

Effect of exercise intensity on the hepcidin response

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ABSTRACT

Introduction: The sympathetic nervous system modulates many metabolic and hormonal responses to exercise. It is not well known its influence on serum levels of peptide hormone hepcidin, a key iron metabolism regulator, during exercise.

Material and methods: This study examined the effects of exercise intensity on the hepcidin response and treated to relate catecholamine and hepcidin responses to exercise. Fifteen trained cyclists and triathletes undertook a maximal stress test on a cycle ergometer followed by two different intensity tests (moderate and high) in random order. The two tests consisted of 30 min of exercise at an intensity 10% lower than that corresponding to the ventilatory threshold (VT) (30-MI) or the respiratory compensation point (RCP) (30-HI).

Results and discussion: Despite higher norepinephrine levels detected after the 30-MI test versus baseline ($p < 0.01$) and after the 30-HI test versus 30-MI ($p < 0.01$), the hepcidin response was unmodified by the intensity of exercise.

Conclusion: Given we were unable to relate the hepcidin response to an exercise known to elicit high sympathetic-adrenal activity, our findings suggest that exercise intensity, and by extension the sympathetic activation, does not modulate the hepcidin response to exercise.

KEYWORDS

Hepcidin, iron, norepinephrine, exercise intensity.

INTRODUCTION

Iron plays an important role as cofactor in mitochondrial energy production, also participates in DNA synthesis and carries out a beneficial prooxidative role¹. Low body-iron stores have been linked to reduced hemoglobin concentrations, red cell volumes and myoglobin levels² limiting the organism's oxygen transport capacity. Reduced oxygen supply to tissues will have detrimental effects on an individual's work capacity and athletic performance^{3,1}. Iron is absorbed by enterocytes, but only about 5-25% of the dietetic iron is typically absorbed⁴ and most of the body's iron stores consist of iron that is recycled by macrophages in the spleen and liver from senescent red blood cells⁵. Hepcidin secreted by hepatocytes⁶ is a major player in regulating systemic iron homeostasis; is encoded by the HAMP gene, whose product is an 80 amino acid-long prohormone. The concentration of circulating hepcidin is determined by the liver⁷. Reduced iron absorption in the duodenum and also impairs iron release from macrophages and the liver by blocking the cell membrane transporter protein ferroprotein⁸. The hepcidin-ferroprotein axis is thus the main feedback mechanism that maintains serum iron levels within a narrow physiological range⁹. Hepcidin expression is regulated by iron signals, erythropoiesis, and inflammation; during inflammation, high plasma hepcidin levels are induced via the IL (interleukin)-6/STAT3 pathway. Increased hepcidin expression represents a hallmark of some anemias⁷.

Iron deficiency is frequently diagnosed among athletes, particularly those trained in endurance sports (Beard and

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Tobin 2000); 11% in men and 35% in women¹⁰. A priority for exercise physiologists and physicians is that adequate blood iron levels should be maintained^{11,12}. Recent research also suggests that altered iron metabolism in athletes may be caused by detrimental effects on the hepcidin regulatory mechanism^{13,14}. Few studies have examined the effects of exercise intensity on hepcidin. Most prior work has focused on the hepcidin response to a single exercise stimulus^{15,16,17,13,18} and transient increases in urinary or serum hepcidin concentrations after one or two bouts of exercise^{16,17,19,20}. Elevations in serum hepcidin levels have been observed approximately three hours after exercise^{21,16,13,18} reported that low-intensity submaximal concentric exercise did not modulate serum or urinary hepcidin concentrations. Inflammatory cytokines and possibly pro-inflammatory cytokines^{22,23} constitute an important signaling pathway regulating hepcidin expression; elevated levels of such cytokines lead to the increased production of hepcidin (Ma et al. 2013). Exercise increases circulating IL-6 levels, and that peak levels are attained immediately after exercise^{24,25}. Plasma concentrations of circulating norepinephrine (NE) reflect the degree of overall sympathetic system activation and are dependent on exercise intensity. During acute incremental exercise, blood catecholamine concentrations rise with increasing exercise intensity, and a marked increase known as the catecholamine threshold is produced at exercise intensities above those triggering the transition from aerobic to anaerobic metabolism^{26,27}. Sympathoadrenal hormone secretion has been positive correlated between circulating IL-6 levels and NE. Interactions between cytokines and neuroendocrine hormones are likely involved in the physiological response to exercise²⁸.

No study has examined the factors involved in the physiological response to different intensity exercise. We hypothesized that if increased sympathetic activity is an important mediator of the IL-6 response to exercise, greater sympathetic outflow, as reflected peripherally by NE release, should precede in time and correlate in incremental amounts with a heightened hepcidin secretory response to exercise.

The present study was therefore designed to identify the relationship between relative exercise intensity and the hepcidin response to exercise and to try to relate sympathetic and hepcidin responses to exercise.

METHODS

Subjects

Participants were 15 male, trained cyclists and triathletes (age 31.7 ± 2.6 yr; body mass 72.4 ± 1.6 kg; BMI 23.2 ± 0.4 kg/m²; height 1.77 ± 0.01 m) recruited from an amateur team. The study protocol fulfilled the tenets of the Helsinki Declaration and was approved by the Review Board of the Universidad Francisco de Vitoria. Written informed consent

was obtained from each subject. All the participants were healthy, and it was checked they had had no signs of infection in the 4 weeks prior to the study. Subjects were also instructed to not take substances known to affect iron metabolism over the 2 weeks leading up to the tests.

Experimental overview

Before the study, subjects were given a detailed description of the whole procedure. Participants attended three laboratory-based testing sessions, each separated by 4 days. No structured exercise was performed during the 24 h before each testing session. Due to diurnal variations in serum hepcidin concentrations, all laboratory tests sessions were carried out in the morning. The participants also refrained from caffeine and alcohol consumption 36 h before each session. The first session included a graded exercise test to determine everyone's cycling $\dot{V}O_2$ max, maximal power output (Wmax), maximal heart rate (HRmax), ventilatory threshold (VT) and respiratory compensation point (RCP). This was followed by two testing sessions conducted in a randomized, counterbalanced order:

- 30-min moderate-intensity continuous cycling at a workload (W) 10% lower than that corresponding to the ventilatory threshold (W-VT) (30-MI).
- 30-min high-intensity continuous cycling at a W 10% lower than that corresponding to the respiratory compensation point (W-RCP) (30-HI).

Sessions were started at 9 a.m. after a 12 h fast. After recording body mass, subjects sat quietly for 10 min before a baseline blood sample was obtained. Each test was performed under comfortable laboratory conditions ($22.6 \pm 0.4^\circ\text{C}$ and $49.1\% \pm 1.9\%$ relative humidity). Water (300 ml volumes) was consumed ad libitum during each trial (Sim et al. 2013).

$\dot{V}O_2$ max assessment

All subjects performed a maximal stress test on a cycle ergometer. Test duration was 8-12 min and workload were set according to the subject's training level. The test protocol on the ergometer (Ergometrics 900; Ergo-line; Barcelona, Spain) was a ramp performed until exhaustion starting at 0 W, and increasing the workload by 25 W/min, while pedaling cadence was kept constant between 70 and 80 rpm. In all cases, the test was ended: 1) if pedaling cadence could not be maintained at least at 65 rpm; 2) at volitional exhaustion; or 3) when the test termination criteria described in the AHA/ACC guidelines (Gibbons et al. 2002) had been met. Gas-exchange data were collected throughout the test using a breath-by-breath metabolic system (ZAN 600 USB CPX, Messgeräte Gmbit, Germany). Maximal oxygen uptake ($\dot{V}O_2$ max) was considered to have been reached if two of

the following occurred: a plateau in VO_2 despite an increased speed; a respiratory exchange ratio (RER) ≥ 1.10 ; a heart rate within 10 beats of the age-predicted maximum ($220 - \text{age}$); or volitional fatigue (Esco et al. 2010). During the tests, heart rate (HR) was constantly monitored via a 12-lead electrocardiogram.

Ventilatory threshold (VT) was recorded when there was an increase in the ventilatory equivalent for oxygen (VE/VO_2) with no increase in the ventilatory equivalent for carbon dioxide (VE/VCO_2) and departure from linearity of VE , whereas the respiratory compensation point (RCP) was defined as an increase in both VE/VO_2 and VE/VCO_2 (Davis 1985). VT and RCP were visually detected by two independent experienced observers. If there was disagreement, the opinion of a third investigator was sought. This non-mathematical method to detect both VT and RCP during a cycle ergometer ramp protocol has been successfully used in several studies conducted at our laboratory²⁹.

Steady state tests

30-MI and 30-HI tests. The 30-MI and 30-HI tests consisted of 30 min continuous cycling at a workload 10% lower than that corresponding to the VT or RCP, respectively. The trials included a 10-min warm-up at 70% VT. Pedaling cadence was kept stable at 70 to 80 rpm. HR was measured using a polar HR monitor (Polar RS 200, Finland), and the rating of perceived exertion (RPE) was scored by each subject using Borg's scale (6 = *no exertion* to 20 = *maximal exertion*). Before the warm-up and immediately upon completion of the 30-min test, venous blood was collected.

Blood collection and analysis

Participants were instructed to lie down for at least 5 min and an indwelling venous cannula was then inserted into a forearm vein for blood collection into two 8.5-ml SST II gel Vacutainers (BD, PL6 7BP, UK) containing an anticoagulant (EDTAK2). Blood samples were obtained at rest and immediately after completing each test. After collection, the blood was left to clot for 60 min at room temperature and then centrifuged at 3,000 rpm for 10 min at 10°C. The serum supernatant was divided into 1-ml aliquots and stored at -20 °C until analysis.

Lactate

Blood lactate concentrations were determined before and after the steady state tests (30-MI and 30-HI) using an electroenzymatic analyzer (YSI 1500; Yellow Springs Instruments, Yellow Springs, Ohio, USA). This determination was performed on 25 μ l samples of venous blood obtained from a fingertip.

Hematological variables

Iron and ferritin levels were measured using the guanine/ferrozine method and an immunoturbidometric method, respectively. A COULTER® LH 750 Hematology Analyzer (Beckman-Coulter, USA) was used to determine red blood cells (RBC) ($10^6 \mu\text{L}$), reticulocytes (%), mean corpuscular volume (MCV) (fL), hematocrit (%) (Hct), hemoglobin (g/dL) (Hb), mean corpuscular hemoglobin (MCH) (pg) and mean corpuscular hemoglobin concentration (MCHC) (g/dL).

Hepcidin

Serum hepcidin concentrations were determined before and after the steady state tests using commercial enzyme immunoassay kits (Demeditec Diagnostic GmbH, Germany) based on the principle of competitive binding. In brief, microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the pro-hepcidin molecule. Endogenous pro-hepcidin in a sample competes with a pro-hepcidin-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed away. The amount of remaining bound biotin conjugate is inversely proportional to the concentration of pro-hepcidin in the sample and this concentration is given by the intensity of color produced after the addition of a substrate solution.

Catecholamines

Norepinephrine concentrations were determined before and after the steady state tests using a commercial enzyme immunoassay kit (Demeditec Diagnostic GmbH, Germany). Briefly, is extracted from a plasma sample using a cis-diol-specific affinity gel. The competitive ELISA kit uses an antigen that is bound to the solid phase of the microtiter plate. The modified standards, controls, and samples and solid phase-bound analytes compete for a fixed number of antiserum binding sites. Free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. Norepinephrine in a sample is then quantified using an absorbance curve prepared using known standard concentrations.

Statistical analysis

Statistical tests were performed using the SPSS statistics 22. Data normality was tested using the Shapiro-Wilks test. Time effects were determined through ANOVA with Greenhouse-Geisser correction. Paired within-subject comparisons were performed among the time points baseline, 30-MI and 30-HI. Relationships between variables were analyzed using Pearson's product moment correlations. Significance was set at 0.05.

RESULTS

All participants completed the study. No adverse events were reported.

Graded exercise test

Variables recorded in the stress tests performed in each subject at maximal effort are provided in Table 1.

Hematological and biochemical variables

The hematological and biochemical variables determined in the venous blood samples are provided in Table 2. All values were within the range of clinical normality.

Table 1. Variables recorded in the graded exercise test.

Variable	Mean ± SD
VO ₂ max (ml/kg/min)	62.20 ± 5.94
Wmax (w)	418.67 ± 48.97
HR max (bpm)	179.40 ± 9.22
% VO ₂ máx-VT (%)	61.98 ± 7.48
W-VT (w)	230.00 ± 43.55
HR-VT (bpm)	134.40 ± 9.93
%VO ₂ max-RCP (%)	84.94 ± 4.90
W-RCP (w)	334.07 ± 40.47
HR-RCP(bpm)	162.47 ± 9.61

Data are expressed as mean ± SD. VO₂max: maximal oxygen consumption, HR: Heart rate, W: watos; VO₂: oxygen consumption; VT: ventilatory threshold; RCP: respiratory compensation point; W-VT: watos reached at ventilatory threshold; HR-VT: heart rate at ventilatory threshold; W-RCP: watos reached at respiratory compensation point; HR-RCP: heart rate at respiratory compensation point.

30-MI and 30-HI tests

The exercise and blood variables recorded with the subject at rest and after the 30-MI or 30-HI tests are shown in Table 3 and Figure 1. Higher values of all the variables examined in response to the 30-HI test compared to the 30-MI test ($p < 0.01$) except hepcidin ($p > 0.05$). Norepinephrine concentrations after the 30-MI test were significantly higher than baseline concentrations ($p < 0.1$). Baseline and post 30-MI test hepcidin levels failed to vary significantly ($p > 0.05$) and were in line with reference normal clinical levels³⁰.

No significant correlation ($r = 0.04$, $p > .05$) was observed between NE and hepcidin responses to the tests.

Table 2. Baseline hematological and biochemical variables. Data are expressed as mean ± SD.

Variable	Mean ± SD
RBC (x10 ⁶ / μL)	5.35 ± 0.41
Reticulocytes (%)	0.82 ± 0.29
MCV (fL)	230.00 ± 43.55
Hemoglobin (g/dL)	15.81 ± 1.16
MCH (pg)	27.71 ± 2.94
HCHC (g/dL)	34.83 ± 0.84
Hematocrit (%)	45.35 ± 2.92
Serum ferritin (ng/mL)	125.06 ± 81.86
Serum iron (μg/dL)	86.39 ± 33.85

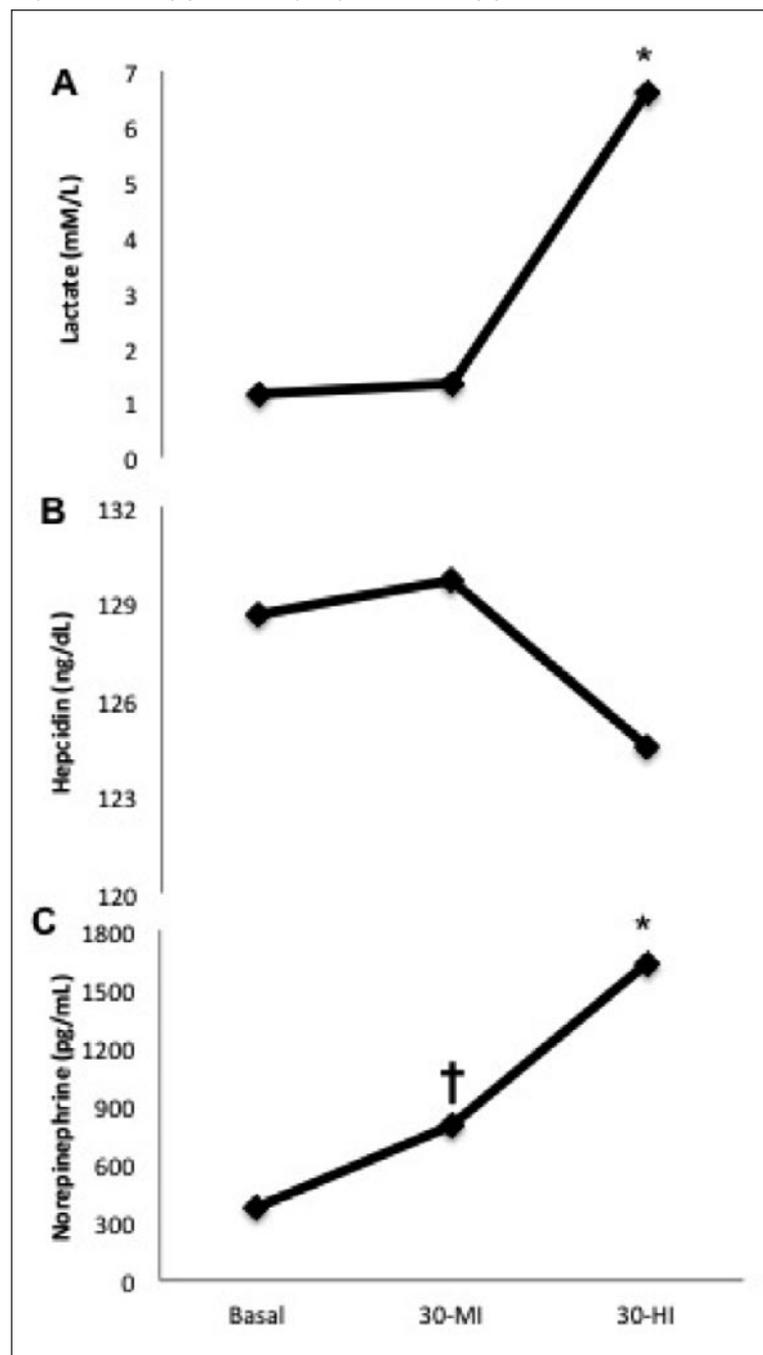
Data are expressed as mean ± SD. RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 3. Exercise and blood variables recorded at baseline and following each of the 30- MI and 30-HI tests. Data are expressed as mean ± SD.

Variable	Basal	30-MI	30-HI
Power output (w)		205.33 ± 40.06	299.33* ± 37.50
Lactate (mM/L)	1.15 ± 0.29	1.34 ± 0.47	6.61* ± 2.94
Herat rate (bpm)		131.73 ± 11.92	169.07* ± 11.23
RPE		12.93 ± 1.49	16.43* ± 1.60
Hepcididin (ng/dL)	128.66 ± 46.15	129.73 ± 42.07	124.55 ± 42.19
Norepinephrine (pg/mL)	367.22 ± 196.75	794.47† ± 292.15	1622.07* ± 452.80

Data are expressed as mean ± SD. RPE: rating of perceived exertion. * Significant difference for 30 HI versus 30-MI ($p < 0.01$); † Significant difference for 30-MI versus baseline ($p < 0.01$).

Figure 1. The effect of 30 minutes at two different intensities of exercise: moderate intensity (30-MI) and high intensity (30-IH) on lactate levels (A), hepcidin levels (B) and norepinephrine levels (C).



* Significant difference for 30 HI versus 30-MI ($p < 0.01$); † Significant difference for 30-MI versus baseline ($p < 0.01$).

DISCUSSION

In response to an increasing intensity of exercise, serum concentrations of most hormones rise. Whereby once the intensity of exercise exceeds that triggering the aerobic-anaerobic transition or lactate threshold, the rate of increasing hor-

more concentrations intensifies. The main finding of our study was that serum hepcidin concentrations failed to vary in response to high-intensity aerobic exercise related to marked sympathetic-adrenal axis activity. This study is the first to examine the acute phase hepcidin response to an intensity of exercise that induces increased sympathetic-adrenal activity, as confirmed by plasma norepinephrine levels. Quantifying the hepcidin response to exercise is important since this hormone plays a vital role in the homeostatic regulation of iron. A submaximal endurance ergocycle exercise test performed by individuals showing normal iron and ferritin levels did not affect serum hepcidin concentrations as an immediate response to high-intensity aerobic exercise as observed by¹⁸. A submaximal cycling test did not cause significant increases in IL-6 or hepcidin levels, probably because of the low intensity of the exercise performed. Muscle iron demands are not sufficiently augmented to induce changes in the metabolic cascade of iron regulation despite conditions of sympathetic-adrenal activation, which does not seem to directly affect the regulation of hepcidin synthesis by liver cells. Hojmann³¹ reported that a protease-dependent was released of IL-6 was initiated by lactate production, linking training intensity and lactate production to IL-6 release during strenuous exercise. Was observed³² no difference between the trials; so endurance exercise at lower running velocity in hypoxic conditions resulted in similar post-exercise hepcidin elevations as higher running velocity in normoxic conditions.

We find inflammation-related regulation, iron-mediated regulation, erythropoietic activity-driven regulation and a mandatory signaling pathway³³. IL-6 is produced in large amounts in contracting skeletal muscles and is released to the circulation. Hepcidin biosynthesis is stimulated by IL-6³⁴. Interestingly, correlational relationships between the levels of plasma NE and plasma IL-6 during exercise have been reported. Skeletal muscles and immune cells express large amounts of β -adrenergic receptors, which are physiologically stimulated by high-intensity exercise¹³. During exercise, plasma epinephrine and norepinephrine levels are elevated, especially at intensities exceeding the aerobic-anaerobic transition or lactate threshold³⁵ (Schneider et al., 2000), and a catecholamine threshold has even been defined³⁶. The intensity of the 30-HI test performed in our study exceeded that corresponding to the previously determined lactate threshold, and although we did not examine the IL-6 response, other authors have correlated elevated plasma catecholamine levels with increased circulating IL-6 levels as a response to high-intensity exercise. As reported³⁷ there was a

significant ($p < 0.05$) increase in hepcidin and interleukin-6 following intense cycling intervals in the participants.

Strenuous exercise raises inflammatory cytokine and acute-phase protein levels (Hoffman-Goetz and Pedersen 1994) and is often used as a model to investigate the acute-phase response. The effect that strenuous exercise has on serum and urine levels of hepcidin has been scarcely addressed. Roecker et al.³⁸ were the first to detect increased urinary hepcidin clearance after running a marathon. Peeling et al.²¹ showed that a 60-min period of high-intensity running (85-90% HRpeak) in moderately endurance-trained participants with serum ferritin levels $>35 \mu\text{g/L}$ led to increased circulating levels of IL-6 (6.9 times) and iron (1.3 times) immediately postexercise. Urine hepcidin levels were significantly increased over time when compared with prerun levels (1.7–3.1 times greater) at 3 h postexercise, but no acute urinary hepcidin response was produced immediately after exercise termination. These results were later reproduced by the same investigators in running sessions of 10 km at 70-80% VO_2 max or interval sessions consisting of 10 x 1000 m at 90-95% VO_2 max, with no effects detected of the type of running surface (asphalt vs grass)²¹. These data of Peeling et al.²¹ are consistent with the hepcidin time-course³⁹, who showed that postexercise hepcidin levels were significantly elevated 3 h after the IL-6 peak, with the highest mean values occurring at 6 h post run.

Robson-Ansley et al.¹⁷ reported significantly increased serum hepcidin levels at the end of a 5 km run, preceded by 120 min of exercise performed at a relative intensity of 60% VO_2 max. Sim et al.¹⁹ observed significant hepcidin increases in response to a cycle ergometry exercise of 120 min of duration performed at 75% VO_2 max.

Recent studies have demonstrated that physical activity causes an increase in the hepcidin response, with peak hormone levels attained at 3 h postexercise^{40,16,41,13}. Our findings indicate that exercise intensity is not a key factor affecting hepcidin production during exercise, at least in the more acute phase of exercise. Consistent with this finding, Newlin et al.¹⁶ showed that if exercise intensity is kept constant but duration extended, postexercise hepcidin responses are exacerbated and female participants performed one of two exercise trials, a 60- or a 120-min run at 65% VO_2 max. Hepcidin levels were significantly elevated 3 h postexercise in both trials but were significantly higher (by $\sim 200\%$) after the 120-min run. Sandström et al.⁴² reported that there was no difference in the occurrence iron deficiency or iron deficiency anaemia and an increase in serum hepcidin in a large group of female athletes was detected and the elevated hepcidin levels may affect the iron balance of the athletes.

Domínguez et al. (2014) reported that hepcidin response to exercise seems to be dependent on a minimum intensity of exercise ($\sim 65\%$ VO_2 max), with maximal levels of the hor-

mones recorded in response to intensities approaching VO_2 max (90%-95% VO_2 max).

Although the mechanisms regulating the hepcidin response to moderate- and high-intensity remain unclear, the results of the different studies seem to incriminate in this response changes in iron metabolism and/or the requirements of tissues induced by exercise more than direct β -adrenergic actions on the liver cell.

PERSPECTIVES

Iron deficiency is common among athletes, particularly those involved in endurance sports. We tried to link increased serum hepcidin to the intensity of exercise since such a link would have useful implications for training intensity recommendations for athletes with chronically low iron levels. Unfortunately, we were unable to detect an acute hepcidin response related to the intensity of the training session. We provide direction for future studies designed to clarify the effects of the intensity and duration of aerobic resistance training in an effort to gain a greater understanding of the mechanisms involved in the development of anemia in athletes, especially endurance athletes.

CONCLUSION

Continuous high-intensity exercise eliciting substantial sympathetic-adrenal activity does not lead to increased serum hepcidin concentrations as an acute response. Accordingly, we were unable to correlate serum norepinephrine and hepcidin levels during the exercise test performed by the study participants.

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