryptophan hydroxilase in the enterochromaffin cells of the gastrointestinal tract. After its release into the blood, 5-HT is taken up by platelets by means of serotonin transporters or else inactivated by monoamine oxidase. Platelets are the main reservoir of 5-HT in the periphery tissues; their storage activity guarantees a low plasma 5-HT concentration. Platelets accumulate, store and release 5-HT in an analogous manner to central serotoninergic synaptosomes [14]. 5-HT released from platelets into the blood activates their aggregation for haemostatic functions and can also be taken up by sympathetic neurons and vascular endothelial cells [13]. Measurement of 5-HT in whole blood gives a reasonable approximation of 5-HT in platelets [3,25] and the free 5-HT/whole blood-5-HT (f-5HT/WB-5-HT) ratio may be a marker of platelet activation [23].

The similarity of platelets and central serotoninergic synaptosomes indicates a reliable surrogate model to study mechanisms and effects of compounds interfering with the storage (5-HT releasers) and the active carrier mechanism (blockers of 5-HT uptake) of 5-HT in neurons [14]. Studies in humans and rats provide good evidence that brain 5-HT activity increases during prolonged exercise and that this response is associated with fatigue. The synthesis and metabolism of 5-HT in the brain increase in response to exercise [12], but the role of 5-HT in the physiological mechanisms of central fatigue are not completely understood and findings about modification of peripheral serotoninergic markers after physical activity are controversial. In humans there is currently no evidence of the influence of plasma TRP concentration on the central serotoninergic system during exercise and the influence of the modification of circulating TRP levels on endurance performance or perception of effort [32,34]. Acute and chronic aerobic exercise significantly affect plasma noradrenalin, but not serum 5-HT [4], and prolonged exercise decreases TRP without changing urinary 5-HT [8]. In rats, an increase in plasma and platelet 5-HT after treadmill exercise was reported [6]. Furthermore, plasma and brain 5-HT concentrations increased after forced swimming tests [31] and pharmacological manipulation of central serotoninergic receptor function can increase or decrease exercise capacity [5]. The evidence for a central component involving the serotoninergic system in the fatigue process in exercise has evolved over the last three decades, but the involvement of increased peripheral TRP availability that guarantees blood brain barrier transport and reflects the brain increase of 5-HT during prolonged exercise [12] remains controversial. In horses, oral TRP supplementation

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had no effect on exercise performance [35], while TRP infusion reduced endurance time [17]. In this species, little information is available on the physiological range of peripheral serotoninergic markers and on their variations in relation to exercise. The aim of the present study was to evaluate the influence of exercise on plasma TRP, f5-HT, WB-5-HT and f5-HT/WB-5-HT ratio, as peripheral markers of serotoninergic activity, in athletic horses.

Materials and Methods

Six Italian Saddle horses (four mares and two geldings), aged between 7 and 13 years old with a mean body weight of 563 ± 13 kg were used. Before the start of the study, each horse underwent a heart exam, respiratory auscultations, and routine haematology at rest (Table 1). All horses were found to be clinically healthy. The horses were usually fed with hay and a mix of cereals (oats and barley), three times a day (08:00 a.m., 12:00 p.m. and 8:00 p.m.) and received water *ad libitum*. Fitness training and general animal care were carried out by professional staff not associated with the research team. Horses completed a standardized obstacle course preceded by a 15 min warm-up consisting of walking, trotting, and galloping, including six jumps of 1.00 ~ 1.25 m height and 1.20 m width. The exercise consisted of a 450 m long trail with ten 1.25 high jumps (5 vertical jumps, 3 long jumps, 1 wall and 1 double long jump). Blood samples were collected at rest 3 p.m.), immediately after exercise (within 1 min) and during the recovery period (30 min after exercise) by means of jugular venipuncture, using vacutainer tubes with K_3-EDTA. All housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 CEE. Treatment of the samples was done immediately after collection to prevent the release of the 5-HT from the platelets into the plasma [23]. Fifty µL of the whole blood specimen was treated with an equal volume of distilled water and 100 µL of an internal standard represented by N-methylserotonin (Chromsystems, Germany) and incubated for 10 min at room temperature. Samples were treated with 100 µL of a precipitation reagent (Chromsystems, Germany) to ensure protein removal. Samples were then vortex-mixed for 30 sec, incubated for 10 min at 4°C and centrifuged to obtain the surnatant that was stored at −20°C and analyzed within one week. The remaining whole blood was centrifuged for 20 min at 1,350 g to obtain plasma for f-5HT and TRP analysis. One hundred µL of plasma was added to an equal volume of N-methylserotonin and of precipitation reagent (Chromsystems, Germany), vortex-mixed for 30 sec, incubated for 10 min at 4°C and centrifuged. The clear surnatants resulting from whole blood and from plasma samples were stored at −20°C and analyzed in HPLC

Table 1. Characteristics, heart rate (HR), packed cell volume (PCV) and blood lactate (BL) of Italian Saddle horse

Horse	Age	Weight	HR (bpm)	PCV (%)	BL (mmol/L)
01	8	600	37	35	0.80
02	7	585	33	33	0.78
03	11	510	34	34	0.67
04	13	520	39	36	0.77
05	12	605	40	28	0.8
06	11	570	41	30	0.70
Mean ± SE	10 ± 0.9	565 ± 13	37.33 ± 1.33	32.67 ± 1.25	0.75 ± 0.02

Table 2. Profiles of plasma tryptophan (TRP), free serotonin (f5-HT), whole blood-5-HT (WB-5-HT), platelet number and f5-HT/WB-5-HT percentage ratio in Italian Saddle horse during experimental conditions

Parameters	Experimental conditions		
	Rest	Immediately after	Recovery period
TRP (µM/L)	41.27 ± 1.65	44.28 ± 3.69	49.06 ± 1.35
f5-HT (µM/L)	0.13 ± 0.01	$0.43 \pm 0.05^{\dagger}$	$0.36 \pm 0.03^{*}$
WB-5-HT (µM/L)	1.16 ± 0.19	$3.83 \pm 0.27^{\dagger}$	$2.65 \pm 0.12^{\ddagger,\S}$
Platelet number K/µL	122 ± 7.7	133 ± 7.7	$142.5 \pm 7.9^{\ddagger}$
f5-HT/WB-5-HT (%)	13.19 ± 2.3	11.52 ± 1.4	13.53 ± 1.1

vs. rest: $^{*}p < 0.01$; $^{\dagger}p < 0.001$; $^{\ddagger}p < 0.05$; *vs*. after exercise: $^{\S}p < 0.05$.

according to a procedure described in detail elsewhere [2]. In order to evaluate the potential modification of parameters due to possible dehydration and splenic contraction after exercise, plasma total protein and packed cell volume (PCV) were determined by spectrophotometer (Biuret method) and microhematocrit centrifuge, respectively. Platelets were counted by an automatic hematology analyzer (HecoVet, Italy). f5-HT/WB-5-HT ratio (%) was calculated with the following formula: f5-HT/WB-5-HT × 100 [23].

All the results were expressed as mean ± SE. One-way repeated measures analysis of variance (ANOVA) was used to determine the statistical effect of experimental conditions on the parameters studied. p values < 0.05 were considered statistically significant. Bonferroni's multiple comparison test was applied for post hoc comparison. A coefficient of linear correlation (r) was computed for values of parameters studied for all sampling times. Data was analysed using the software Statistica 7.0 (StatSoft, USA).

Results

The application of one-way repeated measures ANOVA showed a significant effect of exercise on f5-HT ($F_{(2,10)} = 24.74$; $p < 0.001$), WB-5-HT ($F_{(2,10)} = 34.99$; $p < 0.001$), platelet number ($F_{(2,10)} = 4.25$; $p < 0.05$), total protein ($F_{(2,10)} = 14.30$; $p < 0.01$) and PCV ($F_{(2,10)} = 16.54$; $p < 0.001$). No significant effect of exercise was observed on TRP ($F_{(2,10)} = 2.65$; $p = 0.12$) and on f5-HT/WB-5-HT ratio ($F_{(2,10)} = 0.38$; $p = 0.68$). f5-HT represented almost 13% of whole blood 5-HT at rest; it significantly increased immediately after exercise ($p < 0.01$) and after 30 min ($p < 0.05$). WB-5-HT showed a statistically significant immediately increase after exercise ($p < 0.001$) *vs*. rest. Its level decreased after 30 min compared to immediately after exercise ($p < 0.05$), but was still significantly higher than at rest ($p < 0.01$). Platelet number showed a significant increase 30 min after exercise *vs*. rest ($p < 0.05$). A correlation (r = 0.79; $p < 0.001$) was found between f5-HT and WB-5-HT values (Fig. 1).

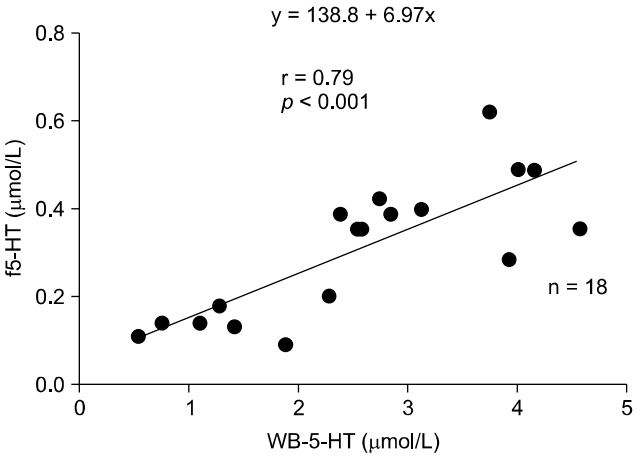

Fig. 1. Linear regression between free serotonin (f5-HT) and whole blood-5-HT (WB-5-HT) in Italian Saddle horse at rest (n = 6), immediately (n = 6) and 30 min (n = 6) after physical exercise (r = 0.75; $p < 0.001$).

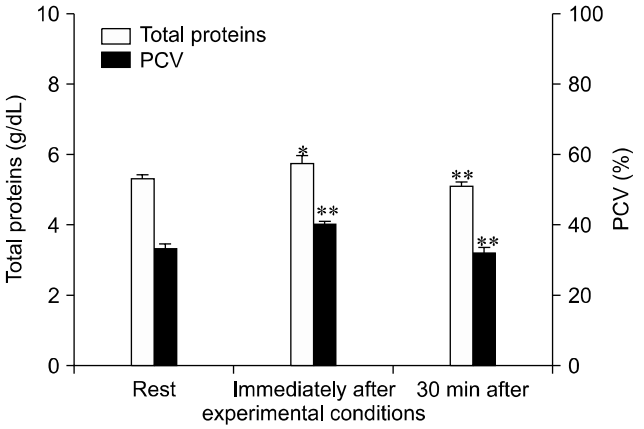

Fig. 2. Total plasma proteins and packed cell volume (PCV) in Italian Saddle horse at rest, immediately and 30 min after physical exercise. Significantly different from previous values *$p < 0.05$, **$p < 0.01$.

Total protein and PCV significantly increases immediately after exercise ($p < 0.05$ and $p < 0.01$ respectively) while significantly decreasing after 30 min compared to immediately after exercise ($p < 0.01$) (Fig. 2).

Discussion

Our results regarding basal f-5HT and TRP are similar to those previously found in trained horses [1]. Circadian rhythms of serum 5-HT [30] and p5-HT [24] have been observed, but no data about WB-5-HT daily variations have been reported. f5-HT values observed at rest were in accordance with daily and seasonal fluctuations [24].

These results showed a significant effect of exercise on f5-HT and WB-5-HT and no effect on the TRP and f5-HT/WB-5-HT ratio. Unlike most amino acids, most plasma TRP are bound to proteins, mainly to albumin [27], with only a small fraction unattached. Unfortunately we only analyzed total TRP. In a previous study on rats, no changes of total plasma TRP were reported after forced treadmill exercise [12]. The increase in brain 5-HT could be due to a rise in brain 5-HT synthesis, turnover and release promoted by exercise. Exercise-induced plasma NEFA concentration increase leads to a rise in free plasma and brain TRP concentration that elicits a rise in 5-HT synthesis [12]. Therefore, many studies demonstrated a dissociation of free TRP and brain TRP pools [18]. The slight increase of TRP levels observed immediately after exercise could be due to dehydration during exercise, demonstrated by increase of total protein and PCV. In contrast, however, a significant decrease of plasma TRP

was observed in Standardbred trotters after short intense exercise [22]. In endurance horses, concentration of TRP significantly increased after a 32 km endurance ride and significantly decreased after a 72 km endurance ride [7]. The behaviour of TRP after different workloads should be better clarified, with due consideration of the fact that the conversion of TRP into 5-HT accounts for only 5% of the total metabolism of TRP [33].

The increase of 5-HT in plasma could be related to higher platelet activation and 5-HT release. Unfortunately, the ratio of f5-HT/WB-5-HT slightly decreased after exercise, indicating a slightly increased reuptake rate of 5-HT in platelets. Our data, showing an increased uptake of 5-HT by platelets, might reflect an increase of the central nervous system serotoninergic activity. The increase of platelet numbers due to dehydration and splenic contraction, as demonstrated by significant increases of total proteins and PCV, could be one of several causes of WB-5-HT increase. Immediately after exercise, the platelet number increased by about 10% and WB-5-HT increased by about 230%. In light of platelets storing but not synthesizing 5-HT and the blood-brain barrier (BBB) being impermeable to 5-HT [37], the source of the additional 5-HT detected after exercise remains unclear. Despite suggestions that the breakdown of BBB may be mediated by 5-HT_2 receptors [31] and that 5-HT may cross the BBB [29], it is unlikely that f5-HT post-exercise could come from the central nervous system. Only a small fraction of 5-HT is produced in the brain-stem neurons of the raphei nuclei [26]. In our opinion, the only possible source of the additional 5-HT is the enterochromaffin cells that are thought to release 5-HT in response to exercise. Our reasoning is as follows: First, enterochromaffin cells synthesize and store about 95% of peripheral 5-HT [19] and in the gastrointestinal tract 5-HT plays a role as a critical signalling molecule [10]. Second, 5-HT is released from enterochromaffin cells in response to acetylcholine, sympathetic nerve stimulation, raised intraluminal pressure and low pH [10]. Third, diet influences f5-HT, but not TRP, in trained horses [1], and food ingestion induces an increase in circulating 5-HT [2]. Last, the ultimate goal of enterochromaffin cell response is to stimulate neurons to initiate peristaltic activity [21]. The importance of this circle involving enterochromaffin cells, platelet and neurons in the control of the active fraction of 5-HT, represented by the circulating f5-HT, has been initially demonstrated *in vitro* by the modulation of equine gastrointestinal motility by drugs targeting the 5-HT receptor [16,36]. Extrinsic neurons lie outside the wall of the gastrointestinal tract and allow communication between the brain and the gastrointestinal tract via parasympathetic and sympathetic innervation [28]. This indication of the connection between gut and brain should be further investigated during physical exercise in order to elucidate the mechanism of central fatigue that was not defined with much precision until recently [15,20].

In conclusion the results obtained in the present study showed a significant involvement of peripheral serotoninergic markers during exercise. Further studies are needed to address whether these modifications could be related to an increase of serotoninergic central activity in horses and if 5-HT released from the gut is related to these modifications.

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