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Original Research

Body Temperature and Plasma Nitric Oxide Metabolites in Response to Standardized Exercise Test in the Athletic Horse

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ABSTRACT

The present study was undertaken to examine the relationship among the circulating levels of nitric oxide metabolites (NOx) and gas parameters and body temperature in horses before and after a standardized exercise test (SET). Total levels of NOx were calculated as the sum of nitrite (NO_3^-) and nitrate (NO_2^-) levels. The results indicate that blood gas parameters were significantly modified at short time after SET (5 minutes), normalizing at 30 minutes after SET. A significant increase of body temperature ($P < .001$) and plasma NOx levels ($P < .05$) was observed at both 5 and 30 minutes after SET. A linear correlation between the postexercise values of NOx and body temperature ($r^2 = 0.501$, $P < .01$) was found. According to these data, plasma NOx could be involved in thermoregulation during physical exercise in horses; further studies are necessary to investigate this association and to understand the systems that permit animals to maintain homeostasis during body exercise.

1. Introduction

Physical exercise is a multifactorial activity that induces changes in every organ and tissue in the body, all aimed to maintain cellular homeostasis during increased energy demand. Regular exercise practicing leads to many health benefits, whereas a sedentary lifestyle is implicated in many chronic health problems. The cardiovascular system responds to the tremendous increase in oxygen and substrate demand by muscles and central nervous system through a selective vasodilation allowing an increase in oxygen and substrate delivery to these districts. At the same time, significant vasoconstriction, initially occurring in skin capillaries, is observed in the enteric district [1].

In general, the control of vasodilation is mainly carried out by some low-molecular-weight, often short-lived, compounds released by vascular endothelial cells, the most effective of which are adenosine and nitric oxide (NO). Nitric oxide is a small and relatively unstable molecule, synthesized from L-arginine by three cell-specific nitric oxide synthase (NOS) isoforms: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS). Although nNOS and eNOS are constitutively expressed, iNOS in aged rats and mice is overexpressed under a variety of acute and chronic pathologic conditions [2]. Because of the very short half-life of NO and its rapid metabolism, direct assessment of NO production is difficult to measure. Nitrite and nitrate (NOx) are the major stable metabolites of endogenous NO and are accessible to quantitative analysis [3]. In relation to physical activity, changes in systemic NOx levels have been

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demonstrated in human species after exercise training and interpreted as a mechanism to improve endothelial functions [4]. Similar results have been obtained in the horse in which the effect of near-maximal physical exercise induced the increase of NO [5]. Evidence for involvement of NO in the homeostatic control of body temperature in homeotherms is accumulating [6]. In a recent study, the effects of increased brain availability of L-arginine on core body temperature (Tcore) and cutaneous heat loss were evaluated in running rats and results suggest that brain L-arginine controls heat loss during exercise [7]. Horse is the only additional athletic species that sweats to thermoregulate in a similar fashion than humans [8] using, however, a greater proportion of its body mass for locomotion than does a human during exercise and having a greater rate of heat production per unity of body mass [9]. Therefore, the present study was undertaken to examine in equines the relationship among the circulating levels of nitric oxide metabolites (Nox), gas parameters, and body temperature in response to a standardized exercise test (SET).

2. Materials and Methods

2.1. Subjects and Management

The study was carried out on eight Thoroughbred horses (four mares and four geldings), averaging 5 ± 1 years of age and 475 ± 28 kg of body mass. Before the start of the study, all subjects underwent a rectal temperature evaluation, heart examination, respiratory auscultations, and routine hematology and plasma biochemistry at rest. Only clinically healthy animals were used. All treatments, housing, and animal care were carried out in accordance with the standards recommended by the EU Directive 2010/63/EU for animal experiments.

Horses were fed with standard rations three times a day (8 AM, 12 PM, 5 PM), in amounts calculated according to the increasing energy needs. The diet consisted of a total mixed ration (Unimix Horses, Bernunzio Feeds Factory, Enna, Italy) composed as follows: 16% crude protein, 10.09% ash, 6% crude fat, 7.35% crude fiber, 0.46% sodium, 0.85% lysine, 0.35% methionine, 0.65% omega-3, at total of 5 ± 1 kg/d. They also received about 5.5 ± 1 kg/horse/d of hay (first-cut meadow hay, sun cured, late cut, 8 kg/horse/d; 6.9% crude protein on average). Water was available *ad libitum*. Ambient temperature and relative humidity were continuously recorded with a data logger (Gelmini, Chichester, West Sussex, UK). Horses were not involved in an exercise or riding curriculum of any kind, before or during the study. Horses were accustomed to individual housing and work on the training mill and treadmill during an adaptation period of 12 weeks, reaching a light-medium fitness level according to previous exercise protocols [10,11]. The daily training during the adaptation period consisted of the following sequence: 5-minute walk (approximate speed = 0.8 m/s); 10-minute trot (approximate speed = 4.0 m/s); 5-minute walk (approximate speed = 0.8 m/s). After every 4 weeks, the trot period was increased by 10 minutes, so that the daily sequence in the last 4 weeks was as follows: 5-minute walk (approximate speed = 0.8 m/s); 30-minute trot (approximate speed = 4.0 m/s); 5-minute walk (approximate speed = 0.8 m/s). During this period, they were familiarized four times per week to the treadmill. After this period, an intermittent exercise protocol on the treadmill was performed. Table 1 shows the protocol of the SET. Ambient temperature and relative humidity remained within a very narrow range, respectively, 19.8 + 0.18°C and 62 + 1%, during the test.

2.2. Parameters Under Evaluation

Because a previous study suggested that rectal temperature can accurately reflect core temperature [12], in this study, each animal underwent rectal temperature measurement, as a representative of body temperature. Temperature was monitored with a digital thermometer (HI92704; Hanna Instruments, Bedfordshire, UK), inserted 15 cm in the rectum. All measurements were taken at rest and at 5 and 30 minutes after SET. At the same time points, blood sample was drawn by means of an external jugular venipuncture and collected into heparinized tubes. Blood samples were cooled in an ice bath, and an aliquot was used for blood gas analysis (3-mL ventilated syringes with a 23-gauge 3.1-inch needle containing freeze-dried lithium heparin) and analyzed by means of a selective ions analyzer (Stat Profile pHOx Analyzer; GEPA, Nova Biomedical Corporation, Waltham, Massachusetts, USA). The following parameters were assessed: hematocrit (Hct), total hemoglobin (Hb), oxyhemoglobin (HbO_2), partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2). The remaining whole blood was centrifuged at 1,350 × *g* for 10 minutes at 4°C, within 40 minutes from withdrawal. The resulting plasma was stored at −80°C until processing for protein removal and subsequent high-performance liquid chromatographic (HPLC) analysis.

2.3. Plasma Sample Processing and High-Performance Liquid Chromatographic Analysis of Nitrite and Nitrate

Deproteinization of plasma was obtained by ultrafiltration as described in detail elsewhere [13]. Briefly, samples (100 μL) were diluted three times with 200 μL double-distilled water (1:5; v:v) and transferred in Eppendorf tubes equipped with a cellulose acetate filtering membrane of 10 kDa cutoff (Nanosep Centrifugal Devices; Pall Gelman Laboratory, Ann Arbor, MI) for a first centrifugation (10,500 × *g*, 20 minutes, 4°C). These ultrafiltrates were collected and subjected to a second centrifugation in tubes with a cellulose acetate filtering membrane of 3 kDa cutoff

Table 1
Protocol of standardized exercise test of moderate intensity performed on a treadmill by eight trained horses.

Pace	Slope (°)	Speed (m/s)	Time (min)
Walk	0	1.8	5
Trot	0	4.0	5
Walk	0	1.8	1
Trot	0	4	2
Trot	10	4	5
Walk	0	1.2	2
Total			20

Table 2
Changes in hematological parameters and in the body temperature of exercised horses at rest and at 5 and 30 minutes after standardized exercise test (SET) of moderate intensity.

	Hct (%)	Hb (g/dL)	Oxy Hb (%)	pCO_2 (mm Hg)	pO_2 (mm Hg)
Rest	34.11 ± 5.67	8.07 ± 1.56	80.93 ± 3.56	46.56 ± 2.49	41.69 ± 3.85
5 min after SET	42.63 ± 2.10[a]	9.66 ± 0.56[a]	88.07 ± 3.28[a]	39.45 ± 2.25[a]	51.15 ± 15.21[a]
30 min after SET	36.07 ± 3.26[b]	8.02 ± 0.88[b]	84.79 ± 2.95[a]	44.08 ± 2.81[b]	44.92 ± 4.52

Abbreviations: Hb, hemoglobin; Hct, hematocrit; Oxy Hb, oxyhemoglobin; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen.
Values are the mean ± standard deviation of eight different exercised animals. Horse training conditions are reported under Materials and Methods. Description of SET is given in Table 1.
[a] Significantly different from rest ($P < .01$).
[b] Significantly different from 5 minutes after SET ($P < .01$).

(10,500 × *g*, 20 minutes, 4°C). The clear protein-free ultrafiltrates were used with no further processing for the direct determination of the compounds of interest.

The determination of nitrite and nitrate in plasma samples was carried according to ion-pairing HPLC methods originally set up in our laboratory [3,14]. The HPLC apparatus consisted of a SpectraSystem P4000 pump and a highly sensitive UV6000LP diode array detector (Thermo-Electron Italia, Rodano, Milan, Italy), equipped with a 5-cm light-path flow cell and set up between 200 and 300 nm wavelength for data acquisition. Data were acquired and analyzed by a PC using the ChromQuest software package provided by the HPLC manufacturer. Separation of NO metabolites was carried out using a Hypersil 250 mm × 4.6 mm, 5-μm particles size column, provided with its own guard column (Thermo-Electron Italia).

Mobile phase and chromatographic conditions allowed the direct separation and quantification of nitrite and nitrate (retention time of nitrite = 11.12 ± 0.07 minutes; retention time of nitrate = 24.74 ± 0.06 minutes). This method to separate nitrite and nitrate has previously been demonstrated to have good characteristics of sensitivity, reproducibility, and recovery comparable to GC/MS and LC/MS methods [3].

Calculations were performed at the wavelengths of 206 nm (nitrite and nitrate), by comparing peak areas in chromatographic run of plasma with those in runs of ultrapure standards with known concentrations.

2.4. Statistical Analysis

All results were expressed as mean ± standard deviation. The analysis of variance (ANOVA) for repeated measures was applied to compare the effect of exercise on parameters of interest. Bonferroni's multiple comparison test was applied for *post hoc* comparison. The relationship between NOx and other parameters (blood gas and body temperatures) was evaluated by Pearson's linear correlation. The significance level was set at $P < .05$. All the data were analyzed using Statistica 8 software (Statsoft Inc, Tulsa, OK).

3. Results

As summarized in Table 2, ANOVA showed a statistical significance of sampling time on Hct ($F_{2,14} = 18.43$, $P < .001$), Hb ($F_{2,14} = 10.42$, $P < .01$), HbO_2 ($F_{2,14} = 11.81$, $P < .001$), pCO_2 ($F_{2,14} = 9.81$, $P < .01$), and pO_2 ($F_{2,14} = 3.81$, $P < .05$).

When prolonging the time of recovery up to 30 minutes, all blood gas parameters except HbO_2 were similar to pre-exercise values, indicating that the duration of the recovery phase was sufficient to ensure the return of the parameters under evaluation within physiological ranges. This assumption was however not applicable when considering the horse body temperature and levels of circulating nitrite + nitrate. In fact, ANOVA showed a significant effect of SET on body temperature ($F_{2,14} = 155$, $P < .001$) and circulating NOx ($F_{2,14} = 6.11$, $P < .05$).

As shown in Fig. 1, significantly higher values of body temperature than those recorded at rest were measured either at 5 (+1.28°C, $P < .001$) or at 30 minutes after SET (+0.72°C, $P < .001$). The values of circulating NOx (expressed by the sum of nitrite + nitrate) determined in plasma before and after SET are illustrated in Fig. 2. With respect to the values measured at rest, moderate physical exercise significantly increased levels of NOx in plasma at both 5 and 30 minutes after exercise ($P < .05$).

To evaluate the potential relationship between the levels of circulating NOx and any of the hematological parameters of Table 2, best-fitting linear regressions were calculated keeping NOx as the dependent variable. No one of the various blood gas parameters correlated with changes in plasma NOx.

Conversely, when plotting the values of plasma NOx as a function of body temperature, the regression model (Fig. 3)

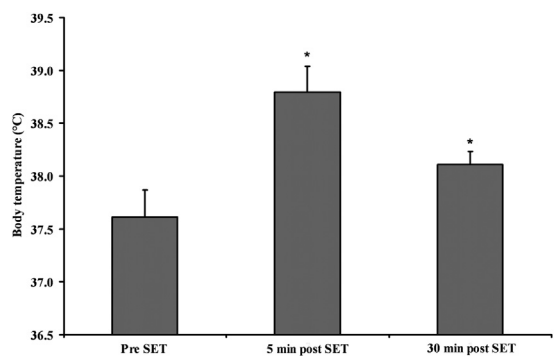

Fig. 1. Changes in rectal temperature determined in exercised horses before and after standardized exercise test (SET) of moderate intensity. Details of the SET protocol are given in Table 1. Values are the mean of eight different animals. Standard deviations are represented by vertical bars. *Significantly different from pre-SET, $P < .01$.

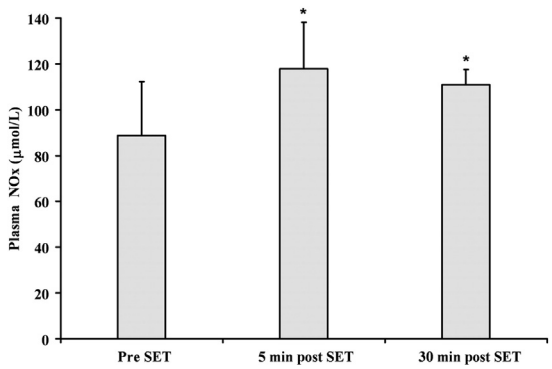

Fig. 2. Changes in the circulating concentrations of nitrite + nitrate, as representative nitric oxide metabolites (NOx) determined in exercised horses before and after standardized exercise test (SET) of moderate intensity. Values are the mean of eight different animals. Standard deviations are represented by vertical bars. *Significantly different from pre-SET, $P < .01$.

gave a positive correlation between rectal temperature and concentration of circulating NOx ($r^2 = 0.501$, $P < .01$).

4. Discussion

Results reported in the present study indicate that in addition to changes in blood gas parameters comparable to SET patterns previously found in the athlete horse [11], moderate physical exercise modulates NO production. Furthermore, NO production and body temperature are correlated after moderate exercise thereby suggesting a complex physiological response aimed at facilitating post-exercise recovery. All the blood parameters evaluated were significantly modified at short times after SET of moderate intensity with most relevant changes observed for Hct. This was probably caused by the combined effects of the increase in sweating and splenic recruitment of erythrocytes occurring during SET.

It has been shown that acute exercise, performed by the contraction of the tibialis anterior rat muscle, increased NO levels in the brain evaluated in terms of the NO metabolites

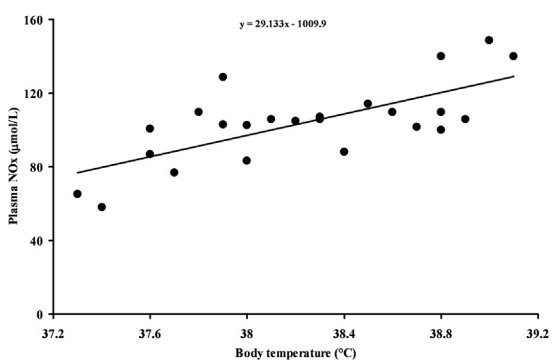

Fig. 3. Plasma nitric oxide (NOx) metabolite concentration (nitrite + nitrate) in horses after moderate standardized exercise test (SET) 5 and 30 minutes after exercise, plotted as a function of body temperature (°C). Value of the correlation coefficient indicates a linear correlation between circulating NOx and body temperature ($r^2 = 0.501$; $P < .01$).

nitrite and nitrate [15]. Additionally, in rheumatoid arthritis patients acute exercise, but not moderate-intensity training, significantly increased circulating NOx levels [16].

Oppositely, the use of a competitive inhibitor of NO synthase (L-nitroarginine methyl ester) in exercising horses diminished sweating rate with elevated core and peripheral temperatures leading to deranged thermoregulation [1]. These effects may be related to peripheral vasoconstriction but suggest possible modulation by NO of the central and peripheral sympathetic control of sweating. The use of L-nitroarginine methyl ester also affected splenic contraction and body temperature, therefore contributing to limit physical performance at higher workloads in Thoroughbred horses [17]. Nitric oxide is thus likely to emerge as an important transmitter between the sympathetic nervous system and the local cutaneous blood vessels in the horse [18]. According to our results, the increase in circulating NOx and the moderate correlation with the increase in body temperature after described physical exercise might be interpreted as a natural response aimed at optimizing the general response to this paraphysiological condition. With the current knowledge, horses with higher NOx because of higher body temperature should probably better perform peripheral vasodilation with important consequences on sweating and loss of exceeding heat. The importance of NO overproduction is reinforced by the remark showing that among the various hematological parameters considered in this study, circulating NOx underwent to the most relevant changes either at shorter or at longer time after SET (Table 2 and Fig. 1). Further study with more time points of temperature and blood samples after exercise should be designed to confirm this hypothesis. From what is reported in this study, the increase in circulating NOx (as an indirect index to measure whole body NO production) is a physiological response of the organism to moderate physical exercise with the main purpose to counteract the increase in body temperature through vasodilation and optimization of heat exchange. It is well known that in the beginning of physical exercise, skin capillaries are vasoconstricted. The subsequent increase in body temperature is accompanied by NO generation, aimed to induce a vasodilatation of skin capillaries. This stimulates skin thermal control center resulting in intense sweating and decrease of body temperature. Previous studies showed that NO promotes skin sudomotor control and increases sweat response to modulate thermoregulation in the exercising horse [19]. The exact mechanisms by which NO intervenes in regulating heat exchange are still unknown. It will be interesting to determine the site or sites of action of NO as a mediator of thermoregulatory sweating in horses as suggested by Joyner [20].

Intermittent exercise protocols can induce more extreme physiological responses (e.g., body temperature increases) than incremental or constant-speed tests performed to fatigue [21]. To our knowledge, this is one of the first studies assessing a moderate correlation between circulating NOx levels and body temperature after exercise in horses. According to the results of the present study, NO appears involved in thermal adjustments during exercise. Its role when environmental conditions may have negative influence in normal thermic homeostasis, such as under

elevated ambient temperature, should be better defined. Further studies with larger groups of animals, maximal-intensity exercise, different environmental conditions, and specific inhibitors of eNOS will be carried out to verify whether decreasing NOx production during exercise uncouples thermal regulation.

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