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Exercise-induced cardiac opioid system activation attenuates apoptosis pathway in obese rats☆

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ABSTRACT

Aim: To compare the effect of 150 min vs. 300 min of weekly moderate intensity exercise training on the activation of the opioid system and apoptosis in the hearts of a diet-induced obesity model.
Methods: Male Wistar rats were fed with either control (CON) or high fat (HF) diet for 32 weeks. At the 20th week, HF group was subdivided into sedentary, low (LEV, 150 min·week^{-1}) or high (HEV, 300 min·week^{-1}) exercise volume. After 12 weeks of exercise, body mass gain, adiposity index, systolic blood pressure, cardiac morphometry, apoptosis biomarkers and opioid system expression were evaluated.
Results: Sedentary animals fed with HF presented pathological cardiac hypertrophy and higher body mass gain, systolic blood pressure and adiposity index than control group. Both exercise volumes induced physiological cardiac hypertrophy, restored systolic blood pressure and improved adiposity index, but only 300 min·week^{-1} reduced body mass gain. HF group exhibited lower proenkephalin, PI3K, ERK and GSK-3β expression, and greater activated caspase-3 expression than control group. Compared to HF, no changes in the cardiac opioid system were observed in the 150 min·week^{-1} of exercise training, while 300 min·week^{-1} showed greater proenkephalin, DOR, KOR, MOR, Akt, ERK and GSK-3β expression, and lower activated caspase-3 expression.
Conclusion: 300 min·week^{-1} of exercise training triggered opioid system activation and provided greater cardioprotection against obesity than 150 min·week^{-1}. Our findings provide translational aspect with clinical relevance about the critical dose of exercise training necessary to reduce cardiovascular risk factors caused by obesity.

1. Introduction

The prevalence of obesity has increased worldwide, reaching epidemic proportions [1]. Obesity is associated with impairment of the structure and function of the heart and increased cardiovascular risk factors, such as hypertension, dyslipidemia and inflammation [2]. Altogether, these conditions lead to the development of cardiovascular diseases, which are the cause of 30% of deaths globally [3,4].

Exercise training is a non-pharmacological strategy capable of reducing cardiovascular risk factors and protecting the heart, as well as

Abbreviations: Δ, delta; Akt, protein kinase B; ANOVA, one-way analysis of variance; BM, body mass; CON, control; DIO, diet-induced obesity; ERK 1/2, extracellular-signal-regulated kinase; GSK-3β, glycogen synthase kinase; HEV, high exercise volume; HF, high fat; LEV, low exercise volume; LV, left ventricles; MERT, maximal exercise running test; mPTP, mitochondrial permeability transition pore; opioid receptors, δ (DOR), κ (KOR) and μ (MOR); PI3K, phosphatidylinositol-3-kinase; RT-qPCR, real-time reverse-transcriptase polymerase chain reaction; SBP, systolic blood pressure; SEM, standard error of the mean

☆ These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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preventing and treating obesity and its metabolic complications [5]. Nonetheless, the adaptive response induced by exercise training is a complex process that depends on several factors, such as exercise duration [6]. For instance, the American College of Sports Medicine recommends a total of at least 150 min of weekly moderate exercise for health benefits like lowering blood pressure, improving lipid profile and enhancing insulin sensitivity [7–9]. However, when it comes to weight management, greater amounts of moderate exercise (250–300 min·week^{-1}) have been associated with a superior weight loss and prevention of weight regain [8–10].

Recently, the opioid system has been included as one of the potential mechanisms involved in the exercise-induced cardioprotection [11]. The three opioid receptors subtypes, δ (DOR), κ (KOR) and μ (MOR), are expressed in the heart and are activated by endogenous peptides, such as enkephalins [12,13]. It has been reported that the opioid system affects the cardiovascular function, leading to anti-apoptotic effects, decreased inflammatory response and modulation of the vascular tone [14,15]. Considering that exercise training increases the expression of opioid system compounds, and pharmacological blockade of opioid receptors abolishes the protection conferred by exercise against cardiac injury, it is well accepted that opioid system is involved in the cardioprotection mediated by exercise [16,17]. Thus, it is feasible that the amount of cardioprotection conferred by opioid system activation is proportional to exercise volume and only achieved above a critical threshold.

To our knowledge, no evidence exists on the effects of different amounts of exercise training on opioid system compounds and its involvement in the exercise-induced cardioprotection against obesity. Therefore, we sought to compare the effect of different exercise volumes on the activation of the opioid system and apoptosis in the hearts of a diet-induced obesity (DIO) model.

2. Methods

2.1. Animals and diet

Male Wistar rats were maintained on a 12:12-h light-dark cycle, in controlled temperature (23 ± 3 °C) and relative humidity (60 ± 5%), with food and water provided ad libitum. Animal care and procedures were in accordance with the conventional guidelines for experimentation with animals (National Institute of Health Guide, 8th edition, 2011). The Ethics Committee on Animal Use of the Fluminense Federal University approved all protocols and procedures used in this study (Protocol number 827).

Diets were offered to the animals during 32 weeks. At 6 weeks of age, animals were randomly allocated into the groups: control (CON) diet (n = 10) or a high-fat (HF) diet (n = 30). At the end of 20th week of diet, animals that received HF diet were randomly subdivided into three experimental groups: 1) HF: high-fat diet and sedentary rats (n = 10); 2) LEV: high-fat diet and low exercise volume (n = 10); and 3) HEV: high-fat diet and high exercise volume (n = 10). The CON group was fed with a standard chow diet and remained without exercise for 32 weeks. The CON diet contained 23% protein, 71% carbohydrates and 6% lipids (17.9 kJ·kg^{-1}); and the HF diet contained 14% protein, 56% carbohydrates and 30% lipids (23 kJ·kg^{-1}) [18].

Body mass (BM) of animals was measured at the beginning and end of the experimental period, and its value is presented as BM gain (i.e. the difference between final and initial BM). The food intake (g) was evaluated daily. Systolic blood pressure (SBP) was measured at the beginning and end of the training protocol by a computerized tail cuff plethysmography system (Visitech Blood Pressure Analysis System, model BP 2000) [19].

2.2. Assessment of maximal aerobic capacity

Initially, all animals were adapted to a motor-driven rodent treadmill (HT 2.0; Hectron Fitness Equipment, Rio de Janeiro, Brazil) at a low velocity (12 m·min^{-1}, 0% inclination, 15 min·day^{-1}, 3 consecutive days). After one week of adaptation, a maximal exercise running test (MERT) was carried out to determine individual maximal exercise capacity. For the MERT, the initial velocity was 10 m·min^{-1}, followed by increments of 3 m·min^{-1} every 3 min, 0% inclination [19]. The test ended when the animals were exhausted, i.e., until they were not able to run and remained at the end of the mat on the shock grid for 5 s. The bars of the shock grid deliver low electrical currents of ~2 mA that cause minor discomfort but no harm to the animals.

The MERT was conducted at pre training (20th week) and at post training (32nd week). During MERT, the maximal velocity was measured. Also, all sedentary rats were adapted to the treadmill and underwent MERT.

2.3. Exercise training protocol

Exercise training began at the 20th week and consisted of treadmill sessions 5 days·week^{-1} with moderate intensity (60% of the maximal velocity obtained in MERT), and 0% inclination for 12 weeks.

The LEV group trained 30 min·day^{-1}, totalizing 150 min·week^{-1}; and the HEV group trained 60 min·day^{-1}, totalizing 300 min·week^{-1} [19]. During exercise training, tail shock was used to induce the rats to run. At mid training (26th week), training rats underwent another MERT to adjust the load of exercise training.

2.4. Tissue extraction

Forty-eight hours after the last training session, the rats were deprived of food for 8 h and then deeply anesthetized (150 mg·kg^{-1} of pentobarbital sodium, ip). Hearts were collected and left ventricles (LV) were isolated. LV were rapidly frozen and stored at −80 °C until protein expression analysis or embedded into freshly prepared fixative (formaldehyde 4% w·v^{-1}, 0.1 M phosphate buffer pH 7.2) for histological analysis. The retroperitoneal and epididymal fat were removed and weighed. Adiposity index was defined as the ratio between the sum of the retroperitoneal and epididymal fat pad weights and total BM.

2.5. Histological analysis

Fixed LV samples were embedded in paraffin and 5-μm-thick sections were stained with hematoxylin and eosin. Digital images were scanned and Cardiomyocyte area was determined using ScanScopeTM CS (Aperio Technologies, CA, USA).

2.6. Immunohistochemistry

LV sections were treated with citrate buffer pH 6.0 for antigen retrieval for 45 min at 96 °C, and then blocked using peroxidase and protein block solution (Novolink Polymer Detection Systems kit, Leica Biosystems, RU). Subsequently, the sections were incubated with the following primary antibodies overnight at 4 °C: DOR, KOR and MOR, and following incubation with post primary and Novolink polymer (Novolink Polymer Detection Systems kit, Leica Biosystems, RU). Positive immunoreaction was detected by diaminobenzidine and the sections were then counterstained with hematoxylin. Negative control was performed by primary antibody omission. Digital images were obtained with ScanScopeTM CS (Aperio Technologies, CA, USA). The immunoreactivity areas of cardiomyocytes were estimated using the Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA), through the density threshold selection tool. The results were presented as a percentage of the CON group [20,21].

2.7. Western blotting

Total LV proteins were extracted in a homogenizing buffer with

protease and phosphatase inhibitors. Protein samples were used for electrophoresis and were transferred to a polyvinylidene difluoride membrane. The blot membrane was incubated overnight at 4 °C with the following primary antibodies: DOR, KOR, MOR, ERK 1/2, phosphorylated ERK $1/2^{Tyr204}$, GSK-3β, phosphorylated GSK-$3β^{Ser9}$, caspase-3, PI3K, Akt, phosphorylated Akt^{Ser473}. Binding of the primary antibodies were detected with the use of secondary antibodies, and ECL Western blotting reagents were used to visualize images of the blots in a ChemiDoc-System (BioRad, Hercules, CA, USA). Cyclophilin was used as a loading control for proteins. The intensity of the chemiluminescent bands was quantified using ImageJ software, version 1.44 (NIH, imagej.nih.gov/ij, USA).

2.8. Real-time reverse-transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was extracted from approximately 40 mg of LV using Trizol reagent (Invitrogen, CA, USA). RNA concentration and integrity were assessed and synthesis of first strand cDNA was performed. RNA samples were quantified by absorbance at 260 nm and 280 nm in order to determine their concentration and purity levels. Quantitative real time PCR was performed using Applied Biosystems StepOne™ Real-Time PCR System (Thermo Fisher Scientific, MA, USA). *Proenkephalin* mRNA expression were assessed by oligonucleotide primers (FW, TCA GGAAAGATTGTCCCTGCTGGT; RV, TTGGAAAGAAGAATGCGCCTG TGG), and TATA box-binding protein — TBP (FW, TACAGGTGGCAGC ATGAAGTGACA; RV, AACCAACAATCACCAGCAGCAGTG) was measured as an internal control for sample variation in RT reaction. All samples were assayed in duplicate. The results were quantified by ΔΔCt method.

2.9. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The homoscedasticity of variances was confirmed and the differences among groups were tested by one-way analysis of variance (ANOVA), followed by Holm-Sidak post hoc test. In all cases, $p < 0.05$ was considered statistically significant. Analysis was performed by GraphPad Prism (version 6.02, La Jolla, CA, USA).

3. Results

3.1. Animals and training

HF group exhibited higher BM gain than CON group (+21.30%, $p < 0.05$), which was reverted by HEV protocol (−23.19%, $p < 0.01$) (Table 1). Food intake was similar among groups (Table 1).

At the end of the experimental protocol, SBP was increased in HF group when compared with the CON group (+9.99%, $p < 0.001$) (Table 1). Both exercise volumes prevented the increase in SBP in relation to HF group (LEV: −13.06%, $p < 0.0001$; HEV: −15.69%, $p < 0.0001$). Moreover, HEV group had an additional decrease in SBP when compared with the CON group (−7.27%, $p < 0.05$).

Sedentary groups (CON and HF) showed significantly lower maximal velocity ($p < 0.01$ in all cases) in relation to both training groups (LEV and HEV). HEV group had increased maximal velocity when compared with LEV group ($p < 0.05$; Table 1).

The HF diet intake led to an increased adiposity index in HF (+116.50%, $p < 0.0001$), LEV (+85.20%, $p < 0.0001$) and HEV (+33.00%, $p < 0.05$) groups when compared to CON group (Table 1). Both exercise protocols induced lower adiposity index in relation to HF group (LEV: −14.46%, $p < 0.05$; HEV: −38.57%, $p < 0.0001$). Furthermore, HEV group showed decreased adiposity index in relation to LEV group (−28.19%, $p < 0.01$).

3.2. Cardiomyocytes hypertrophy

As shown in Fig. 1A and B, HF diet altered the myocardium structure, as both HF and trained groups presented cardiomyocytes hypertrophy in relation to CON group (HF: +45.37%, $p < 0.05$; LEV: +45.24%, $p < 0.05$; HEV: +58.93%; $p < 0.01$; Fig. 1B). However, LEV and HEV groups showed similar pattern to CON group of interstitial space with preserved intramyocardial microvascularization, while HF group showed vacuolated cardiomyocytes, small interstitial space and areas of scarce microvascularization (Fig. 1A).

To evaluate the physiological or pathological feature of the cardiac hypertrophy, PI3K and Akt protein expression were quantified by Western blotting (Fig. 1C and D). PI3K protein expression was decreased in HF group in relation to CON group ($p < 0.05$). The trained groups showed increased pAkt/Akt ratio when compared with both CON ($p < 0.01$ in all cases) and HF ($p < 0.01$ in all cases) groups.

3.3. Opioid receptors

The opioid receptors on heart tissue were assessed by Western blotting and/or immunohistochemistry (Fig. 2). HEV protocol led to increased immunostaining (DOR and KOR: $p < 0.01$; MOR: $p < 0.001$; Fig. 2A, B and C) and protein expression (DOR and KOR: $p < 0.05$; MOR: $p < 0.01$; Fig. 2D) of all three opioid receptors subtypes in relation to HF group. KOR immunostaining and protein expression was increased in HEV group also when compared with LEV group ($p < 0.05$). MOR protein expression was increased in HEV group also when compared with CON group ($p < 0.01$). Moreover, the LEV group presented higher MOR protein expression in relation to both CON ($p < 0.01$) and HF ($p < 0.01$) groups and increased immunostaining when compared with HF group ($p < 0.01$).

3.4. Endogenous opioid peptide

One of the precursors of the opioid system agonist, proenkephalin, was assessed by RT-qPCR. Cardiac proenkephalin mRNA levels were lower in HF group (−91.72%, $p < 0.001$) in relation to CON group, whereas HEV group (+770.05%, $p < 0.05$) presented higher levels when compared to HF group (Fig. 3A).

3.5. Downstream signaling of the opioid system and apoptosis

The HF group exhibited lower protein expression of both pERK/ERK and pGSK-3β/GSK-3β ratios in relation to CON group ($p < 0.05$ in all

Table 1
Biometric, hemodynamic and physical performance data.

Data	CON	HF	LEV	HEV
BM gain (g)	291.90 ± 12.76	354.10 ± 20.30[a]	304.90 ± 17.76	272.00 ± 14.02[b]
Food intake (g/day/animal)	15.83 ± 1.22	15.09 ± 1.39	14.04 ± 1.16	12.46 ± 1.13
SBP (mmHg)	137.20 ± 1.68	150.90 ± 2.06[a]	131.20 ± 2.97[b]	127.20 ± 2.20[a, b]
ΔMaximal velocity (m/min)	−2.00 ± 0.76	−2.00 ± 0.96	6.25 ± 1.71[a, b]	11.50 ± 1.47[a, b, c]
Adiposity index (fold of control)	1.00 ± 0.06	2.17 ± 0.13[a]	1.85 ± 0.10[a, b]	1.33 ± 0.09[a, b, c]

Abbreviations: CON, control; HF, high fat; LEV, low exercise volume; HEV, high exercise volume; BM, body mass; SBP, systolic blood pressure; Δ, delta. Data presented as mean ± SEM. Significant differences between the groups are indicated by symbols ($p < 0.05$): a ≠ CON; b ≠ HF; c ≠ LEV.

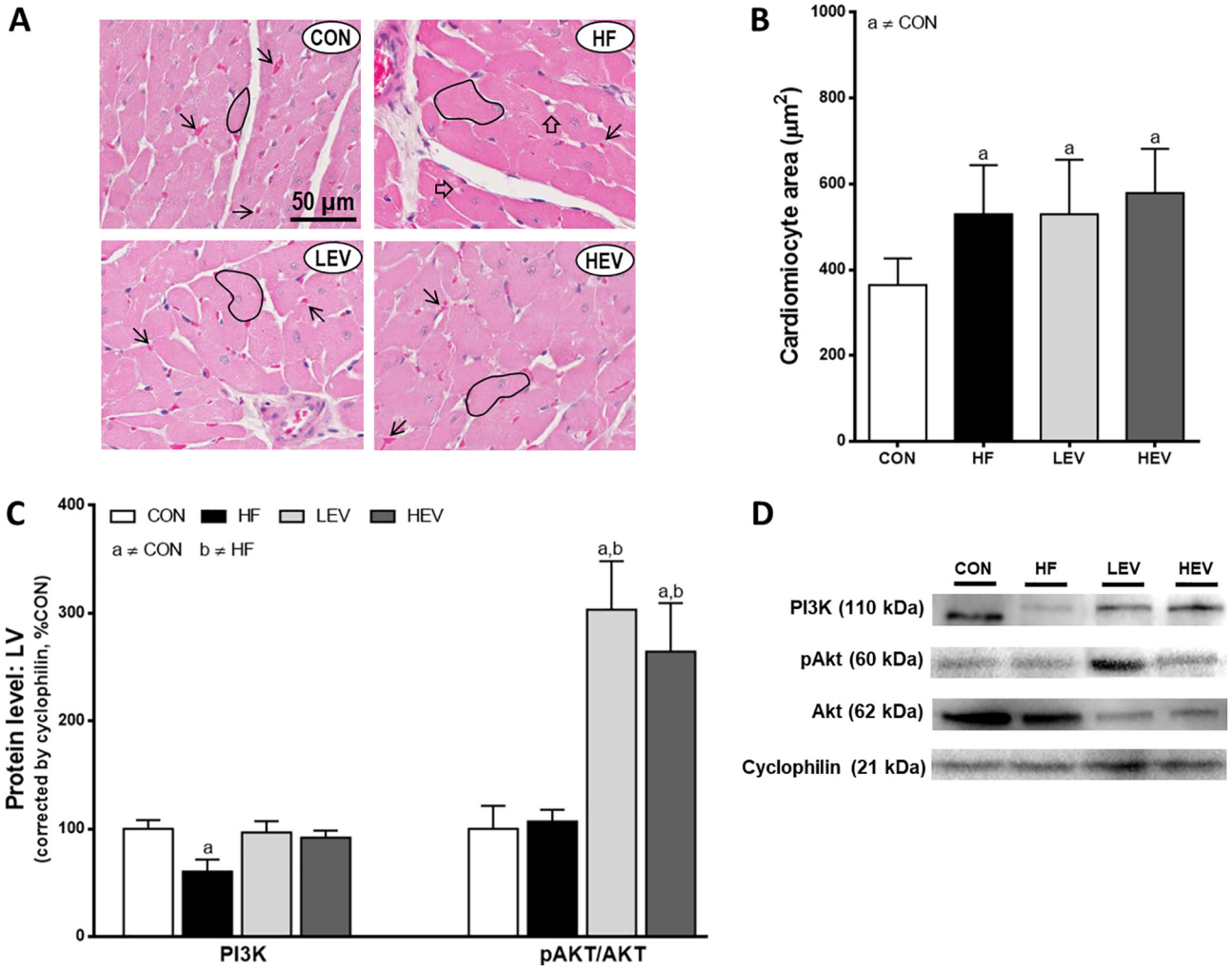

Fig. 1. Cardiomyocyte hypertrophy. (A) Representative photomicrographs showing the myocardium of the left ventricle (stain: hematoxylin & eosin), at the same magnification in all groups (bar = 50 µm). A cardiomyocyte is outlined in each group to compare its differences in area. CON group showing normal pattern of cardiomyocytes and interstitial space with preserved intramyocardial microvascularization (arrows). HF group showing hypertrophied and vacuolated (open arrows) cardiomyocytes, small interstitial space and areas of scarce microvascularization (arrows); LEV and HEV groups showing hypertrophied cardiomyocytes and normal pattern of interstitial space with preserved intramyocardial microvascularization (arrows). (B) Mean cardiomyocyte area, values are expressed in μm^2. (C) PI3K and phosphorylated Akt^{Ser473}/total Akt ratio protein expression measured by Western blotting. Values expressed as percentage of the CON group. (D) Representative Western blotting analysis of proteins in left ventricle. Values presented as means ± SEM, n = 6/group. Significant differences between the groups are indicated by symbols ($p < 0.05$): a ≠ CON; b ≠ HF; c ≠ LEV.

cases; Fig. 3B and D). Furthermore, the HF group had increased activated/inactivated caspase-3 protein expression ratio in relation to CON group ($p < 0.05$). On the contrary, the HEV group increased protein levels of both pERK/ERK and pGSK-3β/GSK-3β ratios when compared with HF group ($p < 0.05$ in all cases), and presented lower activated/inactivated caspase-3 ratio when compared with HF group ($p < 0.01$; Fig. 3C and D; respectively).

4. Discussion

The main goal of the current study was to compare the effect of different exercise volumes on the cardiac morphological changes and activation of the opioid system in a diet-induced obesity (DIO) model. We observed that both exercise training volumes promoted the following benefits: reduction of SBP and adiposity index as well as physiological cardiac hypertrophy, restoring PI3K/Akt signaling pathway. However, the HEV group presented greater reduction in HF diet-induced BM gain than LEV group. Importantly, HEV also demonstrated greater activation of opioid system, not only through the increased expression of endogenous proenkephalin and the three opioid receptors subtypes, but also improving downstream signaling (via ERK/Akt/GSK-3β) and the anti-apoptotic response (activated/inactivated caspase-3 ratio) (Fig. 4).

As previously described, the HF diet intake led to an increased BM gain, adiposity index and SBP [22–24]. Although both exercise volumes were able to reduce adiposity index and SBP, only the HEV protocol decreased BM gain with further reduction of SBP and adiposity index values. These results corroborate with the dose-response relationship between exercise and health outcomes, and reemphasize the importance of exercise as a strategy to treat hypertension and reduce cardiovascular risk factors [9]. Moreover, exercise training improves maximum exercise capacity in a dose-dependent manner [24], as we have here demonstrated that both exercise volumes increased maximum velocity in comparison to sedentary groups, but maximum velocity was even higher in HEV vs. LEV protocol. Therefore, it is clear that although superior amounts of exercise training induce greater benefits, low volumes also produce relevant positive effects, that affect the cellular and molecular pathways to cardiac remodeling.

Obesity is classically related to pathological cardiac hypertrophy, while physiological hypertrophy occurs as a result of exercise training

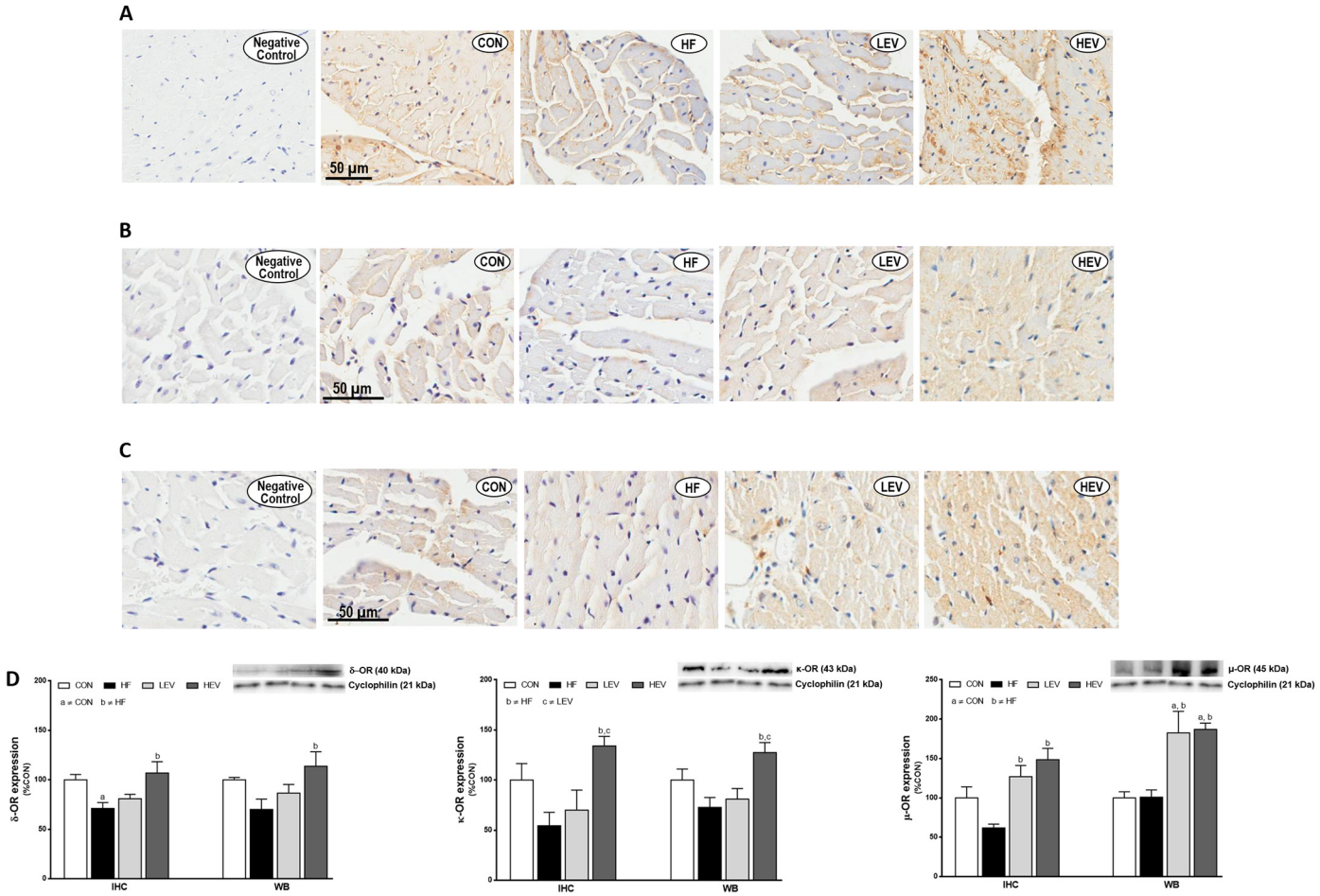

Fig. 2. Opioid receptors in left ventricle. (A–C) Representative photomicrographs showing myocardium with immunoperoxidase (brown)-stained opioid receptors (counterstained with hematoxylin, same magnification in all groups, bar = 50 µm). (D) Opioid receptors protein expression is estimated by immunohistochemistry (IHC) and Western blotting (WB). Values are expressed as percentage of the CON group and presented as means ± SEM, n = 6/group. Significant differences between the groups are indicated by symbols ($p < 0.05$): a ≠ CON; b ≠ HF; c ≠ LEV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[25]. Physiological hypertrophy consists in a standard organization of cardiac structure and improved cardiac function [26]. It has been demonstrated that PI3K/Akt pathway is critical for exercise-induced physiological cardiac hypertrophy, but not pathological hypertrophy [27]. Exercise-induced cardiac hypertrophy is attenuated in Akt knockout models, and pharmacological inhibition of PI3K abolishes the beneficial effects of exercise training [28,29]. Also, previous studies showed that exercise increases Akt expression in an obesity model [30]. In our study, HF sedentary rats showed an increase in SBP and developed a pathological cardiac hypertrophy, an abnormal myocardial interstitium and vacuolization. Altogether, these morphological changes indicate a series of complex cardiac events related to apoptosis and fibrosis, which consequently could lead to adverse remodeling of cardiac structure and impairment in myocardial performance [31]. Even though trained rats were also fed a HF diet, both exercise volumes were able to reduce SBP and induce physiological cardiac hypertrophy, which was confirmed by the favoring of the PI3K/Akt pathway. Therefore, both exercise volumes protocols protected the heart against diet-induced pathological hypertrophy and hypertension.

Our results are in agreement with other studies that reported an opioid system mediated cardioprotective effect of exercise [16,17,32]. A previous study showed that the beneficial effects of exercise training were decreased by a non-selective pharmacological blockade of opioid receptors in a myocardial infarction model [17]. Similarly, a study using an ischemia-reperfusion injury model demonstrated that cardioprotection mediated by acute aerobic exercise is abolished by non-selective pharmacological blockade of opioid receptors [16]. Acute aerobic exercise has been shown to increase opioid system compounds in an ischemia-reperfusion injury model [16,33]. Furthermore, authors have also shown an increased KOR expression after 8 weeks of exercise training in an ischemia/reperfusion model [34]. When comparing different exercise volumes, our results demonstrate that high, but not low, exercise volume increases the cardiac expression of DOR, MOR and KOR. To our knowledge, this is the first study comparing the effect of different exercise volumes on the expression of three subtypes of cardiac opioid receptors in a DIO model.

Moreover, so far there is no information on the effect of exercise training on other components of the opioid system in a DIO model. For example, enkephalins are endogenous opioid peptides that are derived from a pre-proenkephalin precursor protein. The proenkephalin expression encodes a polypeptide precursor, which undergoes translational processing to generate the pentapeptides Met-enkephalin and Leu-enkephalin, that binds to opioid receptors [33]. It has been shown that cardiomyopathy evokes an increase in pre-proenkephalin, and that obesity may change the state of the endogenous opioid system [15]. Our results show for the first time that HF diet intake leads to reduced proenkephalin expression. In agreement with our hypothesis that the opioid system is involved in exercise-induced cardioprotection, only rats exposed to HEV protocol increased proenkephalin expression. Previous studies demonstrated that acute aerobic exercise increased

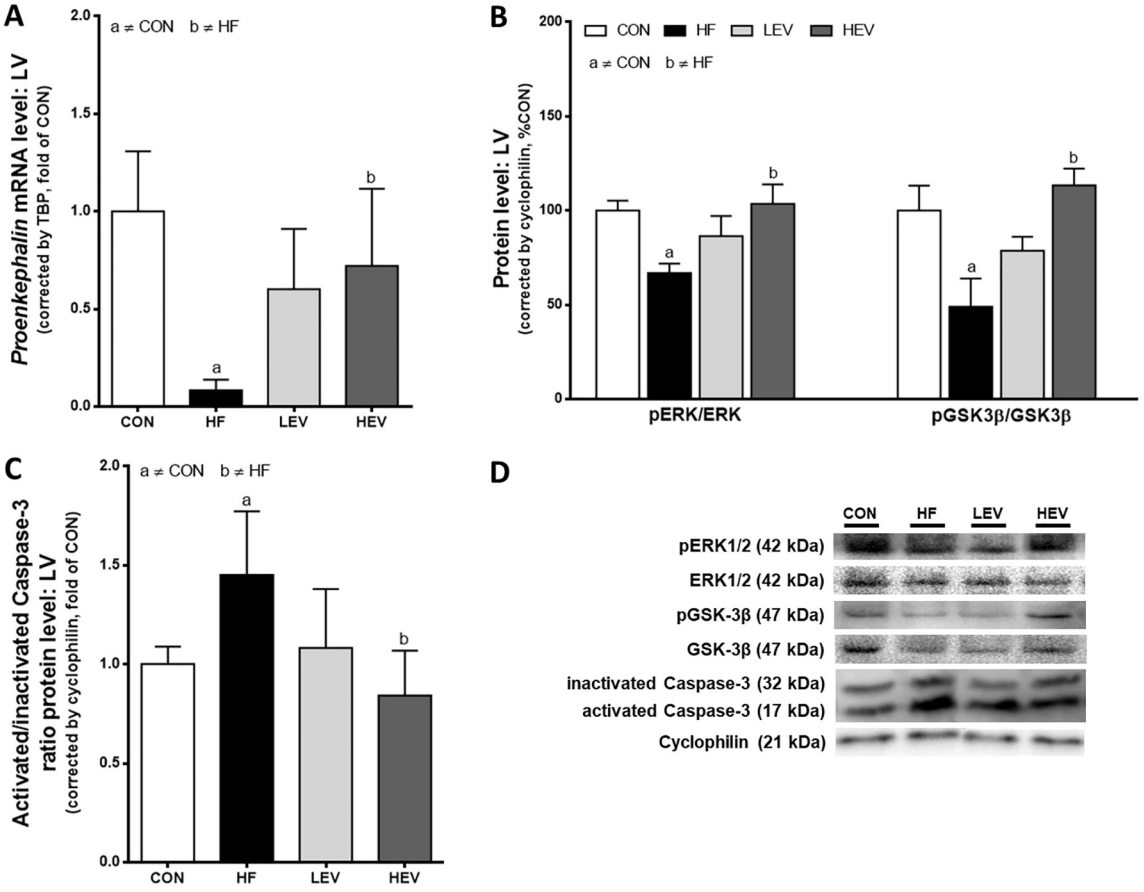

Fig. 3. Peptide and downstream signaling of the opioid system in left ventricle. (A) Cardiac proenkephalin mRNA expression measured via real-time reverse-transcriptase polymerase chain reaction (RT-qPCR). Data are presented as a fold of the control. (B) Phosphorylated ERK 1/2^{Tyr204}/total ERK and phosphorylated GSK-3β^{Ser9}/total GSK-3β ratios protein expression measured by Western blotting. Values are expressed as percentage of the CON group. (C) Activated to inactivated caspase-3 protein expression ratio measured by Western blotting. Data are presented as a fold of the control. (D) Representative Western blotting analysis of proteins. Values are presented as means ± SEM, n = 6/group. Significant differences between the groups are indicated by symbols ($p < 0.05$): a ≠ CON; b ≠ HF; c ≠ LEV.

proenkephalin expression in an ischemia/reperfusion model [33].

The involvement of each opioid receptor subtype in cardioprotection is not completely elucidated. Our study for the first time suggests that DOR, KOR and MOR might be involved in exercise-induced cardioprotection in a DIO model. Several studies suggest that pharmacological activation of DOR by a selective agonist mimics the protective effects of ischemic preconditioning [35–38]. DOR has been shown to be involved in exercise-induced cardioprotection with acute aerobic exercise protocol in ischemia-reperfusion model [33,39]. Although most studies discuss only the role of DOR in cardioprotection, there are evidence indicating the participation of KOR as well. Previous data have demonstrated that the pharmacological activation of KOR by a selective agonist is cardioprotective against ischemia/reperfusion injury model [40]. Furthermore, it has been shown that KOR mediated the beneficial effects of ischemic preconditioning in an ischemia/reperfusion model [41]. Additionally, during exercise training, pharmacological blockade of KOR by a selective antagonist abolished the exercise-induced cardioprotection [34]. The role of MOR in cardioprotection is even less understood, especially considering the existing controversies on its presence in the cardiac tissue [42–44]. Noteworthy, we have reinforced the presence of MOR in cardiac tissue through immunohistochemistry and western blotting analysis. Recently, the involvement of MOR as a cardioprotective mechanism has been suggested, based on its pharmacological activation by a selective agonist in heart failure and ischemia/reperfusion models [37,45].

The pathways involved in the cardioprotection mediated by the opioid system have been shown to include ERK 1/2, GSK-3β, PI3K and Akt [15,37,45]. In our study, although obesity did not change the expression of the opioid receptors, the expression of ERK 1/2, GSK-3β and PI3K were all impaired. On the other hand, ERK 1/2, GSK-3β and Akt were all favored by the HEV protocol. LEV protocol did not change the opioid system compounds expression, suggesting that 150 min·week^{-1} of moderate intensity exercise training are not enough to trigger this protective response. The post-receptor signaling of the opioid system is associated with anti-apoptotic effects [15]. Activation of ERK 1/2, PI3K and Akt converges to GSK-3β, which suppress the opening of mitochondrial permeability transition pore (mPTP) when phosphorylated [45]. This is important because mPTP opening is associated with apoptosis, and consequently with increases in caspase activity [15]. Caspase-3 is a major executioner protein in apoptosis and the terminal factor in the process of apoptotic cell death [46]. Previous studies have also demonstrated that exercise training prevents cardiac apoptosis by reducing caspase-3 activity [46]. However, we have extended the current knowledge by demonstrating that activated caspase-3 expression was increased in obese rats, and that the HEV protocol, not LEV, effectively reduced the activated caspase-3 expression, protecting the heart against diet-induced apoptosis.

The results of the present study should be interpreted while considering some limitations. While we demonstrated the protective effects of exercise in blood pressure and cardiac morphological, we did not assess cardiac function by echocardiography or other method. Furthermore, we determined proenkephalin cardiac expression but did not evaluate the other endogenous opioid agonists (*i.e.* prodynorphin and proopiomelanocortin). Also, we did not have a group of animals

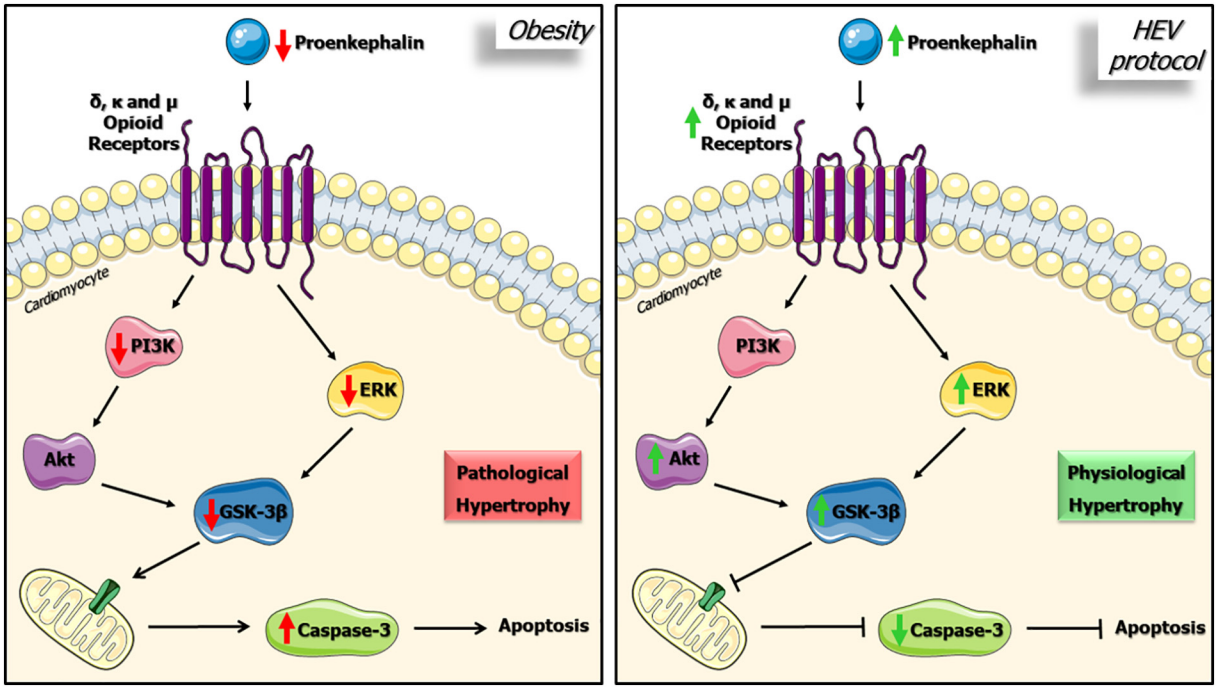

Fig. 4. Effects of obesity and exercise training on cardiac opioid system. Proenkephalin, an endogenous opioid ligand, is reduced in obesity. Although opioid receptors expression is not altered by obesity, the expression of proteins involved in its downstream signaling, such as PI3K, ERK and GSK-3β, is reduced. Decreased GSK-3β leads to mPTP opening and consequently increased activated caspase-3 and apoptosis. Cardiomyocyte hypertrophy presents a pathological character in obesity. On the other hand, the high volume of exercise training upregulates the opioid system. Proenkephalin expression is increased by HEV protocol, which also enhances DOR, KOR and MOR expression. Downstream signaling, i.e. Akt, ERK and GSK-3β, are increased by HEV protocol. Increased GSK-3β leads to mPTP closure and consequently reduced activated caspase-3 and apoptosis. Exercise training induces physiological cardiac hypertrophy. DOR: δ opioid receptor; KOR: κ opioid receptor; MOR: μ opioid receptor; HEV: high exercise volume; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; ERK: extracellular signal-regulated kinases; GSK-3: glycogen synthase kinase 3 beta; mPTP: mitochondrial permeability transition pore.

deficient in opioid receptors, to reinforce the role of opioids in the protection conferred by exercise training.

Taken together, our findings provide new insights with clinical relevance about the critical dose of exercise training necessary to reduce cardiovascular risk factors caused by obesity. We suggest that 150 min·week^{-1} of exercise training is not sufficient to trigger the opioid system-induced protective effect, and that 300 min·week^{-1} provides better cardiovascular benefits through upregulation of opioid receptors and their downstream signaling, attenuating apoptosis pathway in obese rats. In light of our findings, this study may open new perspectives in the treatment of cardiovascular impairments induced by obesity. Further studies are necessary to elucidate the role of each opioid receptor subtype in exercise-induced cardioprotective effects.

Disclosure statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

BAS, MVM, ABV, JPB, ET, ACLN and EDCF were involved in the conception and design of the research; BAS, MVM, LLV, ACM, VSF, ABV, DCM and EDCF collected data, performed the statistical analysis and drafted the paper; MVM, ABV, JPB, ET, DCM, ACLN and EDCF edited and revised manuscript. All authors read and approved the final manuscript.

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Declarations of Competing Interest

None.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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