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Research article

Two protocols of aerobic exercise modulate the counter-regulatory axis of the renin-angiotensin system

Daniel Massote Magalhães [a], Albená Nunes-Silva [b], Guilherme Carvalho Rocha [a], Lucas Neves Vaz [a], Marcelo Henrique Salviano de Faria [a], Erica Leandro Marciano Vieira [a], Natalia Pessoa Rocha [a,c], Ana Cristina Simões e Silva [a,*]

[a] Laboratório Interdisciplinar de Investigação Médica (LIIM), Faculdade de Medicina, Universidade Federal de Minas Gerais (UFMG), Av. Prof. Alfredo Balena, 190, Room 281, Belo Horizonte, Postal Code: 30130-100, MG, Brazil

[b] Department of Physical Education and Sports, Centro Desportivo da Universidade Federal de Ouro Preto (UFOP), Rua Dois, 110, Campus Universitário - Ginásio de Esportes, Ouro Preto, MG, Postal Code: 35400-000, Brazil

[c] Department of Neurology, University of Texas Health Science Center at Houston, USA

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ABSTRACT

Aims: The renin-angiotensin system (RAS) is a dual system with two opposite arms: i) the classical one formed by the angiotensin converting enzyme (ACE), angiotensin (Ang) II and angiotensin type 1 (AT1) receptors; ii) the counter-regulatory arm consisting of ACE2, Ang-(1–7) and Mas receptor. Physical exercise can modulate this system, however, only animal studies have compared the effects of different intensity protocols on the RAS. No data with humans were provided. Therefore, we investigated the acute effect of two protocols of isowork aerobic exercise [High-Intensity Interval Exercise (HIIE) and Moderate-Intensity Continuous Exercise (MICE)] in plasma and urinary levels of RAS components in physically active men.

Main methods: The HIIE protocol included a 5-minute warm-up cycling at 60–70% of heart rate peak (HRp) intensity followed by 10 sets of 30 s above 90% with 1 min of recovery and 3 min of cool down. The MICE protocol was performed at a constant power corresponding to 60–70% of HRp and finalized at the same total work of HIIE. Blood and urine samples were collected before and after the protocols. Plasma and urinary levels of ACE, ACE2, Ang-(1–7) and Ang II were analyzed by enzyme-linked immunoassay.

Key findings: While the HIIE protocol significantly increased urinary levels of ACE and plasma levels of ACE2, the MICE protocol elevated urinary concentrations of ACE2 and of Ang-(1–7). A greater increase of urine concentrations of Ang-(1–7) occurred in the MICE if compared with the HIIE protocol.

Significance: Aerobic physical exercise acutely increases the activity of the counter-regulatory RAS axis, mostly the MICE protocol.

1. Introduction

The last decades were marked by increased knowledge regarding the importance of physical exercise for health (Pedersen and Saltin, 2006). Physical exercise benefits result both from acute exercise (e.g., improving lipid and glucose metabolisms) and cumulative effects (e.g., increasing cross-sectional area and strength of skeletal muscle). However, a great number of people, especially young men, are not motivated to take advantage of physical exercise beneficial effects, mainly because of busy lifestyles (Ashton et al., 2015). In this regard, the High-Intensity Intermittent Exercise (HIIE) appears as an option for people with limited time to exercise. In fact, the biggest advantage of HIIE is to provide maximal health benefits in minimal time: HIIE requires approximately 40% less training time commitment than MICE (Wewege et al., 2017).

The HIIE is based on low volume protocols and 'near maximal' efforts generally performed at an intensity that elicits 80% (but often 85–95%) of maximal heart rate when compared with Moderate Intensity Continuous Exercise (MICE) (MacInnis and Gibala, 2017). Meta-analyses have demonstrated that HIIE can be equally or more effective than standard continuous training for improving body composition measures, maximal oxygen consumption (VO_{2peak}), exercise capacity and metabolic risk factors (Hwang et al., 2011; Milanovic et al., 2015; Sultana et al., 2019;

* Corresponding author.
E-mail address: acssilva@hotmail.com (A.C. Simões e Silva).

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Wen et al., 2019; Wewege et al., 2017). In addition, HIIE has proven to be effective at improving metabolic health, particularly in those at risk of or with type 2 diabetes (Jelleyman et al., 2015). Also, HIIE has been proved to be superior to moderate exercise for increasing VO_{2peak} in stable coronary artery disease patients (Rognmo et al., 2004).

Although there is compelling evidence that HIIE is at least as effective as MICE in improving VO_{2peak} and important clinical indicators including blood pressure and glucose handling (Roy et al., 2018), there are only a few studies investigating the mechanisms by which HIEE exert beneficial effects. Hypothesizing that inflammatory processes might be involved in HIEE-induced responses, Cabral-Santos et al. (2015) designed a study in order to compare the effect of HIIE versus volume matched steady-state exercise (SSE) on inflammatory and metabolic responses. They found that HIIE and volume matched SSE promoted similar inflammatory responses, leading to an anti-inflammatory status. However, the metabolic responses were different. While HIIE promoted greater demands on the anaerobic metabolism (seen by peak lactate) compared to SSE, SSE produced greater demands on the aerobic metabolism (seen by peak non-ester fatty acid) compared with HIIE (Cabral-Santos et al., 2015). In the current study, we are interested in investigating whether HIIE-elicited responses are associated with changes in the renin-angiotensin system (RAS).

The RAS exerts its physiological responses by two opposite arms: (1) a classical one, composed by the angiotensin-converting enzyme (ACE), angiotensin (Ang) II and angiotensin type 1 (AT_1) receptor; and (2) a counter-regulatory arm, comprising an ACE homologue enzyme named ACE2, the heptapeptide Ang-(1–7) and its G-protein coupled receptor, the Mas receptor (Donoghue et al., 2000; Santos et al., 2003; Simoes and Flynn, 2012). Opposing to the classical arm, the ACE2/Ang (1–7)/Mas receptor axis presents anti-inflammatory, vasodilator, antiproliferative, cardioprotective, and renoprotective actions (Santos et al., 2018). Data from studies with animals showed that physical training is capable of downregulating the classical RAS arm and upregulating the counter-regulatory axis components (Frantz et al., 2018; Nunes-Silva et al., 2017). So far, the interaction between the counter-regulatory RAS axis and physical training has not been investigated in humans (Nunes-Silva et al., 2017). In addition to understanding whether there is an acute modulation of physical exercise on the RAS, it would be important to investigate if the intensity of physical exercise can influence the two RAS arms differently. Therefore, this study was designed to investigate the acute effects of two physical exercise protocols (HIIE and MICE), different in volume and intensity but equal in total work [kilo Joules (KJ)], on plasma and urinary concentration of RAS components, in young healthy individuals. We hypothesize that physical exercise results in an acute modulation in the RAS, favoring the activation of the counter-regulatory axis, as evidenced by changes in plasma and urinary levels of RAS components.

2. Subjects and methods

2.1. Subjects and ethical compliance

This study enrolled ten physically active male volunteers aging 20–25 years old. Participants were recruited from fitness centers in Belo Horizonte (Brazil) from July to December 2016. The volunteers were checked for any contraindication to moderate or high-intensity exercises and were classified as physically active based on the International Physical Activity Questionnaire (IPAQ) criteria (Matsudo et al., 2001). Based on the IPAQ classification, eight volunteers were considered very active and two were considered active. All volunteers have trained at moderate to high intensity (strength training and/or non-competitive sports) before enrollment in the study protocol. None of them has practiced sports with bicycles. Subjects trained for about 50 min per day, five times a week.

Participants were excluded if they had any chronic clinical condition, including orthopedic, cardiovascular, metabolic, renal, pulmonary, oncologic or hematologic disorder or any acute infectious or allergic disease during the previous four weeks of study protocol. In addition, cyclists and individuals who have used any medications as central nervous system-stimulant drugs, anabolic steroids, corticosteroids, anti-inflammatory, antibiotics and ACE inhibitor in the past four weeks prior to the study were excluded.

In alignment with the Declaration of Helsinki, all subjects were informed about the risks, discomforts and benefits associated with the protocol and provided written informed consent before admission to the study. The Research Ethics Committee of our institution approved this study under the protocol (60070016.5.0000.5150).

2.2. Study design

Participants were submitted to four supervised sessions conducted with at least 7 days of interval from each other (Figure 1). To avoid the effect of training between sessions, volunteers were instructed not to exercise at moderate or high volumes and intensities between sessions and the interval between sections was always at least of 7 days. In addition, they were instructed to maintain similar eating habits and to avoid caffeine drinks and alcoholic beverages at the day before and at the day of the protocols. To guarantee the adequate hydration, subjects were encouraged to drink 500 mL of fresh water 2 h before collection. The exercise protocols were performed at the same time of the day (between 8 – 11h), the temperature and humidity were maintained between 21 °C–24 °C and 58%–75%, respectively. The HIIE protocol was performed before MICE protocol in order to define the total work. This sequence allowed the equalization of both exercise protocols based on total work. The duration of MICE protocol was defined for the time necessary to achieve the same total work obtained in HIIT protocol. The intensity of MICE protocol was established at a heart rate between 60 to 80% of maximum values, while for the HIIT protocol the heart rate was set at 85%–95% of maximum values. The Borg scale was used to evaluate the intensity of the exercise (Borg, 1982). In MICE protocol, Borg scale was evaluated at every 5 min. For the HIIT protocol, the Borg scale was evaluated before and after the activation phase, before and after each sprint and before and after the cool down.

First session (day 1): The participants were asked to inform about general health conditions and to complete the Physical Activity Readiness Questionnaire (PAR-Q) (Thomas et al., 1992), a short seven-question form that assesses readiness to exercise, and the IPAQ, which quantifies the physical activity performed by the volunteer according to the type, frequency, volume and intensity. After, we measured the body mass of volunteers to estimate the resistance of cycle-ergometer in the Wingate test (Bar-Or, 1987). This test was conducted to estimate the anaerobic capacity of individuals. The volunteer has to pedal for 30 s at maximum speed against a fixed resistance (7.5% of body mass) in order to generate the highest possible power peak and to avoid the decline of the power curve (in the power X time graphic) in that period of time. Although the exercise protocols are both considered aerobic, some moments in the HIIE protocol can present anaerobic characteristics and this test was used to check the homogeneity of the individuals' anaerobic capacity.

Second session (day 2): The anthropometric characteristics were obtained. Body circumferences were measured with an anthropometric tape and the body fat was calculated by the seven skinfold method, according to Jackson and Pollock (1978) (Jackson and Pollock, 1978) using a skinfold caliper (Lange ®). The Body Mass Index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters (Kg/m^2). The aerobic capacity (VO_{2peak}) was measured by a cycle-ergometer progressive exercise-to-fatigue test (Balke and Ware, 1959). The test started at 25W, and the workload increased at a rate of 25W every 2 min, when the rating of perceived effort (RPE) was evaluated (Borg, 1982). The cadence was maintained at 50 rpm. During the entire test, oxygen uptake and carbon dioxide production were evaluated every minute by a gas analyzer (MP-35, BIOPAC® Systems, Goleta, CA), calibrated before each test according to the manufacturer's recommendations. The maximal heart rate measured on this test (HR_{peak}) was

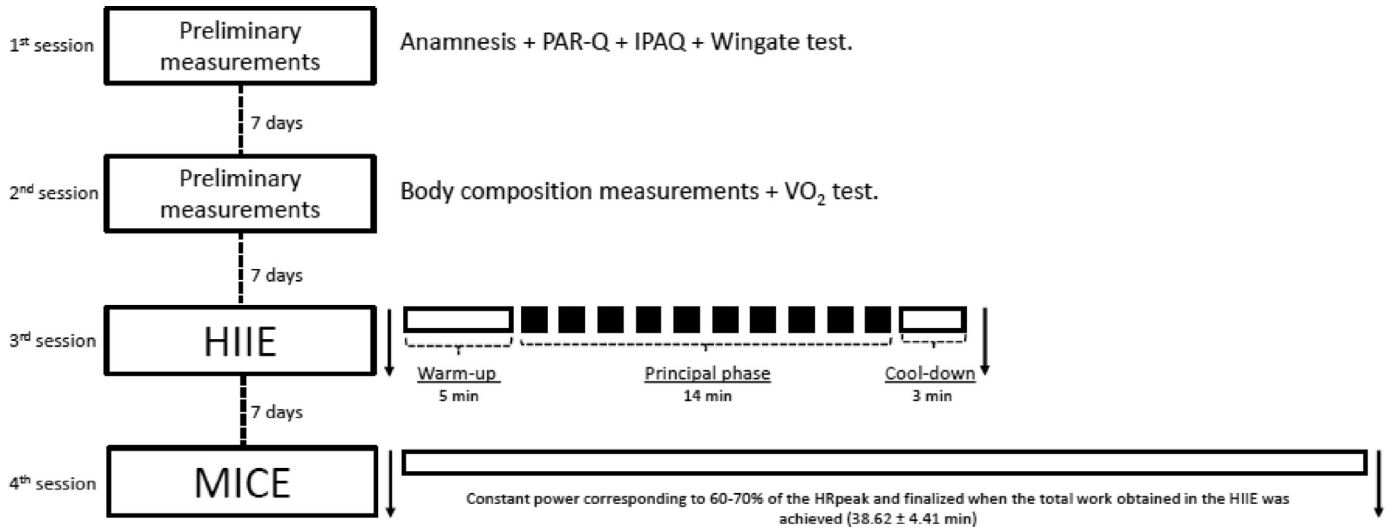

Figure 1. *Schematic view of study design.* Preliminary measurements were performed at the first and second sessions, high intensity interval exercise (HIIE) protocol occurred at session 3 and moderate intensity continuous exercise (MICE) protocol at session 4. ↓ = urine and blood collection.

registered by monitor Polar (Polar Team System, Finland) to use as a control parameter of intensity in both exercise protocols. The test was interrupted when the volunteers are not able to maintain the cadence (50 rpm), configuring fatigue.

Third session (day 3): The HIIE protocol was performed. This protocol included a 5-minute warm-up at a constant power corresponding to 60–70% of the HR_{peak}, followed by 10 bouts of 30 s above 90% of the HR_{peak}. The exercise was then finalized with 1 min of active recovery and 3 min of cool down, both at the same constant warm-up power [watts - W], registered by MCE® software (Staniak, Polônia). The total work [Kilo Joules – KJ] of the HIIE protocol was calculated (the sum of work values in each stage of HIIE, informed by software) in order to obtain the same work in the MICE protocol. The heart rate, RPE, capillary blood glucose and blood pressure were evaluated immediately before and after the HIIE protocol. In addition, heart rate, RPE and power were evaluated before and after each stage of this protocol.

Fourth session (day 4): The MICE isowork protocol was performed. The volunteers cycled at a constant power corresponding to 60–70% of the HR_{peak} and finalized when the total work obtained in the HIIE was achieved. The heart rate, RPE, capillary blood glucose and blood pressure were evaluated immediately before and after the MICE protocol. We also recorded the heart rate, RPE and power in each 5 min of this protocol.

2.3. Biological samples

Urine measurements may reflect temporary alterations in the bloodstream during exercise more accurately than plasma measurements. Urine samples were collected 5 min before and 3 after the exercise protocols. Participants were instructed to obtain midstream clean catch specimens using 20 mL Global Plastic sterile tubes. This sample was kept on ice and processed within 30 min after being obtained. Urine samples were transferred to 15mL plastic tubes and immediately centrifuged (1,800*g*, 5 min, room temperature). The cell-free supernatant was collected, aliquoted and stored at -80 °C freezer until analysis.

Blood samples were collected 3 min before and immediately after the exercise protocols. Ten milliliters of blood were drawn by venipuncture in vacuum tubes containing heparin, kept on ice and processed within 30 min after being obtained. Blood samples were centrifuged at 1,800*g* for 10 min, 4 °C, twice for plasma obtaining. The plasma was collected, aliquoted and stored at −80 °C until assayed.

In order to rule out any confounding factors caused by circadian rhythm, all samples were collected at the same time of the day (between 8 – 10h).

2.4. Assessment of plasma/urinary levels of proteins related to the RAS

Plasma and urine samples were thawed and the levels of Ang II, Ang-(1–7), ACE and ACE2 were measured by Enzyme-Linked Immunosorbent Assay (ELISA), according to the procedures supplied by the manufacturer (MyBioSource, San Diego, CA, USA). All kits applied the sandwich ELISA technique, except for ACE measurement whose kit applied the competitive ELISA method. Concentrations were expressed as pg/mL. The sensitivity of the assays was 1.0 pg/mL for ACE and ACE2; 2.0 pg/mL for Ang-(1–7); and 18.75 pg/mL for Ang II. Experiments were performed blinded regarding exercise protocols.

2.5. Statistical analysis

All variables were tested for Gaussian distribution by the Shapiro-Wilk normality test. The effects of different exercise protocols (HIIE *vs.* MICE) in two different times (before *vs.* after the exercise) on the levels of RAS proteins were compared using repeated-measures two-way ANOVA followed by the Bonferroni post hoc test. We also calculated the ratios of molecules representing the counter-regulatory/classical arms of the RAS [i.e. ACE2/ACE and Ang (1–7)/AngII ratios]. The differences between before *vs.* after training in the ratios of RAS components, the mean arterial pressure [MAP, calculated as: (2 x diastolic pressure) + systolic pressure, divided by 3] and capillary blood glucose were tested using the paired t-test or the Wilcoxon signed rank test, when the variables were normally or non-normally distributed, respectively. These tests were also used to analyze differences between HIIE *vs.* MICE protocols regarding total work, exercise duration, heart rate and power. Spearman's correlation analyses were performed to examine the relationship between biomarkers changes (i.e., the difference between the biomarkers levels obtained after and before the exercises) and age, BMI, body fat, Wingate data (maximum power, average power and total work), VO_{2peak}, glucose, arterial pressure average and exercise total work. All statistical tests were two-tailed and were performed using a significance level of $\alpha = 0.05$. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA), as well as GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, California, EUA).

3. Results

Means and standard deviations of subject's characteristics are shown in Table 1. As expected, the total work was similar in both exercise protocols, which differed in duration and intensity, as showed in Table 2. The capillary blood glucose was significantly reduced in both protocols, HIIE [Before = 98.69

Table 1. Characteristics of 10 healthy male volunteers included in study protocol. Values are expressed as means (±standard deviations).

Variables	Subjects
Age (ears)	21.92 (±1.75)
Weight (Kg)	74.60 (±8.80)
Body Mass Index (BMI)	24.13 (±1.95)
Body Fat (%)	10.27 (±3.50)
Peak of Oxygen Uptake - $VO_{2\ (peak)}$	44.22 mL/kg/min (±5.48)
Wingate test - Anaerobic Power $_{(peak)}$	9.33 W/kg (±0.72)
Wingate test - Anaerobic Power $_{(average)}$	6.95 W/kg (±0.64)
Wingate test - Total Work (KJ)	208.69 kJ (±19.21)

Table 2. Exercise components of high-intensity intermittent exercise (HIIE) and moderate intensity continuous exercise (MICE) protocols. Values are expressed as means (±standard deviations).

Variables	HIIE	MICE	P value
Total work (KJ)	141.5 (±18.33)	142.8KJ (±18.45)	0.114
Duration (min)	21.69 (±1.11)	38.62 (±4.41)	0.001*
Intensity – Heart Rate (bpm)	164.74 (±14.14)	110.5 (±11.60)	0.002*
Intensity – Power (W)	306.22 (±54.71)	62.8 (±0.03)	0.001*

*$p < 0.05$.

(±9.08) mg/dL and After = 86.25 (±8.60) mg/dL; p = 0.004 Bonferroni posttest] and MICE [Before = 100.08 (±12.08) mg/dL and After = 87.54 (±8.66) mg/dL; p = 0.002 Bonferroni posttest]. Two-way ANOVA results for capillary blood glucose = Exercise protocol: $F_{(1,18)} = 0.056$ p = 0.8139; Time: $F_{(1,18)} = 38.252$, p < 0.0001; Exercise Protocol x Time: $F_{(1,18)} = 0.061$, p = 0.807. We did not find any difference between both exercise protocols regarding the mean arterial pressure. The values of systolic, diastolic and mean blood pressure before and after the exercise protocols are given in Table 3.

3.1. Plasma levels of RAS components

The MICE protocol resulted in a decrease in plasma levels of ACE [(Figure 2 A; p < 0.05 Bonferroni post-test). Two-way ANOVA results for plasma ACE = Exercise protocol: $F_{(1,18)} = 0.00$, p = 0.9961; Time: $F_{(1,18)} = 1.00$, p = 0.3299; Exercise Protocol x Time: $F_{(1,18)} = 7.76$, p = 0.0122. On the other hand, the HIIE protocol resulted in a significant increase in ACE2 levels [(Figure 2 B; p < 0.05 Bonferroni post-test). Two-way ANOVA results for plasma ACE2 = Exercise protocol: $F_{(1,18)} = 0.03$, p = 0.8617; Time: $F_{(1,18)} = 7.05$, p = 0.0161; Exercise Protocol x Time: $F_{(1,18)} = 0.84$, p = 0.3710. The plasma levels of Ang II and Ang-(1–7) were not influenced by HIIE or MICE protocols (no significant results were obtained in two-way ANOVA tests). The ratio ACE2/ACE was also altered in MICE protocol [(Figure 3A) before = 0.63 ± 0.41 and after = 0.91 ± 0.60; p < 0,05 paired t-test].

3.2. Urinary levels of RAS components

The urinary levels of ACE2 were increased after MICE protocol [(Figure 4A; p < 0.05 Bonferroni posttest). Two-way ANOVA results for urinary ACE2 = Exercise protocol: $F_{(1,18)} = 0.04$, p = 8390; Time: $F_{(1,18)} = 6.71$, p = 0.0184; Exercise Protocol x Time: $F_{(1,18)} = 6.65$, p = 0.0189)]. The effect of exercise on MICE protocol was also confirmed by difference in the ratio analyses (ACE2/ACE), with an increase of urinary ACE2/ACE ratio after exercise [(Figure 3B) before = 0.52 ± 0.24 and after = 1.04 ± 0.43; p < 0.01 paired t-test]. The urinary levels of Ang II were very low and no difference was found between times or protocols. Both protocols increased Ang-(1–7) levels in the urine [(Figure 4B; p < 0.05 and p < 0.001 for HIIE and MICE, respectively, in the Bonferroni posttest). Two-way ANOVA results for urinary Ang-(1–7) = Exercise protocol: $F_{(1,18)} = 6.84$, p = 0.0175; Time: $F_{(1,18)} = 32.09$, p < 0.0001; Exercise Protocol x Time: $F_{(1,18)} = 4.38$, p = 0.0508). It is worth highlighting that MICE had a larger effect on urinary levels of Ang-(1–7) than the HIIE protocol (Figure 4B).

Plasma and urinary levels of RAS components before and after the exercise protocols are provided in Table 4.

Table 3. Blood pressure measurements before and after exercise protocols.

	Pre-HIIE	Post-HIIE	Pre-MICE	Post-MICE
Diastolic	61.7 ± 9.5	60.4 ± 6.5	62.6 ± 8.5	64.8 ± 7.7
Systolic	117.8 ± 8.1	124.8 ± 14.8	118.5 ± 10.5	112.7 ± 8.6
Mean blood pressure	80.4 ± 8.1	81.8 ± 7.5	81.2 ± 7.9	84.1 ± 5.9

Values are given in mmHg (mean ± standard deviation).
HIIE = High-intensity intermittent exercise; MICE = moderate intensity continuous exercise.

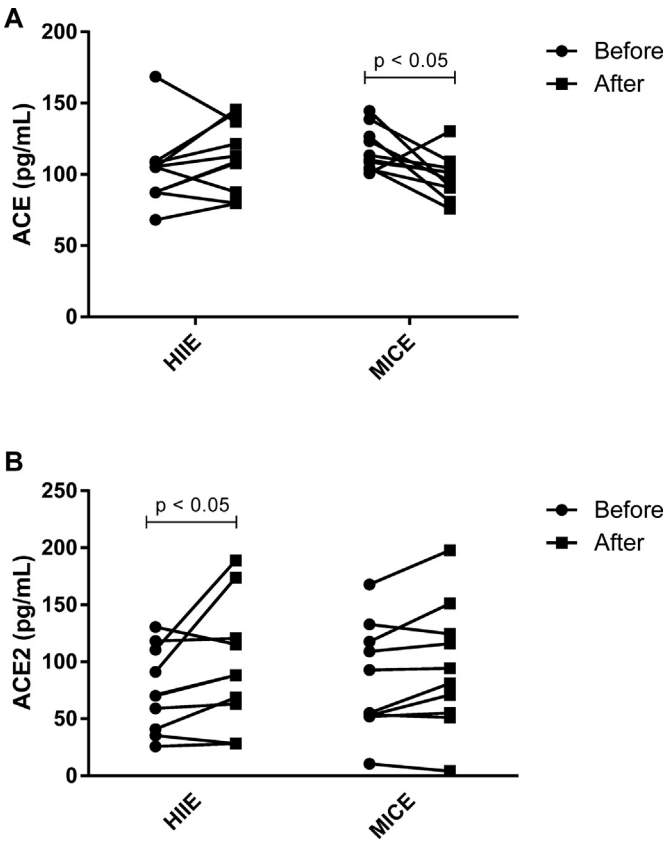

Figure 2. *Plasma levels of renin-angiotensin system components before and after each exercise protocol.* Panel A shows a decrease of plasma levels of ACE in moderate intensity continuous exercise (MICE) protocol and panel B displays an increase of plasma levels of ACE2 after high intensity interval exercise (HIIE) protocol.

4. Discussion

To the best of our knowledge, this is the first study to evaluate the acute effect of aerobic exercise protocols (MICE and HIIE) on plasma and urinary levels of RAS molecules in human samples. Our findings revealed that: (1) acute aerobic exercise can modulate plasma and urinary levels of ACE and ACE2 in healthy individuals; (2) both exercise protocols increased urinary levels of Ang-(1–7), however, the MICE protocol promoted a greater increase of this heptapeptide when compared to HIIE. Taken together, these results suggest an enzymatic modulation in both protocols favoring the balance towards to the activation of the counter-regulatory axis of RAS.

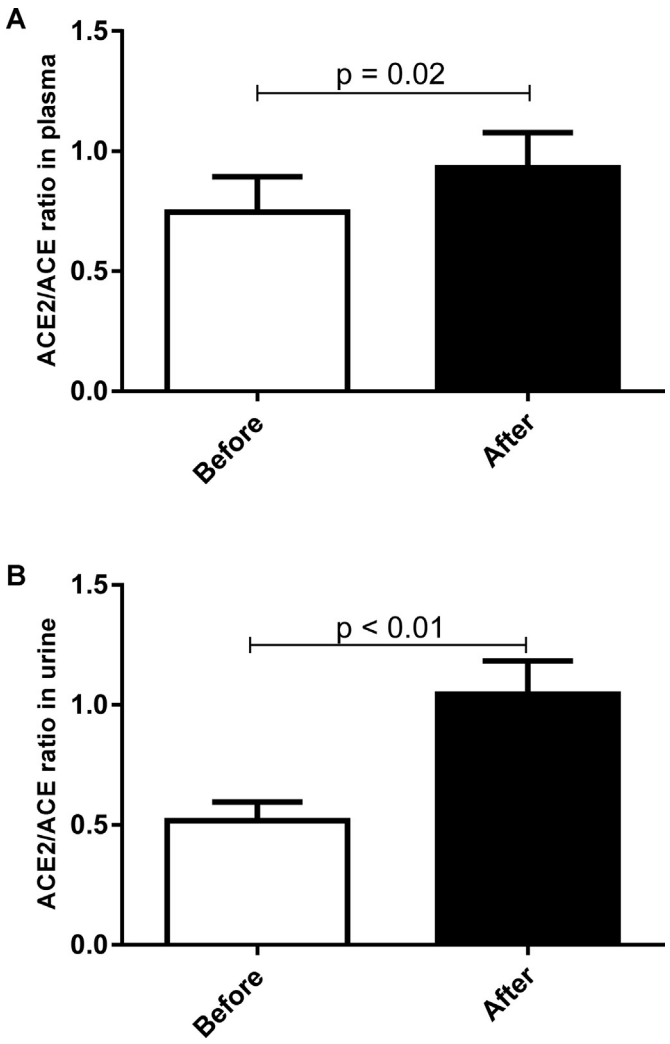

Figure 3. *The ratio between angiotensin converting enzyme (ACE) 2 and ACE obtained before and after moderate intensity continuous exercise (MICE) protocol.* Panel A shows an increase in the ACE2/ACE ratio after in comparison to baseline (before) values in plasma samples. Panel B shows an increase in the ACE2/ACE ratio after in comparison to baseline (before) values in urine samples.

Most of the evidence that physical exercise is associated with the RAS modulation came from genetic studies. The insertion/deletion (I/D) polymorphism within intron 16 of the gene encoding the ACE has been associated with ACE levels, in addition to ACE activity and athletic performance. The I allele rather than the D variant of the ACE gene seems to be related to endurance performance (Collins et al., 2004). The physical performance is probably associated with ACE activity/levels. After an ultra-endurance event (the South African ironman triathlon), individuals homozygous for the I allele (II) displayed a significant decrease in the plasma ACE activity in comparison with the heterozygous ID, whose plasma ACE activity was, in turn, lower than homozygous DD triathletes. There was a significant positive correlation between plasma ACE activity and overall finishing time within the participants who completed the event in 15 h. In addition, plasma ACE activity was strongly associated with plasma ACE concentration (Domingo et al., 2013). The influence of I/D polymorphism on the performance improvement in response to an aerobic training regimen has also been tested. While the ACE II genotype was associated with better improvements in medium duration aerobic endurance performance, the ACE DD polymorphism seems to be more advantageous in performance enhancement in short duration/high intensity endurance activities (Cam et al., 2007).

Although data from human samples are still scarce, studies with animal models corroborated our findings that physical activity is associated with the activation of the RAS counter regulatory axis. In this regard, the beneficial effects of physical training on the cardiovascular system have been associated with ACE2/Ang-(1–7)/Mas receptor signaling. It has been shown that physical training increased Mas receptor expression in aortas of spontaneous hypertensive rats, thereby improving the vasodilator effect of Ang-(1–7) (Silva et al., 2011). Confirming these findings, ACE2 deficiency was related to impaired physical performance and cardiac and skeletal muscle adaptations to exercise in mice (Motta-Santos et al., 2016). Another recent study showed that swimming training improves the oxidative capacity in mice, and the RAS counter-regulatory axis may exert a role in this process (Soares et al., 2019).

We found that both exercise protocols increased urinary levels of Ang-(1–7). Ang-(1–7) seems to induce response similar to those promoted by physical exercise, such as vasodilation (Silva et al., 2011) and the improvement of glucose and lipid metabolism by the phosphorylation of crucial insulin signaling mediators (Akt, GSK-3β and AS160), in liver, skeletal muscle and adipose tissue (Munoz et al., 2010, 2012). Accordingly, both exercise protocols were followed by a significant reduction in capillary blood glucose. A potentially beneficial role of Ang-(1–7) in metabolism was corroborated by the finding that obesity is associated to increased levels of Ang II and decreased concentrations of Ang-(1–7) in plasma and urine of adolescents born prematurely (South et al., 2019).

Another interesting finding of our study is that the modulation of RAS components was not associated with significant changes in blood pressure. Previous studies showed that Ang-(1–7) may contribute to the regulation of blood pressure, especially in patients with essential hypertension (Ferrario et al., 1998; Simoes et al., 2004). In addition, an exaggerated blood pressure response to exercise was associated with augmented rise of Ang II during exercise (Shim et al., 2008). Therefore, our hypothesis is that, in healthy and trained individuals under acute stimulus of exercise protocols, the absence of significant elevation of blood pressure may be, at least in part, due to the elevation of Ang-(1–7) levels that might compensate the cardiovascular effects of Ang II. On the other hand, we might speculate that especially MICE protocol would be beneficial for patients with mild to moderate hypertension. The more intense stimulation of the counter-regulatory RAS axis as a response to MICE protocol may contribute to blood pressure control in hypertensive patients. Long-term studies with hypertensive patients submitted to MICE protocol are necessary to confirm this hypothesis.

We are aware of the limitations of our study. First, the number of participants was relatively small. An increase in sample size would result in more powerful analysis. Despite the relatively small number of volunteers, the homogeneity of our sample resulted in variables with Gaussian distribution, thus allowing the use of parametric methods for statistical analysis. The inclusion of only males also avoided the well-know interferences of menstrual cycles and hormone release of female gender on physical activities and RAS profile (Clotet et al., 2016). On the contrary, the inclusion of only young healthy men did not allow us to infer our findings for women, children, elderly individuals and patients with chronic diseases. Additionally, the regular physical activity of our volunteers could affect both the baseline levels and the RAS components response to exercise. Another limitation is the fact that urinary and plasma levels of RAS components were only evaluated immediately before and after the exercise protocols. Additional time-point evaluations are necessary to better understand the kinetic of RAS components during and after exercise. An investigation of the biological activity of both RAS enzymes (ACE and ACE2) and of the polymorphism for the ACE gene would also provide valuable information. On the other hand, the sample homogeneity, the investigation of the effect of exercise on RAS molecules in both urine and blood samples of humans, and the rigorous protocols of physical exercise and RAS molecules measurements can be regarded as strengths of this study.

Our findings, particularly in those urine samples, may represent an acute modulatory effect of physical exercise on this system in healthy individuals. Increased levels of ACE2 and of Ang-(1–7) may contribute to

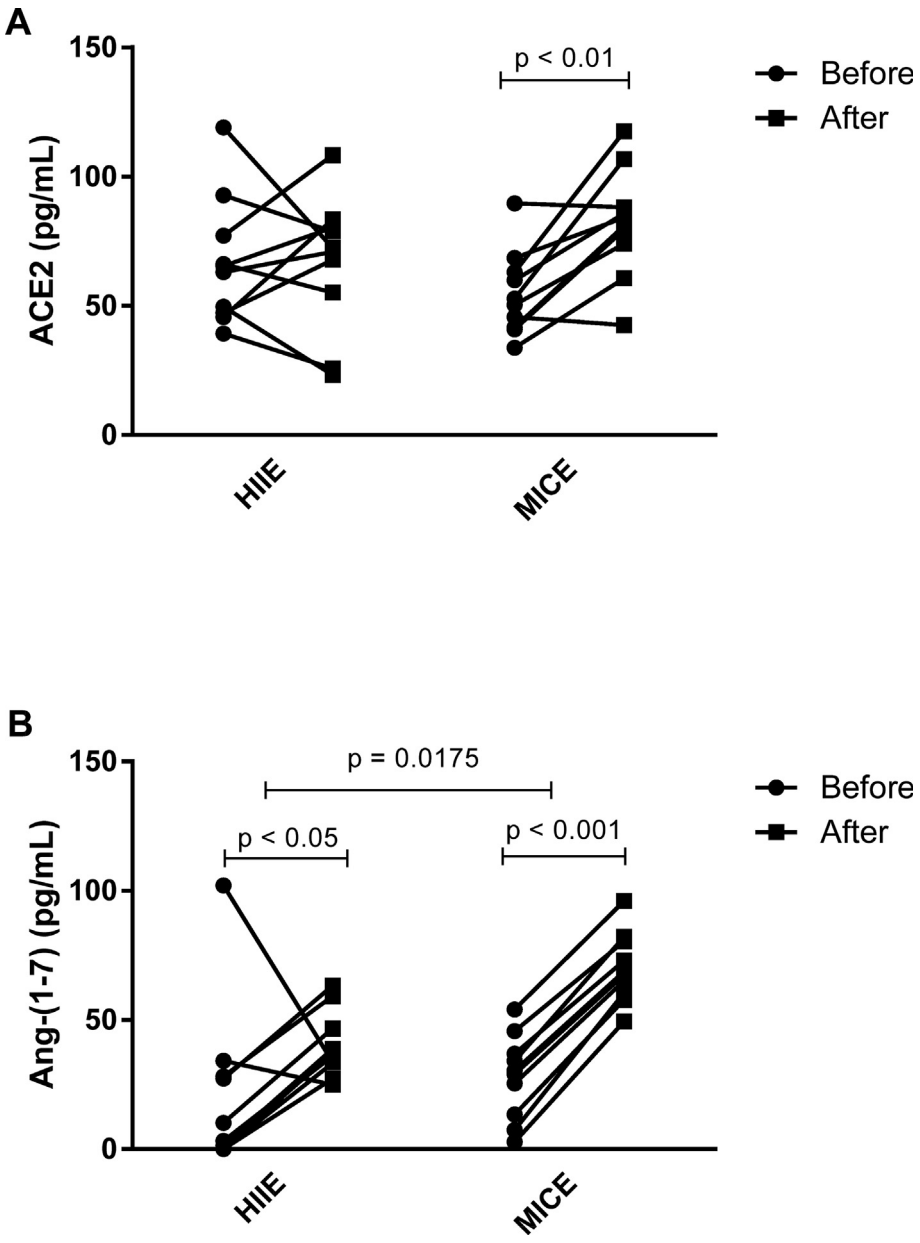

Figure 4. *Urinary levels of renin-angiotensin system components before and after each exercise protocol.* Panel A shows an increase of urinary ACE2 levels after moderate intensity continuous exercise (MICE) protocol. Panel B shows an increase of urinary Ang-(1–7) levels after both exercise protocols. MICE protocol exerts more intense effect on urinary levels of Ang-(1–7) than HIIE protocol ($p = 0.0175$).

Table 4. Plasma and urinary levels of renin-angiotensin system components before and after the exercise protocols.

	Pre-HIIE	Post-HIIE	Pre-MICE	Post-MICE
ACE - plasma	103.2 ± 8.4	112.3 ± 7.8	117.4 ± 4.8	98.0 ± 4.8
ACE2 - plasma	75.3 ± 11.5	96.3 ± 17.3	84.4 ± 15.1	94.6 ± 17.5
Ang-(1–7) - plasma	103.9 ± 44.0	79.4 ± 20.5	154.9 ± 61.4	113.6 ± 28.9
Ang II – plasma	132.2 ± 49.2	97.1 ± 19.5	162.7 ± 67.6	110.8 ± 28.6
ACE – urine	95.7 ± 19.0	140.7 ± 24.1	120.4 ± 13.3	86.3 ± 8.4
ACE2 – urine	66.6 ± 7.8	66.6 ± 8.2	54.7 ± 5.2	82.0 ± 6.7
Ang-(1–7) – urine	20.7 ± 10.0	40.1 ± 4.0	28.0 ± 5.2	70.1 ± 4.3
Ang II - urine	0.0 ± 0.0	17.8 ± 14.7	0.0 ± 0.0	2.7 ± 1.4

Values are given in pg/ml (mean ± standard error of the mean).
ACE = angiotensin-converting enzyme; Ang = angiotensin; HIIE = High-intensity intermittent exercise; MICE = moderate intensity continuous exercise.

beneficial effects of physical exercise and the greater increase of urinary concentrations of Ang-(1–7) obtained in MICE protocol helps raise the question about which training method is more efficient to promote this modulation and its comparative outcomes in the short and long term. In this way, the specific prescription might be more efficient to improve at least cardiovascular, metabolic, and motor functions of healthy individuals. Moreover, in diseases accompanied by the activation of the classical RAS axis, physical exercise should be evaluated as another therapeutic strategy. Further studies are necessary to confirm the potential benefits obtained with physical exercise.

5. Conclusions

In summary, aerobic physical exercise acutely increases components of the counter-regulatory RAS axis. The MICE protocol was associated with a greater increase of these components when compared to HIIE. These findings contribute to our understanding about the role of exercise

on the modulation of the RAS in humans. The stimulation of the counter-regulatory RAS axis may contribute to the beneficial physiological effects of physical exercise. Future studies focusing on the evaluation of chronic effects of different exercise protocols in RAS molecules are necessary.

Declarations

Author contribution statement

D.Magalhães: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

A. Nunes-Silva and N. Rocha: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

G. Rocha: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

L. Vaz, M. Faria and E. Vieira: Performed the experiments; Analyzed and interpreted the data.

Ana. Simoes-e-Silva: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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