High intensity intermittent training is as effective as moderate continuous training, and not deleterious, in cardiomyocyte remodeling of hypertensive rats

KRZESIAK A*, COGNARD C*, SEBILLE S*, CARRE G*, BOSQUET L§ & DELPECH N§1

*Equipe Transferts Ioniques et Rythmicité Cellulaire (TIRC), Lab. Signalisation et Transports Ioniques Membranaires (STIM), Université de Poitiers, EA n°7349, Faculté des Sciences Fondamentales et Appliquées, Pôle Biologie Santé Bât B36/B37
1 rue Georges Bonnet
TSA 51106
86073 Poitiers Cedex 9 - France

§ Laboratoire Mobilité, Vieillissement & Exercice (MOVE), EA 6314, Université de Poitiers n°7368, Faculté des Sciences du Sport, Bât C6
8, allée Jean Monnet
TSA 31113
86073 Poitiers Cedex 9 - France

Running head: Cardiomyocyte remodeling of trained hypertensive rats.

Corresponding author: Laboratoire Mobilité, Vieillissement et Exercices, 8 Allée Jean Monnet, Bât C6, 86000 Poitiers, France. Phone +33549453398, FAX +33549453396, nathalie.delpech@univ-poitiers.fr

Abstract

Exercise training offers possible non-pharmacological therapy for cardiovascular diseases including hypertension. High intensity intermittent exercise (HIIE) training has been shown to have as much or even more beneficial cardiovascular effect in patients with cardiovascular diseases than moderate intensity continuous exercise (CMIE) training. The aim of this study was to investigate the effects of the two types of training on cardiac remodeling of SHR (spontaneously hypertensive rats) induced by hypertension. Eight-week-old male SHRs and normotensive Wistar Kyoto rats (WKY) were divided into four groups: normotensive and
hypertensive control (WKY and SHR-C), hypertensive trained with CMIE (SHR-T CMIE) or HIIE (SHR-T HIIE). After 8 weeks of training or inactivity, maximal running speed (MRS), arterial pressure and heart weight were all assessed. CMIE or HIIE protocols not only increased final MRS and left ventricular weight/body weight ratio, but also reduced mean arterial pressure (MAP) compared to sedentary group. Then, left ventricular tissue was enzymatically dissociated and isolated cardiomyocytes were used to highlight the changes induced by physical activity at morphological, mechanical and molecular levels. Both types of training induced restoration of transverse tubule regularity, decrease in spark site density and reduction in half-relaxation time of calcium transients. HIIE training, in particular, decreased spark amplitude and width, and increased cardiomyocyte contractility and the expression of SERCA and phospholamban phosphorylated on Serine 16.

**New and Noteworthy:** High intensity intermittent exercise training induces beneficial remodeling of the left ventricular cardiomyocytes of hypertensive rats (SHR) at the morphological, mechanical and molecular levels. Results also confirm, at the cellular level, that this type of training, as it appears not to be deleterious, could be applied in rehabilitation of hypertensive patients.

**Keywords:** Exercise training, high intensity intermittent exercise, cardiac remodeling, hypertension, calcium handling.
1. Introduction

Hypertension is a major public-health problem because of its high frequency, (over one billion people worldwide are affected (44) and its associated risk of dementia and cardiovascular diseases. The heart, one of the target organs of hypertension, is remodeled by a compensatory pathologic hypertrophy that leads to heart failure when hypertension is uncontrolled (6). Finding ways of mitigating this process could be a significant solution for such health problems. Among these ways, it is well known that exercise training offers a potential non-pharmacological therapy for cardiovascular diseases, including hypertension. Aerobic exercise training can enhance myocardial function (72), causing changes in cardiomyocyte activity and morphology in healthy subjects (37) and may potentially reverse pathologic cardiac hypertrophy (55, 74). In fact, more and more recommendations are being pronounced by the authorities for exercise training prescriptions in order to prevent many cardiovascular diseases. For example, the American College of Sports Medicine (ACSM) recommends 30 to 60 minutes of moderate intensity exercise five days a week or 20 to 60 minutes of high intensity exercise three days a week (53). A number of publications highlight the beneficial effects of continuous endurance training not only in healthy subjects, but also in hypertensive patients (19, 24). After continuous moderate-intensity exercise (CMIE) training, hypertensive patients presented improvement in blood pressure, maximal oxygen uptake ($$\dot{V}O_{2\text{max}}$$), and heart rate at rest (19). And in this meta-analysis, Cornelissen and Fagard noted decreased of systemic vascular resistance and increased stroke volume.

High intensity intermittent exercise (HIIE) training, which is frequently used in sports training, has also been applied in rehabilitation programs for patients with cardiovascular pathologies including coronary artery disease, heart failure and, more recently, hypertension (25). This type of training is characterized by brief repeated bursts of relatively intense exercise interspersed by periods of recovery. It seems to have as many - or even more -
beneficial effects in hypertensive patients than CMIE training. Indeed, HIIE training causes
greater post-exercise hypotension (systolic and diastolic blood pressure) than CMIE training
(47, 48). Cardiac and endothelial function is also improved after HIIE training in hypertensive
patients. Molmen-Hansen and colleagues (47) have shown an increase in stroke volume and
in endothelium-dependent flow-mediated dilatation of the brachial artery. And to a greater
extent than CMIE training, HIIE training favors an improvement in aerobic fitness, with
increased $\dot{V}O_2_{max}$ in hypertensive patients (48) or at high risk of hypertension (17).

While studies, aimed at understanding the mechanisms responsible for cardiac
remodeling after HIIE training in heart failure rats, have previously been carried out (73), they
have not been performed in hypertensive rats. While some of them were focused on cardiac
remodeling induced by training in spontaneously hypertensive rats (SHR), only moderate
continuous, not high intensity intermittent training, was used to demonstrate inversion from
pathological to physiological cardiomyocyte phenotype. For example, in SHR, Garciarena and
colleagues showed an increase in cardiomyocyte cross-sectional area and myocardial capillary
density as well as a reduction of cardiac fibrosis after CMIE training (22). Another study
noted, in the same murine model, an improvement of contractility and intracellular calcium
transient in ventricular myocytes following continuous endurance training (13). However,
some challenges and questions remain: 1/ to ensure that HIIE training is not deleterious at
cellular level; 2/ is HIIE training as effective or even more effective than CMIE as a means of
reversing cardiomyocyte remodeling? The purpose of this study was to compare the effects
of CMIE and HIIE training on cardiomyocyte remodeling in spontaneously hypertensive rats.
We were particularly interested in the elements contributing to the process of excitation-
contraction coupling and calcium mobilization. First, an architectural study on the transverse
tubules was carried out, given that disorganization of these structures has been repeatedly
demonstrated in pathologies involving the cardiovascular system (40, 43, 60, 71). Second, a
molecular study on calcium at rest and in response to electrical stimulation was performed, since this ion homeostasis deregulation is frequently described during hypertension (13, 33, 61). As calcium playing an important role in excitation-contraction coupling, cardiomyocyte contractility (7, 9) was also studied in this paper. Lastly, the expressions of the SERCA and phospholamban proteins were studied by western blot to explain some of our results.

2. Materials and methods

2.1. Animals

Eight-week-old male (eighteen weeks at the time of rat sacrifice) SHR and normotensive Wistar Kyoto rats (Janvier, Genest St Isles, France) were housed individually, with water ad libitum and access to normal rat chow (20g per day). They were kept on a 12 hour light/dark reverse cycle (dark: 8 am to 8 pm) in a temperature-controlled room (22°C). Four experimental groups were established: normotensive and hypertensive control (WKY and SHR-C), hypertensive trained with CMIE (SHR-T CMIE) and hypertensive trained with HIIE (SHR-T HIIE). Body weight (BW) was measured every week; mean arterial pressure (MAP) and heart rate (HR) were recorded three days post exercise training protocol by the tail cuff method (CODA, EMKA Technology, Prague, Czech Republic). Experimental protocols were approved by the French Ethics Committee in Animal Experimentations (Apafis #3071-2015092511304016).

2.2. Exercise training protocols

The two types of exercise training were performed on a treadmill (Exer3/6 Treadmill, Columbus Instruments, Columbus, USA), 5 days a week for 8 weeks. CMIE protocol was continuous exercise training at 70% of maximal running speed (MRS) for 36 minutes. HIIE protocol was intermittent exercise training, 3 minutes at 90% of MRS and 3 minutes at 50% of MRS, repeated 6 times. The week before the beginning of the first set of training protocols, a treadmill habituation session was performed, starting with a zero degree slope and a 15
m·min\(^{-1}\) speed. Then the grade was increased by 5 degrees every two days, the speed by 2 m·min\(^{-1}\) and the duration by two minutes daily. Two days after habituation, maximal running speed (MRS) was measured in hypertensive rats. The test consisted of five minutes of running at 13 m·min\(^{-1}\) at a grade of 10 degrees, then the speed increased by 3.6 m·min\(^{-1}\) every two minutes, until the animal was exhausted (19, 20). MRS was repeated every two weeks and at the end of the exercise training protocol, in order to adapt the training intensity to the upgraded performance of the animal. The average running speeds is 24.5 ± 3.36 m·min\(^{-1}\) for CMIE (minimum: 17.9 m·min\(^{-1}\), maximum: 33.1 m·min\(^{-1}\)) and for HIIE, 18.3 ± 3.2 m·min\(^{-1}\) and 32.9 ± 5.7 m·min\(^{-1}\) respectively at 50% (minimum: 12.9 m·min\(^{-1}\), maximum: 26.1 m·min\(^{-1}\)) and 70% (minimum: 23.2 m·min\(^{-1}\), maximum: 47.1 m·min\(^{-1}\)).

2.3. Interstitial collagen

LV sections were stained with picrosirius red for quantifying collagen volume fraction (CVF) as previously described (75). Images were captured using a slide scanning microscope (Olympus VS 120) and analyzed with the ImageJ software.

2.4. Cell isolation

Three days after the end of the whole training protocol, the animals were euthanized with pentobarbital (40 mg/kg, Ceva Santé Animale, Libourne, France) and their heart was quickly removed, followed by enzymatic dissociation of the left ventricular tissue as previously described (13). The digested heart was then removed from the cannula and weighed, and the left ventricle was separated, weighed, cut into small pieces and gently triturated using Pasteur pipettes. Cells were re-suspended in Tyrode and plated on Petri dishes previously coated with matrigel (BD Biosciences, Bedford, USA). Tyrode contained (in mM): 140 NaCl, 5.4 KCl, 1.8 MgCl\(_2\), 1.8 CaCl\(_2\), 10 Hepes, 11 glucose, 11 NaHCO\(_3\), pH 7.4.
2.5. Transverse tubules

To analyze T-tubule configurations, ventricular myocytes were loaded with 10 μM of di-8-ANEPPS (Invitrogen, California, USA) during 15 min in Tyrode at room temperature. Images were acquired through a confocal microscope (Olympus FV1000). The probe was excited with the 488nm laser line of an argon/krypton laser and the fluorescence detected in front of a band filter centered at 522nm. Results were analyzed with the plugin of ImageJ named Transverse tubular system regularity analysis (52). The analysis was divided into three steps, first a Fast Fourier Transform (FFT) was computed, then the background noise of the FFT spectrum was removed, and finally the peak on the frequency interval was sought. The peak amplitude was taken as an index of regular distribution of TT network named TT power. Cardiomyocyte images were also used to measure cell length and width at rest.

2.6. Spontaneous calcium sparks

Left ventricular cardiomyocytes were incubated with 5 μM of fluo-8-AM (Invitrogen, California, USA) during 15 min in Tyrode at room temperature. Calcium sparks were observed through a 60x water immersion lens (1.2 NA) and images acquired first in “XY” mode taking an image of the whole cell and then in “Xt” mode recording time fluorescence variations along a given space line (1 line/2 ms, resulting in 512x512 pixels images). Sparks were analyzed using the HARVELE software (59), developed in our laboratory under IDL 6.3, which automatically detects calcium release events and measures five parameters: Amplitude, FDHM (full duration at half maximum) in ms, FWHM (full width at half maximum) in μm, time to peak in ms and occurrence frequency in sparks/s. For XY standard deviation images (Fig.4) analysis, 60 fluorescence images were acquired at a rate of 1 image/300 ms.

2.7. Stimulated intracellular calcium measurements
To evaluate global intracellular calcium transients, isolated cardiomyocytes were loaded with 5 µM fluo-8-AM (Invitrogen) for 15 min at room temperature in Tyrode solution. Cells were stimulated by the field-stimulating method through a pair of platinum electrodes at 1Hz and 20mA. Fluorescence images (512x512 pixels) were recorded by a Bio-Rad MRC 1024 Confocal Laser Scanning Microscopy (Bio-Rad Laboratories, California, USA). Fluorophore was excited with the 488nm laser line of an argon/krypton laser and the emitted fluorescence was detected at 522 nm. Three parameters were analyzed: amplitude (ΔF/F0), time to peak and time to 1/2 decay in ms.

2.8. Cell contractility

Freshly isolated cardiomyocytes were placed in a field-stimulating chamber (Series 40 Quick Change Imaging Chambers, RC-46SLP, Warner Instruments, Connecticut, USA) on an inverted microscope (Olympus IX 70) and perfused with Tyrode solution at room temperature. Cells were stimulated at 1 Hz with an intensity of 20 mA delivered through a STM4 constant current stimulator (Bionic Instruments, France) and visualized on a PC monitor with a camera (Myocam, Ionoptix, Massachusetts, USA). The images of cardiomyocytes obtained (IonWizard 6.2, Ionoptix, Massachusetts, USA) were used to measure sarcomere length at rest and during stimulation. Three parameters were then calculated: cell shortening (expressed as a percentage of resting sarcomere length), maximal velocity of contraction, and relaxation.

2.9. Patch clamp measurements

The whole-cell mode of the patch-clamp technique was used to record and measure L type calcium current (ICaL) on isolated left ventricular cardiomyocytes used in this work through an Axopatch 200B amplifier with a CV 202AU headstage (Molecular Devices, Sunnyvale, CA, USA). Cells were seeded in 35 mm diameter culture dishes for 4 h prior to the patch-
clamp experiments. Voltage-clamp was generated by a personal computer equipped with an analogue-digital converter (Digidata 1322a, Molecular Devices) and using the v10 version of the pClamp software from Molecular Devices. The composition of the bath solution was (in mM): TEACl 20, NMDG 130, MgCl\textsubscript{2} 1.4, CaCl\textsubscript{2} 1.8, D-Glucose 10, HEPES 10, taurine 10, creatine 10 (pH adjusted to 7.4 with NaOH). The intra-pipette solution contained (in mM): TEACl 20, CsCl 110, Na\textsubscript{2}ATP 2, NaGTP 0.3, EGTA 10, MgATP 5, HEPES 10 (pH adjusted to 7.2 with KOH). All the measurements were obtained at room temperature (20–22 °C).

Pipettes (borosilicate GC150-T glass microelectrodes) were manufactured on a vertical twin heating puller (Narishige). Their resistance was between 2 and 5 MΩ when filled with the intrapipette solution. After obtaining a gigaseal, a suction pulse was applied to establish the whole-cell mode. In order to stabilize ionic currents, recordings were performed 5 min after entering the whole-cell configuration. Series resistances were compensated by 70–90%.

Analyses were performed using pClampfit 10 software (Molecular Devices). Cell capacitance was measured by integrating the area under the capacitive transient elicited by 5 mV depolarizing steps from a holding potential of 0 mV. For adrenergic response measurements, a 1 µM final concentration of Isoproterenol (Sigma-Aldrich) was superfused for 2 minutes.

2.10. Western blotting

Left ventricular cells samples were homogenized in a lysis buffer containing phosphatase and protease cocktail inhibitors (Sigma, Missouri, USA). Protein samples were separated by SDS-PAGE in polyacrylamide gel (8 % or 10 % depending on the protein type). Proteins were transferred to nitrocellulose membranes (Millipore, 0.22 µm) after electrophoresis. Membranes were blocked for 2 h at room temperature in TBS-Tween 20 with 5 % non-fat dry milk and then incubated with specific primary antibodies at 4°C overnight. The following antibodies were used: monoclonal antibodies for sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA: 1/500, Santa Cruz, Texas, USA), total phospholamban (PLBt: 1/200, Santa Cruz,
Texas, USA), Junctophilin-2 (JP-2: 1/500, Santa Cruz, Texas, USA), and GAPDH (1/100, HyTest, Turku, Finland); polyclonal antibody for phosphorylated phospholamban at serine 16 (PLBser16: 1/500, Abcam, Cambridge, UK). Membranes were incubated with a second antibody conjugated with peroxidase (mouse for SERCA2, PLBt, JP-2 and GAPDH, rabbit for PLBser16, 1/5000 Interchim, Montluçon, France) at room temperature for 2h. Proteins were detected by chemiluminescence (ECL Prime kit, GE healthcare, Amersham, UK) in GeneGnome (SynGene, Cambridge, UK). Densitometric analyses of immune-reactive band were carried out with GeneTools software (SynGen, Cambridge, UK).

2.11. Statistical analysis

Statistical analysis was performed by means of GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data are means ± SE, n is the number of cells and N is the number of animals. Unpaired T-test was used to compare SHR-C and WKY. One-way ANOVA with Newman-Keuls post-hoc testing was used to compare the effects of both types of exercise training on hypertension. WKY data are presented for reference and, in figures, appear as dashed outlined bars.

3. Results

3.1. General characteristics and physical capacity.

Before addressing cellular effects, observations were first carried out on the overall characteristics of the animals. Morphological data, arterial blood pressure and physical capacity from each experimental group are reported in Table 1. Initial BW was similar in the four groups and increased at the end of the protocol for all groups. Final BW was significantly higher in normotensive rats (WKY) compared to hypertensive untrained rats (SHR-C). There was no difference in the body weight of hypertensive rats, whether or not they were trained. An increase of the heart weight/body weight ratio was observed in all SHR groups especially
in the rats trained with continuous exercises. Moreover, in our study, hypertension induced left ventricular hypertrophy, as an increase of left ventricular weight/body weight ratio was detected in all groups. Our results did not show significant difference between groups regarding the collagen volume fraction (CVF). Notwithstanding the absence of significant difference at p≤0.05 the percentage of fibrosis increased between WKY and SHR-C (4.29 ± 1.63 % to 5.63 ± 1.66 %, Table 1). Values after both types of training (5.14 ± 1.97 and 4.68 ± 1.67 for SHR-T CMIE and SHR-T HIIE respectively) were between normotensive and sedentary hypertensive rats. Arterial blood pressure was higher in SHR-C compared to WKY, nevertheless, the two protocols of training seemed to mitigate this increase, with values lower than in sedentary hypertensive rats. Heart rate (HR) was also changed in the hypertensive trained groups, with a reduction of about 10 and 8 % in SHR-T CMIE and SHR-T HIIE respectively with regard to SHR-C. The initial values of MRS were similar in the three hypertensive groups with a mean approximating 30 m·min⁻¹. However, after 8 weeks of training, the physical capacity (final MRS) of SHR-C had decreased while the physical capacity of rats trained with CMIE or HIIE had drastically increased.

3.2. Cellular morphology.

Knowing that ventricular mass increases with training, the question that arose was: what type (pathological and/or physiological) of hypertrophy was involved? In order to answer this question, the morphology of cardiac cells was studied. No significant differences were observed in cell length at rest (Fig. 1A) either between WKY and SHR-C groups or between SHR-C and both SHR trained groups, whereas cell width (Fig. 1B) increased in SHR-C (24.3 ± 0.4 µm) compared to WKY (22.9 ± 0.5 µm). Cell width was also higher in SHR-T CMIE (25.6 ± 0.6 µm) than in SHR-C and SHR-T HIIE (23.7 ± 0.4 µm).

3.3. Transverse tubules
Analysis of cardiomyocyte morphology was also performed by studying the plasma membrane invaginations, named transverse tubules (TT), through Fast Fourier Transform (FFT) processing. The disruption of TT was previously studied in view of observing the evolution of hypertension (60) and it seems that prevention of remodeling during hypertrophy could be clinically important as a way of delaying progression to heart failure (71). As illustrated in Figs. 2A and B, hypertension led to various degrees of disorganization of the TT network. TT power (Fig. 2C), which is a TT regularity parameter was measured as the peak value (see black arrow in Fig. 2B) from power spectra (see section 2.4), decreased in SHR-C compared to WKY. Training seemed to mitigate the loss of organization, as the TT power value was higher in trained rats (CMIE: 55.9 ± 0.5 A.U. and HIIE: 56.0 ± 0.5 A.U.), with intermediate values between SHR-C (53.6 ± 0.5 A.U.) and WKY (59.2 ± 0.6 A.U.). Measurement of the period (Fig. 2D) is related to sarcomere length. The period of TT in cardiomyocytes at rest was greater than 1.7 µm, in all rat groups, (Fig. 2D) which corresponds to the distance between tubules (eventually the sarcomere length at rest) classically reported (between 1.7 and 2 µm). We observed a significant decrease of the period in hypertensive rats (SHR-C: 1.753 ± 0.007 µm), compared to normotensive ones (1.786 ± 0.005 µm). There was no difference between TT period of SHR-C and both trained SHR groups (SHR-CMIE: 1.749 ± 0.007 µm, SHR-HIIE: 1.733 ± 0.007 µm).

3.4. Spontaneous calcium sparks

The line scan images (X,t) of which three examples are presented in Figs. 3A, B and C, made it possible to study the time and space shapes of sparks (illustrated in Figs. 3D, E and F). From the table in Fig 3, a general picture emerges: the values of FDHM, FWHM and Time to peak showed that, compared to normotensive rats (WKY whose values are present in the figure legend), hypertension (SHR-C) led to increases in all of these parameters. By contrast, the two training protocols both tended to mitigate or cancel this effect (SHR-T CMIE and
SHR-T HIIE). Similar behavior was observed in the occurrence frequencies of sparks. Regarding the amplitude parameter, hypertension did not lead to variations, except for the fact that HIIE training could significantly decrease this amplitude (SHR-T HIIE).

Fig. 4A shows representative standard deviation images of left ventricular cardiomyocytes sites where sparks arose (calcium release sites). In SHR-C, spark site density (Fig. 4B) was significantly higher (0.0054 ± 0.0005 sparks/µm²) than in normotensive rats (0.0037 ± 0.0003 sparks/µm²). Both training protocols tend to decrease spark site density (CMIE: 0.0045 ± 0.0003 sparks/µm²; HIIE: 0.0035 ± 0.0004 sparks/µm²) towards values close to those of healthy ones (WKY) or even below, as regards HIIE training. Some cells displayed calcium whole-cell waves and their percentage over the total of studied cells increased with hypertension (Fig. 4C), from 0% in the WKY group to 12% in the SHR-C group. Moreover, training seemed to reduce calcium wave frequency, especially in the SHR-T HIIE group, for which the frequency was divided by 2 compared to the SHR-C group.

3.5. Calcium transients.

The changes in the shape and characteristics of calcium transients in response to electrical field stimulation are illustrated in Fig. 5A. Notwithstanding the absence of significant differences at p<0.05, the amplitude of transients tended to decrease in SHR-C (26.4 ± 2.9 versus 34.2 ± 3.3 A.U. in WKY, p=0.4, Fig. 5B), while the two types of training protocols both tended to mitigate this reduction (27.6 ± 3.5 and 30.0 ± 2.3 A.U. for CMIE and HIIE respectively). The measurements (Fig. 5C) of times-to-peak did not reveal significant differences either between hypertensive and normotensive rats (SHR-C: 39.8 ± 5.6 and WKY: 33.5 ± 3.8 ms, p=0.5) or between sedentary and trained rats (SHR-T CMIE: 37.1 ± 2.9 ms) despite a decreasing trend of time-to-peak for rats trained by HIIE (30.0 ± 2.5 ms, p=0.3). By contrast, the relaxation phase (measured as the time-to-half-decay) appeared slower in
sedentary hypertensive rats (68.3 ± 7.6 ms) compared to the WKY group (54.5 ± 6.5 ms). The training protocols significantly sped up this phase (52.6 ± 4.7 and 44.1 ± 3.2 ms for SHR-T CMIE and SHR-T HIIE respectively, Figs. 5A and 5D), particularly in the HIIE group.


Resting sarcomeric length was measured at 1.66 ± 0.09 µm. Comparison between normotensive (WKY) and hypertensive untrained rats (SHR-C) showed no significant difference of the shortening amplitude (7.2 ± 0.3 and 7.1 ± 0.5 % respectively) in response to electrical field stimulation (representative trace examples in Fig. 6A and data in Fig. 6B). The same observations were made for the maximal velocity of shortening (1.03 ± 0.11 vs 1.01 ± 0.12 µm/ms, Fig. 6C). On the other hand, the maximal velocity of relaxation was significantly higher in normotensive rats than in hypertensive untrained rats (0.99 ± 0.12 vs 0.68 ± 0.08 µm/ms respectively, Fig. 6D). As regards the effect of exercise training, there was a significant increase in percentage of shortening for SHR-T HIIE (9.2 ± 0.5 %, Figs. 6A and B), indicating an increase in amplitude of contraction and consequently an improvement in contractility. There was also a significant increase in the maximal velocity of shortening (about a 57% increase for both group of trained rats, Fig. 6C) and relaxation (SHR-T CMIE: 63% and SHR-T HIIE: 87%, Fig. 6D) compared to SHR-C.

3.7. Patch clamp

Since voltage dependent L-type calcium (LTC) current (ICaL) plays a significant role in ECC of cardiomyocytes patch-clamp experiments were performed to address possible effect on this ionic current. In resting conditions (without stimulation of β-adrenergic system) hypertension and both types of training did not impact this calcium current density (WKY: - 4.5 ± 0.2 pA/pF, SHR-C: -4.9 ± 0.1 pA/pF, SHR-T CMIE: -4.7 ± 0.1 pA/pF, SHR-T HIIE: -4.6 ± 0.2 pA/pF for example for a pulse to 0mV). On the other hand, LTC channel response to β-
adrenergic stimulation is reported in Fig. 7 and we observed that the percentage of increase of
ICaL density was lower in SHR-C (73.3 ± 2.6 %) compared to normotensive ones (119.0 ± 5.3 %). Both types of training seemed to mitigate this decrease with intermediate values (98.5 ± 3.1 and 98.1 ± 2.7 % respectively for SHR-T CMIE and SHR-T HIIE). Observed effects were clearly due to the superfusion of isoproterenol since washout suppressed these effects.

3.8. Calcium regulatory proteins.

In order to explain the data obtained during the recordings of calcium signaling and cellular contractility, the expression of two proteins, SERCA2a and phospholamban, each of which plays a major role in calcium recapture, was subsequently determined. A representative western blot of SERCA2a, total phospholamban and phospholamban phosphorylated at serine 16 (PLBser\textsuperscript{16}) is presented in Fig. 8A. No significant differences were observed in SERCA2a (Fig. 8B), total phospholamban (PLBtot, Fig. 8C) and PLBser\textsuperscript{16} (Fig. 8D) expression in cardiomyocytes obtained from SHR-C compared to WKY. However, training seemed to play a major role in the change of expression of these two proteins. Indeed, with regard to SHR-C, an increase in SERCA2a expression level was observed after the two types of training, CMIE and HIIE (the values reached 148 and 296% of the SHR-C value respectively, Fig. 8B). Training also induced a significant increase (the values were multiplied by around 10 and 16 for SHR-T CMIE and SHR-T HIIE respectively, Fig. 8D) in the phosphorylation degree of PLBser\textsuperscript{16}, contrary to that of total phospholamban, which was not affected (Fig. 8C). In addition Junctophilin-2 expression (43, 66), a protein thought to be involved in maintaining of TT arrangement, was addressed and was apparent for normotensive rats (Fig. 8A) but not for the SHR-C group. However both training types did not allow restoring this expression.
4. Discussion

The aim of this study was to investigate the effects of two types of physical exercise training (continuous at moderate intensity and intermittent at high intensity) on the remodeling of left ventricular cardiomyocytes isolated from hypertensive rats. We demonstrate that HIIE training as well as CMIE training induce significant cardiovascular changes not only at the morphological and mechanical levels but also at the molecular level.

Overall we observed improved cardiovascular function through decreased MAP in the trained rats. While the beneficial effects of training on blood pressure had previously been shown in SHR (8, 11, 41, 56) our study shows for the first time that HIIE training also induces a hypotensive effect. To our knowledge, only one study (28) has compared high intensity intermittent and low intensity endurance trainings on hypertensive rats. Contrary to our results, an increase in MAP after 4 weeks of both types of training was observed in this study. Nevertheless, we cannot accurately compare these data with our results because a different hypertensive rat model (Dahl salt rats) was used by the other group, and there is little information on the modalities of performed intermittent training. Another study (4) has shown that high intensity training does not correct hypertension in hypertensive rats (SHR), but the used exercise modalities were continuous rather than intermittent. In humans, the amplitude of hypotensive effects remains variable according to different training arrangements (63), although their existence appears to have led to a consensus. And while the mechanisms underlying post-training hypotension have yet to be fully ascertained, some of them have been suggested. Increased parasympathetic tone of the heart (19, 51) and/or improved vasodilator effect of the endothelial function can occur (5, 26). In our study, rest heart rate reduction after training suggests involvement of sympathetic activity in hypotensive effect mitigation (20). Indeed, mean arterial pressure of SHR-T rats still higher than normotensive rats despite a decrease of values compared with SHR-C, but many other studies with different types of
physical exercise shown same results (29, 32, 38, 39, 62). We also observed an increase in the LVW/BW ratio in hypertensive rats, especially when they were trained. Ventricular hypertrophy had previously been demonstrated in untrained hypertensive rats (45) or those trained with continuous exercises (22). The increased ventricular size induced by physical training, called eccentric hypertrophy, is generally characterized by an increased cardiomyocyte size (addition of sarcomers in series) enhancing cardiac output and meeting energy needs (70). However, in our study we did not observe any effect of exercise training on cardiomyocyte length and we cannot draw a conclusion on the type of hypertrophy. This may be explained by two hypotheses: first, effects differ according to the myocardial region from which the cells come (36); second, pathological hypertrophy, at the morphological level, is preserved in trained hypertensive rats despite improved cardiac function (contractility and calcium transient), as shown by our results and those of Carneiro et al (13). Measurement of cardiac fibrosis percentage is another way to determine the type of hypertrophy. Our results concerning the CVF did not highlight a significant difference between all groups. These results are not in agreement with previous studies showing an improvement of the myocardial fibrosis after exercise training in SHR (22, 39, 50). However, comparison is difficult because Garciaarena et al and Locatelli et al used swimming training and rats are older with fibrosis more developed in study of Pagan and coworkers. On the contrary, Schreckenberg et al (58) showed the development of fibrosis induced by 6 months running training of SHR. Here again, modalities of training were very different: free running wheel with a running distance per week ten times higher than in our study. To our knowledge, two studies have used HIIT training: Brown et al (10) show an improvement of the percentage of cardiac fibrosis and Holloway et al (28) no effect. Unfortunately, models of rats were different from SHR. Taken together the different data are in favor of a no deleterious effect of training on the extent of fibrosis.
Our results demonstrate that hypertension induced a reduction in the index of TT regularity (TTpower) observed in cardiovascular diseases (57, 60, 71). Moreover, this loss of organization was in our experiments clearly mitigated by CMIE or HIIE training. The TT level of organization for trained-rats was found to be intermediate between those of hypertensive and healthy rats. To date, there have been no other data in this field on hypertensive animals, but one study in post-infarct rat model showed an improvement in TT density after 8 weeks of training by continuous exercise at 85-90% of $\dot{V}O_2$max (34). From the TT organization point of view, training seems to delay deleterious evolution of cardiovascular pathologies. Indeed, Wei et al (71) and more recently Shobesberger et al (57) suggested that prevention of TT remodeling (decrease of TT regularity and maintain of the transverse axial tubule system) during hypertrophy may be clinically important in delaying progression toward heart failure.

In addition to the effects observed on the morphology of cardiomyocytes, we found significant changes in calcium homeostasis. Our results indicate that training reduced the deleterious effects of hypertension by decreasing calcium leakage through ryanodin receptor (decrease in spark site density for HIIE) and by improving calcium uptake, resulting in temporal and spatial shortening of sparks (FDHM and FWHM were decreased). To our knowledge, only one study on the effect of training on spontaneous calcium release during hypertension has been published (12). For the four parameters studied previously, however the reported results of this study are contradictory with ours: an increase in amplitude, FDHM, FWHM and peak time after 8 weeks of training was observed. Outside of the rats ages (24 weeks versus 18 in our study), the method of acquisition of sparks may explain this discrepancy. In their experiments, sparks were measured on the cells "at rest" but previously stimulated (1 Hz), and consequently the mechanisms underlying the release and recapture of calcium ions may be different (15, 27).
Knowing that calcium ions play a pivotal role in excitation-contraction coupling, and especially as regards cardiomyocyte contractility, the effects of training on calcium homeostasis may explain the results observed for cardiomyocyte contractile activity. Indeed, increased rates of contraction and relaxation of cardiomyocytes for the two types of training and enhancement in shortening amplitude after HIIE training were recorded. These observations seem to be consistent with data already published. Carneiro and colleagues reported an increase in the maximum rate of contraction and relaxation in SHR rats as a result of continuous training of moderate intensity (11), which is in line with our conclusions for the CMIE group. Another group has demonstrated a reduction in half-relaxation and half-contraction time following HIIE, but it occurred in a heart failure model (73). We also noticed a longer time to half decay in SHR sedentary group but physical training, and particularly HIIE decrease this parameter. A recent study (54), also observed this effect in SHR rats after continuous training at moderate intensity. All these results suggest that training (CMIE and HIIE) induce higher availability of Ca^{2+} in the cytosol and a faster removal of calcium from the cytosol, which in turn leads to relaxation.

In order to control intracellular calcium concentration, numerous proteins (calcium channels, exchangers or pumps), finely regulate intracellular calcium transients and excitation contraction coupling (7, 21). Here we demonstrate that both types of training increased the expression of SERCA and phospholamban phosphorylated at serine 16 (PLBser16) without altering total PLB (PLBtot). These findings are in accordance with other studies (11, 13, 18, 22, 41, 73) using different animal models (SHR, Wistar Kyoto and rat model of myocardial infarction) and training modalities. The effect of training on the expression of these two proteins, which is essential for calcium reuptake by sarcoplasmic reticulum (SR), may explain our results on cardiac calcium homeostasis and contractility. Indeed, SERCA is inhibited when interacting with dephosphorylated PLB and then, PLB phosphorylation causes
activation of SERCA, which allows calcium reuptake (42). When PLBser16 is increased, SERCA becomes more active and more calcium is pumped into the sarcoplasmic reticulum, resulting in a more rapid decrease in intracytosolic calcium. These proteins activations could explain the positive lusitropic effect and the positive inotropic effect observed in the trained groups.

Among the hypotheses that would explain increased PLB phosphorylation, cardiac β-adrenergic responsiveness appears as a credible candidate. It is well known that hypertension impairs cardiac β-adrenergic signaling (1, 49). GRK2 and calcineurin have been shown to be involved in blunting β-adrenergic receptor signaling in pressure overload hypertrophy (16). Moreover, studies in both normotensive and hypertensive animals have shown that exercise training improve β-adrenergic receptor responsiveness (2, 41, 46, 64).

In our study, we have tested the beta-adrenergic responsiveness indirectly through the response of L-type calcium channels to isoproterenol. While β adrenergic responsiveness is altered in hypertensive rats, it appears to be improved by both type of training. This may explain increased PLB phosphorylation. Others studies with different modalities of exercise had also highlighted a decrease of β adrenergic response in SHR model and an improvement of the responsiveness after exercise training. These effects would be due to a change of GRK2 protein expression that would be increased in hypertensive sedentary rats and decrease with training (31, 41).

In summary, even if many aspects remain to be explored, we are proposing on the basis of our experimentation a hypothetical diagram describing the possible mechanisms of intracellular remodeling (Fig.9). In this diagram, three conditions are represented: healthy, hypertensive and hypertensive-trained. We observed no change in expression for SERCA and PLB in hypertensive rats relative to healthy rats (WKY). On the contrary, training clearly
causes overexpression of SERCA and increase of PLBser16, more markedly for HIIE, without PLBtot modification. If the PLBser16 is increased, then SERCA is more active and allows more calcium to be pumped into the SR, resulting in a more rapid decrease of calcium present in the cytosol. We also hypothesized that the improved regularity of the transverse tubules following training could be due to a restoration of the expression of junctophilin-2 (JP-2) which is known to be involved in the remodeling of the internal structures of the cardiomyocytes (43). Indeed, previous studies have shown a link between loss of TT regularity in cardiovascular pathologies such as heart failure, and decreased expression of junctophilin-2 (71). Moreover, this anchoring protein linking the TT and the sarcoplasmic reticulum membranes could play a major role in restoration of efficient calcium cycling. In acute junctophilin knockdown mice, spark density increase (68), changes in their morphology such as increased FDHM, FWHM and amplitude (69), and calcium transient alteration (14) have all been shown to occur. Unfortunately, we rule out this hypothesis because junctophilin expression is not restored after the both types of training. It would be interesting to pursue investigations to understand underlying mechanisms of these improvements.

5. Limitations and future directions

First, our results did not allow us to characterize the type of cardiac hypertrophy observed in hypertensive rats trained or not. Measurements of ANF, skeletal alpha-actin or alpha/beta-MHC (Myosin Heavy Chain) ratio (makers of pathological cardiac hypertrophy) which could have clarified the question of the type of hypertrophy observed. Second, our study was voluntarily mainly focused in cardiac remodeling at the cellular level. So we have not made echocardiographic measurements to observe the effects of both trainings to elucidate the putative interaction between the above cellular observations and left ventricular function. Third, the use of telemetry system, for the measurement of arterial pressure would have been advantageous because data are collected throughout all the experiment. Unfortunately, these
two types of equipment (echocardiograph and telemetry system) were not available in the laboratory.

Our work questioned the effects of both types of training (one of which was HIIE) on left ventricular cardiomyocytes in the early compensated phase of hypertension. We demonstrated that CMIE training and HIIE training mitigate deleterious remodeling of left ventricular cardiomyocytes from structural, functional and molecular standpoint. The question that still arises is HIIE training effects on the transition from compensated cardiac hypertrophy to decompensated heart failure. Echocardiography will make it possible to monitor structural and functional cardiac remodeling induced by HIIE training during the different phases of this transition. This could be the purpose of a future study.

5. Conclusion

Training, whether continuous at moderate intensity or intermittent at high intensity, mitigates the cardiac remodeling induced by hypertension. Furthermore, high intensity intermittent training does not cause any deleterious effects and even seems to improve contractility and some associated calcium mechanisms.

If we extrapolate these results to humans, it seems appropriate to use both types of training, without discrimination, in rehabilitation of patients suffering from cardiac pathologies. In addition to beneficially modifying intracellular mechanisms, HIIE training has a second advantage, a better patient adhesion (3), since it reduces training time and thereby counteracts one of the causes of lack of activity, "lack of time" (23, 67). Our results linked with studies in humans (30) support the idea to not limited the prescription at one modality of training (low or moderate continuous intensity) because HIIE does not cause deleterious
effects. However it’s important to note among other things that HIIE had more effect in the increasing of MRS since performance seems to be a good indicator of mortality risk (35, 65)
Acknowledgments: All microscopy experiments were performed on the ImageUP platform.

We wish to thank the platform Prebios for animal care.

Grants: Amandine Krzesiak was the recipient of a doctoral scholarship from the Poitou-Charentes region (now Région Nouvelle Aquitaine).

Disclosures: The authors have no conflicting interests to disclose in relation to this work.


Figure legends:

Table 1: WKY: normotensive control. SHR-C: hypertensive control. SHR-T CMIE: hypertensive trained with CMIE (continuous moderate intensity exercise). SHR-T HIIE: hypertensive trained with HIIE (high intensity interval exercise). Each rat has 18 weeks at the end of the protocol. BW: body weight. HW: heart weight. LVW: left ventricular weight. CVF: collagen volume fraction. MAP: mean arterial pressure. HR: heart rate. MRS: maximal running speed.* significantly different from baseline value within the same group. § significantly different from hypertensive group (SHR-C). # significantly different from SHR-T CMIE. † significantly different from WKY. Data are mean ± SE *,$,# p ≤0.05, **,$§ p ≤ 0.01,

1: For fibrosis, N= 3 and n= 50 fields in all groups.

**Figure 1:** A: Cell length at rest. B: Cell width at rest. † significantly different from WKY.

Data are mean ± SE * †p ≤ 0.05, ††p ≤ 0.01. WKY: n = 89 N = 4. SHR-C: n = 106, N = 6.


**Figure 2:** A: Crops of representative images of cardiomyocytes with transverse tubules (TT) di-8-ANEPPS staining (Scale bar: 2µm). B: Power versus spatial frequency (c/µm: cycle/µm) from TT staining (from A). Black arrow: see text. C: TT peak power. D: Period of TT network. † significantly different from WKY. Data are mean ± SE, ††p ≤ 0.01, *** †††p ≤ 0.001. WKY: n = 121, N = 4. SHR-C: n= 139, N = 6. SHR-T CMIE: n = 141, N = 6. SHR-T HIIE: n = 121, N = 6.

**Figure 3:** Calcium spark examples (A, B and C) of line scan images (2 ms/line; 512 pixels/line) recorded in SHR-C, SHR-T CMIE and SHR-T HIIE cardiomyocytes. D to F: Plot profile examples of sparks expressed as the intensity of fluorescence (IF) versus time of line scan. G: Spontaneous calcium spark characteristics. For reference, values of WKY:

Amplitude: 0.569± 0.006, FDHM: 13.28± 0.10, FWHM: 1.37± 0.01, Time-to-peak: 10.12± 0.14, Frequency: 0.130±0.005. FDHM: full duration at half maximum. FWHM: full width at half maximum. § significantly different from hypertensive controlled group.* significantly different from SHR-T CMIE. Data are mean ± SE *§p ≤ 0.05 **§§p ≤ 0.01 ***§§§p ≤ 0.001. WKY: n = 3119 sparks, N =4. SHR-C: n= 3427 sparks, N = 6. SHR-T CMIE: n = 4632 sparks, N = 6. SHR-T HIIE: n = 3354 sparks, N = 6.

**Figure 4:** Sequences of images in fast mode were analyzed with a program allowing us to calculate the standard deviation of the recorded fluorescence at each pixel location. A: Images resulting from this analysis. (Scale bar: 40 μm). White arrows indicate some spots of release.
Through that process, the sites were numbered and calcium release site density (number of sites/μm²) was computed. B: Spark site density. C: Calcium wave frequency expressed as the percentage of cells presenting calcium waves. Data are mean ± SE *p ≤ 0.05 **p ≤ 0.01. WKY: n = 3119 sparks, N = 3. SHR-C: n= 3427 sparks, N = 6. SHR-T CMIE: n = 4632 sparks, N = 6. SHR-T HIIE: n = 3354 sparks, N = 6.

**Figure 5:** A: Normalized representative images of calcium transient responses obtained during field-stimulation at 1Hz. B: Peak amplitude of calcium transients C: Time-to-peak. D: Time-to-½ decay. Data are mean ± SE *p ≤ 0.05, **p ≤ 0.01, ns: non-significant. WKY: n = 30, N = 3. SHR-C: n = 30, N = 3, SHR-T CMIE: n = 40, N = 5, SHR-T HIIE: n = 60, N = 4.

**Figure 6:** A: Representative recordings of cardiomyocyte contraction elicited by field-stimulation at 1Hz (Scale bar: 1s). Data are expressed relative to resting sarcomere length (rsl). B: Cardiomyocyte shortening (% of rsl). C: Maximal velocity of shortening. D: Maximal velocity of relengthening. † significantly different from WKY. Data are mean ± SE *†p ≤ 0.05, **†p ≤ 0.01, ***p ≤ 0.001. WKY: n = 30, N = 4. SHR-C: n = 30, N = 3. SHR-T CMIE: n = 40, N = 5. SHR-T HIIE: n = 60, N = 4.

**Figure 7:** Effect of isoproterenol 1μM on beta-adrenergic response on cardiomyocytes ICaL. † significantly different from WKY. Results are expressed as a percentage of increase of the current compared to the state of rest (without isoproterenol). Data are mean ± SE ***:†††p ≤ 0.001. WKY: n = 12, N = 4. SHR-C: n= 12, N = 4. SHR-T CMIE: n = 12, N = 4. SHR-T HIIE: n = 12, N = 4.

**Figure 8:** Calcium regulatory proteins. A: Representative western blot of SERCA2a, Phospholamban, Junctophilin-2 and GAPDH. B: SERCA2a expression relative to GAPDH. C: Total phospholamban expression relative to GAPDH. D: Phospholamban phosphorylated at serine 16 expression relative to total phospholamban. Junctophilin-2 expression relative to
GAPDH is not presented in the figure because there is no expression for the three groups of hypertensive rats. † significantly different from WKY. Data are mean ± SE normalized by SHR-C * † p ≤ 0.05, ** †† p ≤ 0.01. WKY: n = 8, N = 4. SHR-C: n = 6, N = 3. SHR-T CMIE: n = 6, N = 3. SHR-T HIIE: n = 8, N = 4.

**Figure 9:** Diagram summarizing observed effects of arterial hypertension and combined arterial hypertension training on some intracellular mechanisms. TT: transverse tubule, LTCC: L-type calcium channel, P: phosphorylation, JP2: junctophilin-2, Ca2+: calcium, SERCA: sarco/endoplasmic reticulum calcium ATPase, PLB: phospholamban, SR: sarcoplasmic reticulum, PKA: protein kinase A activated (act) or inactivated (ina), AMPc: cyclic adenosine monophosphate, ATP: adenosine triphosphate, AC: adenylyl cyclase. β2 adr: beta 2 adrenergic receptor.
| Table 1: Morphometric data, cardiovascular parameters and physical capacity. |
|-----------------------------------------------|--------------|-----------------|-----------------|-----------------|
|                                | WKY         | SHR-C           | SHR-T CMIE      | SHR-T HBE       |
| Initial BW (g)                 | 246 ± 11    | 237 ± 18        | 243 ± 13        | 251 ± 27        |
| Final BW (g)                   | 411 ± 5**   | 384 ± 8**       | 362 ± 8**       | 357 ± 12**      |
| HWBW (mg/g)                    | 4.44 ± 0.391 | 5.61 ± 0.241    | 6.47 ± 0.126**  | 6.66 ± 0.201**  |
| LVW/BW (mg/g)                  | 2.82 ± 0.073 | 3.74 ± 0.111111 | 4.48 ± 0.125**  | 4.36 ± 0.125**  |
| CVF (%)                        | 4.29 ± 1.63 | 5.03 ± 1.06     | 5.14 ± 1.07     | 4.58 ± 1.67     |
| MAP (mmHg)                     | 92.3 ± 2.2   | 145.4 ± 4.5111  | 119.4 ± 2.725** | 130.8 ± 2.85111 |
| HR (bpm)                       | 320.7 ± 9.8  | 345.2 ± 6.8     | 311.6 ± 5.6111  | 317.1 ± 6.111   |
| Initial MRs (mmHg)             | /            | 29.2 ± 2.5      | 30.7 ± 1.9      | 30.2 ± 2.8      |
| Final MRs (mmHg)               | /            | 24.5 ± 2.5**    | 37.1 ± 4.9**    | 40.3 ± 6.8**    |
Figure 2

A

WKY  
SHR-T CMIE  
SHR-T HIIE  
SHR-C

2μm

B

Relative power (A.U.)

C

TT Power (A.U.)

D

TT Period (μm)

WKY  SHR-C  SHR-T CMIE  SHR-T HIIE
Figure 3

- **A** SHR-C
- **B** SHR-T CMIE
- **C** SHR-T HIIE

### Table

<table>
<thead>
<tr>
<th>Metric</th>
<th>SHR-C</th>
<th>SHR-T CMIE</th>
<th>SHR-T HIIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (A.U.)</td>
<td>0.568 ± 0.005</td>
<td>0.572 ± 0.004</td>
<td>0.488 ± 0.005</td>
</tr>
<tr>
<td>FDHM (ms)</td>
<td>15.50 ± 0.16</td>
<td>13.04 ± 0.18</td>
<td>13.55 ± 0.13</td>
</tr>
<tr>
<td>FWHM (μm)</td>
<td>1.47 ± 0.01</td>
<td>1.42 ± 0.011</td>
<td>1.38 ± 0.016</td>
</tr>
<tr>
<td>Time to peak (ms)</td>
<td>12.84 ± 0.21</td>
<td>11.12 ± 0.19</td>
<td>10.59 ± 0.26</td>
</tr>
<tr>
<td>Frequency (sparks/s)</td>
<td>0.146 ± 0.006</td>
<td>0.133 ± 0.004</td>
<td>0.115 ± 0.007</td>
</tr>
</tbody>
</table>

Downloaded from www.physiology.org/journal/jappl at Macquarie Univ (137.111.162.020) on February 12, 2019.
Figure 4
Figure 6
Figure 7