Detectable levels of eHSP72 in plasma are associated with physical activity and antioxidant enzyme activity levels in hypertensive subjects

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Abstract

Previous studies reported that extracellular HSP72 (eHSP72) correlates with poor prognosis, markers of vascular dysfunction, and the severity of cardiovascular diseases, associated with a systemic oxidative and inflammatory profile. On the other hand, eHSP72 may represent immune-regulatory signaling that is related to exercise benefits, but the association between physical activity levels and eHSP72 levels is not established. Thus, since regular physical activity may avoid oxidative stress and inflammation, we investigate whether detectable levels of eHSP72 in plasma are associated with physical activity and antioxidant enzyme activity levels in hypertensive subjects. Physical activity levels of hypertensive subjects ($n = 140$) were measured by tri-axial movement sensor pedometer for 24 h during 5 consecutive days. One day after, blood was collected into heparinized tubes for oxidative stress analyses (catalase—CAT and superoxide dismutase—SOD activities and malondialdehyde levels) or in disodium EDTA tubes for eHSP72 assays. Thus, hypertensive subjects were classified as physically inactive ($< 10,000$ foot-steps/day) or active ($>$ than 10,000 foot-steps/day) and according detectable or not detectable eHSP72 levels in plasma, performing the inactive/eHSP72[−], active/eHSP72[−], inactive/eHSP72⁺, and active/eHSP72⁺ groups. We found that detectable levels of eHSP72 in plasma were associated with physical activity levels and low oxidative stress profile (Higher CAT and SOD activities and low malondialdehyde levels). eHSP72 levels can be used as a biomarker of the amount of physical activity necessary to improve antioxidant defense and thus cardiovascular health in hypertensive subjects.

Keywords Physical activity · eHSP72 · Oxidative stress · Hypertension

Introduction

Cardiovascular diseases (CVD) are the major causes of mortality worldwide, and hypertension contributes to the high prevalence of CVD (Roth et al. 2015). Moderate to high levels

of physical activity is recommended as a low-cost and non-pharmacological strategy in hypertension management (Ciola et al. 2009; Guimaraes et al. 2010; Ibrahim and Damasceno 2012). Besides the blood pressure and metabolic benefits related to physical activity levels, the pro-inflammatory and pro-oxidant profile observed in hypertensive subjects can be prevented by regular physical activities (Molmen-Hansen et al. 2012).

The induction of stress-regulated 72-kDa heat shock proteins (encoded by HSPA1A gene, formerly known as HSP72) by exercise has been described in studies that addressed its molecular chaperone action, with many cytoprotection properties in CVD. It is well established that these proteins in the intracellular space (iHSP72) have essential roles in cardiovascular protection, in both myocardial and endothelial cells (Pockley et al. 2009; Rodriguez-Iturbe et al. 2003).

More recently, exercise is considered a stress-like event that induces HSP72 release from many cells and tissues to

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extracellular *millie* (eHSP72) related to immune/metabolic signaling (Heck et al. 2017; Radons 2016; Krause et al. 2015). Since eHSP72 can bind to cell surface receptors with high specificity and affinity to many cells, including Toll-like receptors 2 and 4 and others, the binding of eHSP72 to these surface receptors specifically activates intracellular signaling cascades, which in turn exert immunoregulatory effector functions, a process known as the chaperokine activity (Asea 2005).

On the other hand, blood levels of eHSP72 are correlated with inflammatory markers in essential hypertension (Srivastava et al. 2016), and its chaperokine activity is becoming apparent that there is an inflammatory component to the vascular disease. The pieces of evidence are also accumulating to suggest that the immunological component to the development of atherosclerosis may, at least in part, involve the reactivity to eHSP72 (Wright et al. 2000; Pockley et al. 2002, 2009; Zhang et al. 2010). In this way, eHSP72 levels are elevated in transient hypertension of pregnancy, preeclampsia, and superimposed preeclampsia, representing not only a biomarker for these conditions but also playing a role in their pathogenesis (Molvarec et al. 2011). Also, higher eHSP72 levels were correlated with poor prognosis in patients suffering from several diseases (Gelain et al. 2011; Molvarec et al. 2011; Zhang et al. 2010).

Remained a controversy regarding the role of eHSP72 in CDV: At the same time that eHSP72 levels are biomarkers of inflammatory process and complications in several diseases, the heat shock response (HSR) that involves the ability to synthesize and release HSPs is considered a highly conserved system which many organisms require to the maintenance of health (Miragem and Homem de Bittencourt 2017). In this way, impaired HSR is a common feature of several chronic degenerative conditions associated with unresolved inflammation, such as coronary and peripheral artery diseases (Jenei et al. 2013a, b), obesity and type 2 diabetes mellitus (T2DM) (Hooper et al. 2014; Krause et al. 2015; Krause et al. 2014), aging (Miragem and Homem de Bittencourt 2017; Njemini et al. 2004), and neurodegenerative diseases (Porto et al. 2018).

Besides the controversy about the role of eHSP72 in CVD listed above, the eHSP72 levels may predict the development of atherosclerosis in subjects with established hypertension, at the same time that may represent protection against the progression of atherosclerosis in this subject group (Pockley et al. 2003). Thus, since regular physical activity may avoid oxidative stress and inflammation, we hypothesized that detectable eHSP72 levels might be associated with mild stress promoted by physical activity which in turns promoted antioxidant defense and that detectable eHSP72 levels may indicate the amount of physical activity necessary to improve health. Thus, herein, we investigate whether detectable levels of eHSP72 in plasma are associated with physical activity levels

and oxidative stress profile in hypertensive subjects. To the best of our knowledge, this is the first study to report the association between higher levels of physical activity and detectable levels of eHSP72 in plasma and its association with an antioxidant profile (higher antioxidant enzymes activities and lower malondialdehyde levels in plasma) in hypertensive subjects.

Methods

Study population

In this cross-sectional study, 140 consecutive hypertensive subjects (primary hypertension) were recruited at the Health Care Base Unit from Luzerna - Santa Catarina, Brazil. This particular city has 100% of all subjects (5,581 inhabitants) registered by the Family Health Care Strategy Program in 2015. Thus, according to the government data, 921 subjects were classified as hypertensive subjects according to previous medical diagnosis. The sample size for the present study was calculated by taking the Brazilian prevalence of hypertension of 24.9% in 2015, the estimation of error of 8.4% (absolute precision calculated as the difference between the Brazilian prevalence and the local prevalence: $24.9\% - 16.5\% = 8.4\%$), with 95% confidence interval and the minimum sample size was calculated as 102 subjects. Considering the design effect and nonresponse rate, the sample size was further increased by 40%. The final sample size in the study was fixed as 140. A total of 302 subjects were consecutively invited to participate in the study until the sample size was completed. Consecutive subjects registered as hypertensive by medical diagnosis, with more than 18 years old and at least 3 months of medical follow-up in the Health Care Unit, were considered for inclusion. Medical diagnosis was defined according to the 7th Brazilian Guideline of Arterial Hypertension (Systolic and diastolic blood pressure levels described respectively): (1) office blood pressure ≥ 140 and/or 90 mmHg; ambulatory blood pressure monitoring ≥ 130 and/or 80 mmHg; (3) home blood pressure monitoring ≥ 135 and/or ≥ 85 mmHg (Malachias et al. 2016). Subjects who had a cognitive impairment to answer the questionnaire or muscle skeletal/neurological limitations to walk were excluded in this study. Subjects under cancer treatment or mental illness were also excluded. The full clinical record of the subjects was registered for inclusion with the detailed physical status and routine clinical laboratory tests. Blood samples for the measurement of eHSP72 all biochemical parameters were also collected at inclusion. The study was carried out in 6 consecutive months, with the inclusion of the hypertensive subjects and all procedures made once a week. All details of the study are in accordance of the Helsinki declaration at the Universidade do Estado de Santa Catarina-UDESC in collaboration with the

Department of Life Science UNIJUI University based on a study protocol approved by the Institutional Committee of Science and Research Ethics protocol number 689798/2014. All patients provided written informed consent.

Design of the study

The hypertensive subjects were stratified regarding physical activity and detectable levels of eHSP72 as follows: First, the hypertensive subjects were evaluated regarding physical activity levels, measured by tri-axial movement sensor pedometer. Second, the subjects were stratified as physically inactive (< 10,000 footsteps/day) or active (> than 10,000 footsteps/day). Third, we used a high-sensitivity ELISA kit for eHSP72 detection in plasma samples; thus, the hypertensive subjects were stratified in four groups by physical activity and eHSP72 levels as follows: inactive/eHSP72⁻; active/eHSP72⁻; inactive/eHSP72⁺; active/eHSP72⁺ (Fig. 1).

Clinical data and physical activity levels classification

All subjects had a medical history interview with blinding health professionals and students. A study questionnaire was used for recording the relevant demographic and clinical data (age, weight, height, smoking habit, medications, and concomitant disease). All subjects were instructed to go to the Health Care Base Unit in fasting (12 h) condition and with adequate clothes. First, the subjects remained in rest condition for 5 min seated on a chair with back support, in a calm

environment office, with the legs maintained uncrossed, and with the feet on the floor. Subjects were instructed not to speak during the blood pressure measurements, which were always performed by a trained professional (nurse). The blood pressure was measured three times with a 1-min interval between the verifications. A manual sphygmomanometer (Premium®) validated and calibrated annually according to INMETRO (National Institute of Metrology, Quality and Technology) recommendations was used: The indication of pressure in the sphygmomanometer cannot show alterations greater than 3 mmHg (0.4 kPa) over its entire measuring range after being subjected to 10,000 pressure cycles, and when pressurized, the sphygmomanometer cannot present air leakage greater than 4.0 mmHg/min (0.5 kPa/min).

Physical activity levels were measured by tri-axial movement sensor pedometer (PW-610/611-Power Walker TM®). Energy expenditure, number of footsteps, and distance performed in each 24 h for 5 consecutive days (Monday–Friday) were recorded. The pedometer installation and uninstallation were performed in the morning (8–9 a.m.). Hypertensive subjects that presented less than 10,000 footsteps/day were classified as “physically inactive” and more than 10,000 footsteps/day as “active” (Tudor-Locke and Bassett 2004).

Blood sample

Blood was collected into heparinized (30 IU/mL final volume) tubes (for redox status and metabolite measurements) or in disodium EDTA (2 mg/mL final volume) tubes (for eHSP72 assays), and after separation, plasma samples were frozen.

Plasma HSP72 (eHSP72) quantification

A highly sensitive EIA method (EKS-715 Stressgen, Victoria, BC, Canada, with a detection range between 0.20 and 12.5 ng/ml) was used to determine the amount of eHSP72 protein in plasma. Absorbance was measured at 450 nm, and a standard curve constructed from known dilutions of HSP72 recombinant protein to allow quantitative assessment of eHSP72 plasma concentration in duplicates. Quantification was made using a microplate reader (Mindray MR-96A) (Goettems-Fiorin et al. 2016; Mai et al. 2017).

Oxidative stress analyses

Superoxide dismutase

Total superoxide dismutase (SOD) activity was performed by inhibition of auto-oxidation of pyrogallol in duplicates. Briefly, in a cuvette, 930 µL of 50 mM Tris/1 mM EDTA buffer (pH 8.2), 4 µL of catalase (30 µM), and 50 µL of homogenate were added and mixed. After pyrogallol (24 mM in HCl 10 mM) was added and SOD activity determined at 25 °C in

Fig. 1 Experimental design. The hypertensive subjects were classified as physically inactive (< 10,000 footsteps/day) or active (> than 10,000 footsteps/day) and according to detectable or not detectable eHSP72 levels in plasma, performing the inactive/eHSP72⁻, active/eHSP72⁻, inactive/eHSP72⁺ and active/eHSP72⁺ groups. A highly sensitive EIA method (EKS-715 Stressgen, Victoria, BC, Canada, with detection range between 0.20 and 12.5 ng/ml) was used to detect eHSP72 in plasma samples. Mean bias ± s.d. (absorbance units—AU at 450 nm) was 0.0013 ± 0.048 AU (95% IC = −0.010 and 0.008)

a spectrophotometer (420 nm) for 120 s. Results were expressed in units of SOD mg of protein⁻¹ (Marklund and Marklund 1974).

Catalase

Catalase (CAT) activity was performed accordingly to Aebi (1984). In a quartz cuvette, 30 µL of homogenate and 2865 µL of phosphate buffer (50 mM, pH 7.4) were mixed, and after, 105 µL of hydrogen peroxide (0.01 M) was added and mixed. The decomposition of hydrogen peroxide by CAT activity was determined at 25 °C in a spectrophotometer (240 nm) for 120 s in duplicates. The CAT activity was calculated from delta absorbance during measured time interval [$(\Delta \text{Abs}_{240\text{nm}} = (\text{Abs}_{\text{Final}} - \text{Abs}_{\text{Initial}}) / \text{measured time interval})$], where 39.4 is the molar extinction coefficient for H₂O₂ using the following expression: $[\text{H}_2\text{O}_2 \text{ mM}] = ((\Delta \text{Abs}_{240\text{nm}} \times 1000) / 39.4) / \text{protein concentration}$. The results were expressed in pmol s⁻¹ mg of protein⁻¹.

Malondialdehyde

Malondialdehyde as an indirect indicator of lipid peroxidation levels was assessed by the method of thiobarbituric acid reactive substances (TBARS). Briefly, 50 µL of plasma in duplicates was precipitated with 10% (w/v) trichloroacetic acid (TCA), centrifuged, and incubated with TBA for 15 min at 100 °C. TBARS were extracted using n-butanol (1:1). Afterwards, the absorbance was recorded by spectrophotometry at 535 nm using malondialdehyde standard prepared from 1,1,3,3-tetramethoxypropane (Buege and Aust 1978). All oxidative stress variables were normalized by the total protein content of each sample (Bradford 1976).

Statistical analyses

All data were tested for normality by Kolmogorov–Smirnov test. Thus, statistical analysis was developed using one-way analysis of variance (ANOVA). Post hoc multiple comparisons among groups were performed with the Student–Newman–Keuls test. All statistical analyzes were performed using SPSS for Windows, version 20.0. Data are presented as means ± SD. The level of statistical significance was set at a value of $P < 0.05$. All procedures were performed in duplicate and the inter- and intra-rate coefficient (Bland–Altman analysis) of the main results (PAD, PAS, eHSP72, CAT, SOD, and TBARS) was described as the difference between the two measurements (duplicates) as a function of the average of the two measurements of each sample (average bias) as mean bias ± standard deviation of bias and 95% confidence intervals – limits of agreement (Bland and Altman 1986).

Results

The 140 hypertensive subjects studied here presented a mean of 61.3 ± 11.0 years old, (37 men, 26.4%; 103 women, 73.6%). The mean time of hypertension diagnosis was 11.6 ± 9.7 years. Additional descriptive data about comorbidities, cardiovascular risk factors, and pharmacological characteristics are presented in Table 1.

First, the hypertensive subjects were evaluated regarding physical activity levels, measured by tri-axial movement sensor pedometer and presented a daily mean of 8090 ± 4288 foot-steps, with 5246 ± 3101 m traveled and an estimated energy expenditure of 406 ± 199 kcal daily. Second, the subjects were stratified as physically inactive (< 10,000 footsteps/day, 57.9% of the subjects) or active (> than 10,000 footsteps/day, 42.1% of subjects). Third, we found that 46 (32.9%) hypertensive subjects had detectable levels of these proteins into the bloodstream and presented a mean eHSP72 concentration of 1.57 ± 3.97 ng/ml, while 94 (67.1%) hypertensive subjects showed no detectable levels in plasma (<0.2 ng/ml). Thus, the hypertensive subjects were stratified in four groups by physical activity and eHSP72 levels as follows: inactive/eHSP72⁻ ($n = 52$); active/eHSP72⁻ ($n = 42$); inactive/eHSP72⁺ ($n = 29$);

Table 1 Comorbidities, cardiovascular risk factors, and pharmacological characteristics of hypertensive subjects ($n = 140$)

	<i>n</i>	%
Dyslipidemia	99	70.7
Family history	79	56.4
Type 2 diabetes mellitus	21	15
Coronary artery disease	17	12.1
Heart failure	8	5.7
Smoke	7	5
Stroke	7	5
Peripheral artery disease	7	5
Kidney disease	5	3.6
Retinopathy	5	3.6
Type 1 diabetes mellitus	2	1.4
Myocardial infarct	1	0.7
Diuretics	69	49.3
Inhibitor of angiotensin-converting enzyme	60	42.9
Angiotensin II receptor antagonist	38	27.1
Calcium blockers	16	11.4
Adrenergic blockers	45	32.1
Renin inhibitors	1	0.7
Number of antihypertensive drugs		
0	16	11.4
1	50	35.7
2	51	36.4
3	19	13.6
4	4	2.9

Table 2 Comorbidities, cardiovascular risk factors, and pharmacological characteristics of hypertensive subjects separated by physical activity levels and detectable eHSP72 levels

	Inactive eHSP72 ⁻ (n = 52)	Active eHSP72 ⁻ (n = 42)	Inactive eHSP72 ⁺ (n = 29)	Active eHSP72 ⁺ (n = 17)	ANOVA (P value)
Dyslipidemia	38 (73.1%)	30 (71.4%)	21 (72.4%)	10 (58.8%)	0.723
Family history	28 (53.8%)	23 (54.8%)	15 (51.7%)	13 (76.5%)	0.433
Type 2 diabetes mellitus	4 (7.7%)	7 (16.2%)	7 (24.1%)	3 (17.6%)	0.239
Coronary artery disease	6 (11.5%)	6 (14.3%)	2 (6.9%)	3 (17.6%)	0.705
Heart failure	2 (3.8%)	3 (7.1%)	2 (6.9%)	1 (5.9%)	0.906
Smoke	2 (3.8%)	2 (4.8%)	2 (6.9%)	1 (5.9%)	0.235
Stroke	3 (5.8%)	4 (9.5%)	—	—	0.943
Peripheral artery disease	3 (5.8%)	1 (2.4%)	1 (3.4%)	2 (11.8%)	0.491
Kidney disease	3 (5.8%)	—	1 (3.4%)	1 (5.9%)	0.474
Retinopathy	2 (3.8%)	2 (4.8%)	1 (3.4)	—	0.850
Type 1 diabetes mellitus	1 (1.9%)	1 (2.4%)	—	—	0.800
Myocardial infarct	—	1 (2.4%)	—	—	0.510
Diuretics	20 (38.5%)	25 (59.5%)	17 (58.6%)	7 (41.2%)	0.131
Inhibitor of angiotensin converting enzyme	25 (48.1%)	14 (33.3%)	15 (51.7%)	6 (35.3%)	0.373
Angiotensin II receptor antagonist	12 (23.1%)	15 (35.7%)	6 (20.7%)	5 (29.4%)	0.456
Calcium blockers	3 (5.8%)	5 (11.9%)	4 (13.8%)	4 (23.5%)	0.237
Adrenergic blockers	14 (26.9%)	16 (38.1%)	10 (34.5%)	5 (29.4%)	0.698
Renin inhibitors	—	1 (2.4%)	—	—	0.510
Number of antihypertensive drugs					0.176
0	5 (9.6%)	5 (11.9%)	2 (6.9%)	3 (17.6%)	0.166*
1	26 (50%)	11 (26.2%)	9 (31%)	5 (29.4%)	
2	16 (30.8%)	14 (33.3%)	13 (44.8%)	8 (47.1%)	
3	4 (7.7%)	11 (26.2%)	4 (13.8%)	—	
4	1 (1.9)	1 (2.4%)	1 (3.4%)	1 (5.9%)	

*Chi-square test for trend

Table 3 - Lipid profile, blood glucose, and hepatic and renal blood biomarker characteristics of hypertensive subjects separated by physical activity levels and detectable eHSP72 levels

	Inactive eHSP72 ⁻ (n = 52)	Active eHSP72 ⁻ (n = 42)	Inactive eHSP72 ⁺ (n = 29)	Active eHSP72 ⁺ (n = 17)	ANOVA (P value)
LDL	103.5 ± 38.7	112.4 ± 33.9	108.6 ± 35.3	109.1 ± 29.6	0.798
HDL	47.9 ± 11.7	48 ± 15.5	46.3 ± 11.2	40.19 ± 7.6	0.267
Triglycerides	143.3 ± 48.6	121.2 ± 58.1	142.5 ± 54.6	150.6 ± 85.7	0.139
Cholesterol	180 ± 37.8	184.9 ± 37	183.4 ± 36.3	180.1 ± 27.2	0.949
Glucose	105.1 ± 23.9	99.9 ± 31.6	103.5 ± 17.8	103.0 ± 21.1	0.865
TGO	33.8 ± 15	30.1 ± 13.1	27 ± 8.7	31.2 ± 9.4	0.601
TGP	14.9 ± 9.1	14.8 ± 12.9	10.3 ± 5.2	15.1 ± 10.1	0.446
GGT	41.9 ± 77.3	27.7 ± 21.3	33.1 ± 39.4	19.3 ± 8.7	0.496
Total protein	6.8 ± 0.7	6.6 ± 0.9	6.7 ± 0.8	6.9 ± 0.8	0.607
Creatinine	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	0.570
Urea	39.4 ± 21.6	39.9 ± 19.1	31.6 ± 11.9	30 ± 17.8	0.142
Uric acid	5.1 ± 0.1	5.0 ± 1.1	5.5 ± 1.2	5.8 ± 2.1	0.072

Fig. 2 Effects of physical activity levels and eHSP72 detectable levels on blood pressure of hypertensive subjects. Systolic arterial pressure (SAP) (a) and diastolic arterial pressure (DAP) (b) in hypertensive subjects stratified in inactive/eHSP72⁻ ($n = 52$), active/eHSP72⁻ ($n = 42$),

inactive/eHSP72⁺ ($n = 29$) and active eHSP72⁺ ($n = 17$) groups. Mean bias $\pm$ s.d. in the blood pressure measurements were as follows: SAP = 0.60 ± 4.27 mmHg (95% IC = -8.97 and 7.76) and DAP = 0.18 ± 3.76 mmHg (95% IC = -7.19 and 7.55)

active/eHSP72⁺ ($n = 17$). Descriptive data about the stratified groups are presented in Table 2. Descriptive data about the stratified groups are presented in Tables 2 and 3. There was no difference among the groups in terms of comorbidities, cardiovascular risk factors, and pharmacological characteristics (Table 2). Also, there was no difference regarding blood lipid profile, glycemia, and levels of hepatic and renal blood biomarkers (Table 3).

Physical activity levels and presence of eHSP72 in plasma was not associated with systolic or diastolic arterial pressure (Fig. 2a–b). However, we found that the active/eHSP72⁺ group presented higher levels of plasma antioxidant defense than the active/eHSP72⁻ group, as observed by SOD (Fig. 3a) and CAT (Fig. 3b) enzyme activities. Also, the active/eHSP72⁺ group presented higher CAT activity in comparison with all groups (Fig. 3b). Additionally, the active/eHSP72⁺ group presented lower levels of plasma malondialdehyde (Fig. 3c) than the active/eHSP72⁻ group.

Discussion

In our study, we found that active hypertensive subjects with detectable levels of eHSP72 in plasma have antioxidative stress profile, demonstrated by higher antioxidant enzyme

activities and lower malondialdehyde levels in plasma. Although physical activity is well known as a non-pharmacological strategy for improvement of the antioxidant defense, the association among physical activity, eHSP72 levels, and antioxidant stress profile was not described before. Our results support the hypothesis that detectable eHSP72 levels may be associated with mild stress promoted by physical activity which in turn promoted antioxidant defense and that the amount of physical activity necessary to improve health may be different among subjects.

In the last decade, a sum of evidence indicates that eHSP72 is associated with pro-inflammatory signaling (Borges et al. 2012). Thus, a subclinical pro-inflammatory state, as observed in hypertension by increased oxidative stress profile and pro-inflammatory cytokine serum concentration (Crowley 2014), may be associated with higher eHSP72 levels and worse prognosis. However, clinical and experimental pieces of evidence suggest that the presence of eHSP72 in plasma at lower levels are related to health status and longevity while higher levels are associated with disease progression and systemic damage (Zhang et al. 2010; Gelain et al. 2011). Some studies showed protective roles of eHSP72 mainly in the cardiovascular system (Pockley et al. 2002, 2009; Wright et al. 2000).

It is well known that physical activity represents a protector factor against oxidative stress (Cracowski et al. 2003; Pialoux

Fig. 3 Effects of physical activity levels and eHSP72 detectable levels on oxidative stress profile of hypertensive subjects. Plasma SOD (a) and CAT activity (b), and MDA levels (c) in hypertensive subjects stratified in inactive/eHSP72⁻ ($n = 52$), active/eHSP72⁻ ($n = 42$), inactive/eHSP72⁺ ($n = 29$) or active eHSP72⁺ ($n = 17$) groups. Mean bias $\pm$ s.d.

in the essays were as follows: SOD = 0.005 ± 0.017 U SOD/mg protein (95% IC = -0.027 and 0.039); CAT = 0.003 ± 0.06 U CAT/mg protein (95% IC = -0.12 and 0.012) and MDA = 0.009 ± 0.08 AU (95% IC = -0.18 and 0.16)

et al. 2009; Campos et al. 2015). Physical exercise may increase antioxidant defenses and decrease oxidant effects in hypertensive subjects (Kumral et al. 2016). These effects may be mediated by preserved nitric oxide (NO) function, which is a crucial factor of vascular homeostasis, by vasodilatation effects and oxidative stress modulation. Therefore, is possible to suggest that physical activity, since it increases eHSP72 levels, may protect endothelial function, by eHSP72 interaction with endothelial and immune cell surface receptors (Pockley et al. 2009) and by regulation of ROS production. The shear stress, central nervous system, and renin-angiotensin axis modulation by exercise may be responsible for the observed effects. Also, for cardiovascular diseases, exercise can be simultaneously related to the recovery of eHSP72, iHSP72, and antioxidant levels (Heck et al. 2017; Lawler et al. 2006; Mai et al. 2017).

Here, we found an association between physical activity, eHSP72 levels, and SOD activity. There is no study nowadays exploring this relation. Effects on eHSP72 or SOD activity was investigated after acute or chronic exercise challenges, not with habitual physical activity levels. In an animal model, 24 weeks of exercise (but not 12 weeks) was associated with a 3-fold increase in heart HSP72 levels and also increased SOD levels. However, eHSP72 and SOD were not evaluated in extracellular (plasma) (Moran et al. 2004), and these effects of regular exercise were associated with the anti-inflammatory profile. Muscle contraction oxidant production may explain these adaptations by increased mitochondrial activity. The oxidative challenge imposed by exercise is related to bio disponibility of NO that, in turn, regulates vascular oxidative stress and endothelial function positively, an essential benefit for hypertensive subjects (Muller et al. 1994; Higashi et al. 1999; Kostic et al. 2009). Also, dyslipidemia and lipid peroxidation levels are increased in hypertensive subjects while antioxidant defenses (CAT, SOD, and GPx) are decreased ((Rodriguez-Iturbe et al. 2003; Redon et al. 2003). The reduction of antioxidant enzyme activities can be related to constant oxidative challenge imposed by hypertension that induces downregulation of antioxidant gene expression (Kedziora-Komatuska et al. 2004; Simic et al. 2006). Thus, upregulation of antioxidant enzyme activity observed in our study may be related to sufficient physical activity challenge to the human body, marked by increased eHSP72 levels.

The interpretation of eHSP72 detectable levels as a biomarker of health benefits of physical activity is also supported by the decreased oxidative stress profile in our data and previous works. eHSP72 levels may represent a danger signal if it is associated with increased oxidative pattern (Gelain et al. 2011). But here, we found an association between physical activity and eHSP72 levels, with improved antioxidant profile and decreased lipid peroxidation profile. In the same way, more oxidative plasma profile is associated with the decrease in eHSP72 immune detection (Grunwald et al. 2014) as observed in our data. Since more active hypertensive subjects presented eHSP72 detectable levels together with an antioxidant profile,

the eHSP72 levels represent a sufficient and adequate challenge promoted by physical activity and not a cardiovascular risk. Also, while exercise benefits include a decrease in oxidative stress in different organs that are at the controlled oxidative situation and thus may not require a higher amount of intracellular HSP70 expression (De Moraes et al. 2018), in our work, it was demonstrated that higher physical activity levels may also promote antioxidant benefits systemically, preserving eHSP72 functions in circulation. Since eHSP72 in the bloodstream may represent an immune signaling (Asea 2005) and that more oxidative plasma profile may induce the oxidation of eHSP70 and promote a functional impairment and lack of stimulatory capacity of these proteins (Grunwald et al. 2014), our data indicate that higher levels of physical activity promotes antioxidant benefits and may preserve eHSP70 immune-related benefits.

In conclusion, detectable levels of eHSP72 in plasma are associated with physical activity levels and low oxidative stress profile. eHSP72 levels can be used as a biomarker of the amount of physical activity necessary to improve antioxidant defense and thus cardiovascular health in hypertensive subjects. As limitations of our study, we cited that 89% of hypertensive subjects used antihypertensive medicine that may inhibit the free radical production or can have antioxidant activity. Also, other factors may affect the oxidative profile such as eating and drinking behavior, emotional stress, non-diagnosed diseases, and comorbidities. Regarding laboratory limitations, we assessed only lipid peroxidation levels by TBARS method and only CAT and SOD antioxidant enzyme activities to provide an oxidative profile of hypertensive subjects. An analysis of more oxidative markers may provide further information and complete the oxidative profile and its association with eHSP72 levels. In perspective, future studies may associate physical activity levels separately by intensity, duration, or type of physical activity.

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Author contributions ETM, RZS performed all clinical procedures (subject selection, questionnaire application, blood pressure measurements, blood sample collection, and physical activity levels classification). ABS and PBGF performed OS analyses. MNS and YPS performed biochemical and analyses. TGH and ETM performed eHSP72 and statistical analyses. ETM, RZS, MB, TGH co-wrote the paper. TGH, MSL, and MB designed the study, provided experimental advice and helped with manuscript revision. All the authors had final approval of the submitted and published versions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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