Mechanisms underlying cardioprotection induced by exercise training in animal models of acute and chronic cardiac pressure overload

Daniel Moreira Gonçalves
Porto, 2011
University of Porto
Faculty of Sport
Research Centre in Physical Activity, Health and Leisure (CIAFEL)

Mechanisms underlying cardioprotection induced by exercise training in animal models of acute and chronic cardiac pressure overload

Academic dissertation submitted with the purpose of obtaining a doctoral degree in Physical Activity and Health, according to the Law 74/2006 from March 24th.

Supervisor:
Professor José Alberto Ramos Duarte
Faculty of Sport, University of Porto, Porto, Portugal

Co-Supervisor:
Professor Adelino Leite Moreira
Faculty of Medicine, University of Porto, Porto, Portugal

Daniel Moreira Gonçalves
Porto, 2011
Moreira-Gonçalves D (2011). Mechanisms underlying cardioprotection induced by exercise training in animal models of acute and chronic cardiac pressure overload. Academic dissertation submitted with the purpose of obtaining a doctoral degree in Physical Activity and Health. Faculty of Sport, University of Porto, Portugal.

**Key words:** Exercise training; mechanisms of cardioprotection; acute and chronic pressure overload; cardiac tolerance
FUNDING SOURCES

The author of this thesis was supported by an individual grant from the Portuguese Foundation for Science and Technology (SFRH / BD / 33123 / 2007).

The experimental studies included in this thesis were supported by a grant from the Portuguese Foundation for Science and Technology (PTDC/DES/104567/2008).

This dissertation was conducted in the Research Centre in Physical Activity, Health and Leisure (CIAFEL), a research unit housed in the Faculty of Sport, University of Porto, Portugal.
To my parents
and my brother
ACKNOWLEDGMENTS

“Coming together is a beginning; keeping together is progress; working together is success”

Henry Ford

The four years that I dedicated to this work were abundant of battles against disappointment and frustration like I never felt before. I was fortunate to have several persons that walked by my side during this long journey and helped me to transform those moments in joy, victory and success. There are no words capable to express how much grateful I am to all of you, who made this thesis possible:

To my supervisor Professor José Alberto Duarte, I wish to express my most sincere gratitude for your scientific guidance, support and growing friendship. Your critical spirit, vast scientific knowledge and work capacity were encouraging for me. I hope to be always capable to correspond to the expectations and trust that you deposited on me.

To my co-supervisor Professor Adelino Leite Moreira, thank you so much for the opportunity you gave me almost 8 years ago to initiate research in cardiovascular physiology with your group. Your scientific advise, trust, enthusiasm and endless supply of positive reinforcement were definitely crucial for me. I’m eternally thankful.
To Tiago HenRIques Coelho, I truly appreciate your dedication, enthusiasm and promptness in all moments of this work (even when you were almost without time to breath). Your competence, modesty and integrity are really inspiring for me. Definitely, you are an example to follow. Thank you for everything my friend.

To Hélder, my “brother in arms”, with who I shared so many concerns during this long journey. I’m sincerely grateful for all your valuable help, support and encouraging words when I most needed. You were always an example of dedication and amazing intellectual shrewdness. I hope that the “osteoblasts” of our friendship remain highly active for the coming years.

To Rita Ferreira, I am eternally tankful for your friendship and for what you have done for me and for this work. You are certainly one of the most enthusiastic persons that I ever met. I really do not know how, but I hope that someday I can return to you the precious help that you provided to me.

To Professor Scott Powers, from the University of Florida, thank you so much for hosting me in your lab, I enjoyed every moment that I spent there. To meet you in person was the concretization of a dream. It was amazing to realize that behind such a big name was an incredibly humble person.

To Miss Celeste Resende, a million thanks for what you have been for me since I was a grad student in 2002. Your contribution as a lab technician and assistance with animals’ care and training was priceless. For several times you were my
partner, confidant and teacher (and always ready to cover my backs) but most importantly, you became a friend for life.

To Nádia Gonçalves and Inês Pires, for your true friendship, promptness in helping me with everything that was on your reach and for the countless time that we spent together in the hemodynamic room. I truly appreciate every single minute that you dedicated to listen to me… every word of incitement…every single encouraging gesture.

To Teresa Baltazar and Ana Oliveira, I’m grateful for the friendship that we have been developing over these years and for the priceless support and motivation that you both provided to me. Thanks for worrying so much about me.

To Professor José Oliveira, for our long conversations on his office at the end of the day, valuable advices and suggestions, positive reinforcements and friendship.

To Ana Isabel Padrão, your help was determinant and I hope to be able to return all the time and effort that you dedicated to this work. I’m so sorry because of those awkward names that I used to label the samples.

To Sara Vieira, the best trainee in the world. Your readiness to help me in every task that you had chance was amazing.

To Rodney Paixão, Joana Fonseca and Carla Santos for their valuable help with animals’ training.
To my friends Alberto Alves, Fernando Ribeiro, Ana Carvalho, Eduardo Teixeira, Norton Oliveira, Lucieli Cambri, Eliane Dallegreve, and Ricardo Arantes, thanks for those exceptional moments during our lab “scientific meetings”.

To Dra. Antónia Teles, Mrs. Armando Jorge, Miss Rosinha, Miss Francelina, Miss Laurinda, Marta Oliveira, Mizé Mendes, Manuel Pinto, Marina Neto, Mário Santos, Silvia Oliveira and Francisco Nóvoa, from the Department of Physiology and Cardiothoracic Surgery, Faculty of Medicine from the University of Porto, thanks for the great working relationship and environment.

To the technical staff from the Faculty of Sport, namely to Mrs. Rui Biscaia, Mrs. Fernando Marinho, Miss Ana, Miss Tina, Miss Mafalda, Miss Virgínia, Dr. Pedro Novais, Dr. Nuno Reis, Miss Manuela Santos, Miss Manuela Guimarães, Miss Teresa Santos, Miss Carla Pereira, Miss Carla Dias and Mrs. João, I’m grateful for your friendship, our long conversations and your promptness in providing any support.

To my great friend André Soares, your incessant support, pragmatic advices and words of incitement were very important.

To my friend António Quintela, thanks for earing my concerns, your constant support and motivation were determinant mainly during the writing process of this thesis.
To João Coelho André Teixeira, Hélder Fonseca and Joaquim Fontoura, for their friendship and comprehension when I could not be present.

And finally…
Para os meus pais e irmão, a quem dedico todo este percurso. Como sempre, vocês foram incansáveis no apoio e motivação constante ao longo destes anos. Sem vocês ao meu lado, jamais teria conseguido. Agradeço-vos profundamente a vossa compreensão (reconheço que não fui fácil) e peço-vos desculpa pelos dias de mau humor e pelas presenças físicas acompanhadas de pensamento distante. É a vocês que devo tudo o que sou hoje. Obrigado.
1 INTRODUCTION

1.1 Modulation of cardiac phenotype by load

1.1.1 The duration and intensity of the stimuli as possible determinants of the divergent phenotypes 2

1.1.2 Mechanisms underlying cardiac adaptive and maladaptive phenotype 6

1.1.2.1 Cardiomyocyte 6

1.1.2.1.1 Growth pathways 7

1.1.2.1.2 Myosin heavy chain 10

1.1.2.1.3 Excitation-contraction coupling disturbances 12

1.1.2.1.4 Energy and metabolism 13

1.1.2.1.5 Death and renewal 14

1.1.2.2 Extracellular Matrix Remodeling 17

1.1.2.3 Cardiac vascularity 18

1.2 Exercise training-induced cardiac protection 19
1.2.1  Brief historical perspective  19
1.2.2  Exercise training and cardiac tolerance to pressure overload  21

2 AIMS  25

3 RESULTS  27

   Paper I: Moderate exercise training provides left ventricular tolerance to acute pressure overload  29

   Paper II: Intermittent beta-adrenergic stimulation mimics exercise-induced cardiac phenotype and protects against left ventricular pressure overload insult  41

   Paper III: Cardioprotective effects of exercise training at different time points during the development of experimental pulmonary arterial hypertension  85

4 GENERAL DISCUSSION  129

5 MAIN CONCLUSIONS  137

6 REFERENCES  139
RESUMO

A sobrecarga cardíaca tem um efeito determinante na modulação do fenótipo cardíaco. Em resposta a um aumento sustentado da carga, o coração desenvolve um conjunto de adaptações inicialmente compensatórias que visam normalizar a tensão imposta sobre a parede ventricular e garantir a perfusão de órgãos vitais. No entanto, se a tensão persistir de forma sustentada, poderá ocorrer uma resposta descompensatória, que poderá levar ao desenvolvimento de insuficiência cardíaca. Por sua vez, a sobrecarga cardíaca intermitente imposta pelo exercício físico induz um conjunto de adaptações compensatórias que parecem conferir proteção contra inúmeros estímulos deletérios. Neste sentido, o principal objetivo do presente trabalho foi verificar se o exercício físico crônico seria capaz de aumentar a tolerância cardíaca à sobrecarga de pressão aguda (estudos I e II) e crónica (estudo III). Adicionalmente, pretendeu-se averiguar se a aplicação de um estímulo de natureza diferente à do exercício físico (estimulação com dobutamina), mas capaz de mimetizar algumas das suas características hemodinâmicas, nomeadamente a duração e magnitude da sobrecarga, poderia resultar igualmente num fenótipo cardioprotetor (estudo II). Os estudos I e II incidiram sobre o ventrículo esquerdo (VE) enquanto que o estudo III se debruçou sobre o ventrículo direito (VD). Os resultados destes estudos indicam que o exercício físico crônico previne a disfunção cardíaca induzida pela sobrecarga aguda de pressão, acompanhada por uma redução da lesão ultra-estrutural, da expressão da forma ativa da caspase-3 e do Nf-KB, bem como de menores níveis de dano oxidativo. A sobrecarga crónica intermitente induzida pela estimulação beta-adrenérgica com dobutamina também protegeu o VE contra a sobrecarga aguda de pressão e mimetizou em vários aspectos o fenótipo protetor induzido pelo exercício físico. Relativamente à sobrecarga crónica contínua, os dados obtidos sugerem que o
precondicionamento com exercício físico, assim como o exercício realizado durante ou após o estabelecimento da sobrecarga crónica de pressão sobre o VD, previne a disfunção cardíaca e aumenta a sobrevida. Esta melhoria parece estar associada à normalização de alterações na cinética do cálcio e da transição da expressão da isoforma das cadeias pesadas de miosina alfa para beta, redução da ativação neurohumoral, deposição de colagénio e inflamação, preservação da funcionalidade mitocondrial e diminuição do dano oxidativo. Como conclusão geral, os resultados do presente estudo sugerem que o exercício físico crónico aumenta a tolerância à sobrecarga aguda e crónica de pressão, previne a disfunção e diminui a probabilidade de desenvolvimento de insuficiência cardíaca.

**Palavras Chave:** exercício físico crónico; mecanismos de cardioprotecção; sobrecarga de pressão aguda e crónica; tolerância cardíaca.
ABSTRACT

Cardiac overload is considered an important modulator of the cardiac phenotype. In response to sustained cardiac overload, the heart develops a series of compensatory adaptations in order to normalize wall stress and guarantee the perfusion of vital organs. However, this seems to be only a short-term solution once if the stress is sustained, a decompensatory response occurs and HF will develop. In contrast, the intermittent cardiac overload induced by exercise results in several compensatory adaptations that translate into an improved cardiac phenotype that provides cardiac protection against several cardiac insults. In this sense, the main purpose of the present work was to address whether exercise training could enhance the ability of the heart to support acute (studies I and II) and chronic (study III) pressure overload and thus, prevent cardiac dysfunction and failure. Additionally, we evaluated if a stimulus, other than exercise (dobutamine stimulation), but mimicking the duration and magnitude of the exercise-induced cardiac overload, could similarly induce a cardioprotective phenotype (study II). In studies I and II the focus was on the left ventricle (LV) while in study III it was on the right ventricle (RV). Our results show that exercise training may prevent cardiac dysfunction induced by acute pressure overload, an observation that was paralleled by reduced ultra-structural damage, decreased expression of the active form of caspase-3 and NF-kB, and lower levels of oxidative damage. Chronic intermittent overload by beta-adrenergic stimulation with dobutamine also protected against acute pressure overload induced injury, and mimicked several aspects of the cardioprotective phenotype induced by exercise training. Regarding chronic pressure overload, our findings indicate that exercise preconditioning, as well as exercise performed during or after the establishment of RV chronic pressure overload prevents dysfunction and enhances survival. This
improved outcome was associated with normalization of calcium handling disturbances, alpha to beta-MHC expression shift, decreased neurohumoral activation, collagen deposition and inflammation, and preserved mitochondrial function and oxidative damage. The overall conclusion of our work is that exercise training increases the tolerance to both acute and chronic pressure overload, and may prevent from cardiac dysfunction and failure.

**Key Words:** Exercise training; mechanisms of cardioprotection; acute and chronic pressure overload; cardiac tolerance
LIST OF ABBREVIATIONS

Akt: protein kinase B
ANP: atrial natriuretic peptide
Ang-II: angiotensin-II
ATP: adenosine triphosphate
ADP: adenosine diphosphate
BNP: brain natriuretic peptide
CVD: cardiovascular disease
CSC: cardiac stem cell
CaMKII: calmodulin-dependent protein kinase II
DNA: deoxyribonucleic acid
ET-1: endothelin-1
ECM: extracellular matrix
ERK: extracellular signal-regulated kinases
GSK: glycogen synthase kinase
GPCR: G-protein coupled receptors
GH: growth hormone
HF: heart failure
iTAC: intermittent transverse aortic constriction
IGF-1: insulin-like growth factor-1
IL: interleukin
I-R: ischemia-reperfusion
JNK: c-Jun amino-terminal kinase

LV: left ventricle

LTCC: L-type calcium channels

MMP: metalloproteinase

MURF1: muscle specific ring finger protein 1

MHC: myosin heavy chain

MAPK: mitogen activated protein kinase

MI: myocardial infarction

m: meters

min: minutes

mTORC2: mammalian target of rapamycin complex 2

NFAT: nuclear factor of activated T cells

NCX: sodium/calcium exchanger

OPN: osteopontin

PI3K: phosphoinositide 3-kinase

PDK1: phosphoinositide-dependent kinase-1

PLN: phospholamban

RV: right ventricle

RyR: ryanodine receptors

RNA: ribonucleic acid

Ser: serine

SERCA2a: sarcoplasmic reticulum calcium-ATPase

Thr: threonine
TGF-beta: transforming growth factor-beta

TNF-alpha: tumor necrosis factor-alpha

TIMP: tissue inhibitor of metalloproteinases

VEGF: vascular endothelial growth factor
1. Introduction

1.1. Modulation of cardiac phenotype by load

The heart has a remarkable adaptive ability, allowing it to continuously adjust its function to different challenges imposed by diverse stimuli throughout the life span (95, 207, 241). In order to respond to these continuous challenges the heart can reversibly adapt its function by activating intracellular signaling cascades, mainly anchored in the beta-adrenergic system (242). Under prolonged demands, its ability to maintain cardiac function within a physiological/homeostatic range is limited by restricted boundaries, which when surpassed will result in a maladaptive phenotype. This can be illustrated by the chronic (but transient) elevation of workload imposed to the heart by exercise training or by the chronic (but sustained) overload imposed by a disease state (e.g. pulmonary or systemic hypertension, valve dysfunction). In both circumstances, the heart will develop hypertrophy, a compensatory adaptation thought to provide mechanical advantages as it normalizes wall stress and decreases oxygen consumption, but ultimately divergent fates will occur (73, 241). In fact, participation in regular exercise is related with the development of mild to moderate left ventricular hypertrophy accompanied by enhanced cardiac performance (18, 34, 169, 189, 215). Importantly, no signs of deterioration in cardiac function or occurrence of cardiovascular symptoms or events, were detected even after long periods of time (up to 17 years) of uninterrupted and intense training (206), though this is currently a topic of debate. On its turn, hypertrophy developed in response to an overloading disease setting is commonly recognized as a major independent risk factor for morbidity and mortality (153, 174) and strong data collectively provide evidence that modulating the hypertrophic growth of the heart ameliorates both left and right ventricular dysfunction (20, 52, 59, 73, 196, 272). Indeed, if the inciting stimulus is
not relieved, the initially compensatory hypertrophy progresses to heart failure (HF) through a series of molecular, cellular, and interstitial changes that remain poorly understood (60, 73, 172, 184, 241). The recognition of a continuous progression from compensated hypertrophy toward HF substantiates the interpretation of cardiac hypertrophy as an early therapeutic target (52, 73, 207).

1.1.1. The duration and intensity of the stimuli as possible determinants of the divergent phenotypes

The reason why certain stimuli promote an adaptive cardiac phenotype while others originate a maladaptive one remains unknown. For a long time, it has been considered that the duration of the overload was determinant, since physiological overloads such as exercise are intermittent, while pathological overloads such as hypertension are sustained (207). In order to address whether the maladaptive phenotype is determined by the nature of the stress rather than its duration, Perrino and collaborators (207) developed a mouse model of intermittent transverse aortic constriction (iTAC) that allowed to deliver pressure overload, transiently and reversibly. iTAC was induced for 90 minutes, two times per day, which was the duration and frequency of the swimming protocol that was used to induce adaptive remodeling. Comparison of the resultant phenotypes revealed mild hypertrophy with preserved systolic function and fetal gene expression in the iTAC group that resembled the exercised group. Nevertheless, iTAC also developed diastolic and beta-adrenergic dysfunction, cardiomyocyte apoptosis and vascular rarefaction (207). It was therefore proposed that it is the nature of the stimuli (physiological vs. pathological), and not its duration, the responsible for triggering maladaptation. However, the magnitude of the overload was not controlled in that experiment and
thus, the observed disturbances may fairly be a consequence of the cumulative damage induced by the severity of each episode of overload. Remarkably, it seems that exercise is not always favorable and may indeed be harmful if performed above certain limits. Recent human data suggest that the right ventricle (RV), but apparently not the left ventricle (LV) (206), may develop ventricular dysfunction, fibrosis and arrhythmias as a consequence of extreme exercise regimens (13, 42, 61, 141, 186, 192, 261, 263). These effects may be the result of cumulative and consecutive prolonged bouts of exercise. Indeed, some studies show that extreme exercise such as marathon or ultra-marathon running is associated with transient RV dilation and dysfunction, as well as with the release of several biochemical markers of cardiac injury such as brain natriuretic peptide (BNP) and cardiac troponin T (186, 192, 201). The decline in cardiac function in response to prolonged acute intense exercise (150 minutes at 80% of maximal oxygen consumption) has also been associated with decreased beta-adrenergic sensitivity in trained individuals (10). It is interesting to note that these alterations were reported in endurance athletes, who are the more susceptible athletes to the development of overtraining, a syndrome that results from an imbalance between excessively great volumes of training without sufficient rest and recovery between each exercise session, ultimately affecting athletic performance (166). Additionally, increased apoptotic markers, metalloproteinase (MMP)-9 activity and mitochondrial DNA damage were reported in the LV of rats after running a bout of exercise until exhaustion (treadmill running with 10% grade at a speed of 30m/min) (107). The subsequent repetition of bouts inducing such alterations may induce cumulative damage. For instance, it was shown that rats submitted to sustained intensive exercise training (16 weeks, 5 days/week, 60 min/day, 36 m/min) developed features of maladaptive remodeling in the RV such as cardiac fibrosis (and
elevated pro-fibrotic mediators), cardiac dysfunction and increased susceptibility to arrhythmia (13). Of note, these changes were reversed after cessation of exercise training. In another report, rats were submitted to 6 weeks of prolonged (stepwise increased, reaching a maximum of 2h20min per day in the 4th week) and intense exercise (35 m/min) (119). At the end, animals presented reduced exercise capacity and evidences of degeneration of the cardiomyocyte structure, such as myofilaments degradation, cellular swelling, appearance of peroxisomes, and decreased rate of oxidative phosphorylation (119). Whether these changes affected cardiac function is unknown since no hemodynamic data was presented. Also, strenuous exercise (90min/day at 26.8 m/min on a 15% slope treadmill, 5days/week, for 7 weeks) has been shown to induce cardiomyocyte growth with little or no growth adaptation of the capillary vasculature, as well as an increase in the average maximum distance from the capillary wall to the mitochondria of cardiomyocytes, possibly compromising oxygen delivery and diffusion (4, 5). In face of these evidences, its seems reasonable to speculate that the exercise benefits may be “dose-dependent”, with elevated “doses” of endurance exercise eventually leading to deleterious cardiac adaptations in the long term (77). These observations claim for confirmation with more studies in order to verify whether “too much of a good thing” is actually bad/deleterious (140). In the meantime, the amount and intensity of exercise to reach such potential “overdose” level is far from representing a threat for the great majority of the population since they do not even meet the minimal amount of exercise recommended by the guidelines (77). Altogether, these data suggest that cardiac adaptive or maladaptive phenotype can be a consequence of the severity and/or duration of the stimuli together with an improper recovery between exercise bouts. When the imposed stress is too severe or prolonged, the cells might not be able to recover
Figure 1- Progression from normal to adaptive and maladaptive remodeling. In response to an elevated workload, a series of compensatory adaptations are triggered in order to preserve myocardial structural and functional integrity. If the workload is sustained (e.g. induced by strenuous exercise or cardiac diseases) the initially balanced and adapted phenotype progressively develops structural and functional disturbances that lead to cardiac maladaptation and dysfunction.

homeostasis, their integrity can be compromised and cellular death pathways might be favored, progressively contributing to maladaptation (40, 75, 137, 138, 171). Even the regenerative capacity recently recognized to the heart may be impaired in this situation, with cardiomyocyte death exceeding renewal, further compromising cardiac recovery (152, 170, 252). On its turn, if there is a perfect match between the stress demands and the cellular ability to cope with it, pro-survival pathways are preferentially activated and an improved homeostatic capacity (increased tolerance) can be attained (40). Thus, the cell’s lack of an appropriate recovery period, together with a progressive or sustained elevated functional demand, may be the main reason
why stimuli like hypertension or aortic stenosis (and also prolonged exercise) lead to cardiac dysfunction, whereas intermittent cardiac overloads during repeated shorter bouts of mild, moderate and intense exercise (and perhaps the early phases of increased pressure and volume overload) promote an adaptive phenotype. These ideas are illustrated in Figure 1.

1.1.2. Mechanisms underlying cardiac adaptive and maladaptive remodeling

Great efforts have been made to identify the basic mechanisms that differentiate adaptive from maladaptive remodeling in order to promote the former and avoid/modulate the latter. Cardiac remodeling is thought to encompass modifications at the level of cardiomyocyte, vascularity and extracellular matrix components of the myocardium (85, 115).

1.1.2.1. Cardiomyocyte

The normal adult myocardium is composed of billions of cardiomyocytes which are characterized by structural and functional heterogeneity that becomes more obvious when the heart is challenged by demanding situations (170, 194, 204, 223). In response to the same amount of stimuli, some cardiomyocytes may experience significant homeostatic disruption and damage with subsequent elimination by cellular death processes when tolerance limits are exceeded [reviewed by references (53, 75, 259)]. Until a certain point, the heart compensates this loss with the formation of new cardiomyocytes (11, 62, 121, 122). The surviving cardiomyocytes may present an enhanced function, at least temporally, through a series of intrinsic compensatory adaptations.
1.1.2.1.1. Growth pathways

The growth of cardiomyocytes is characterized by the activation of complex signaling pathways, some of which have been identified and associated to the development of an adaptive or maladaptive phenotype [reviewed by references (16, 57, 74, 104)]. Activation of the insulin-like growth factor (IGF)-1/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is considered a hallmark of adaptive growth of cardiomyocytes, typical from normal postnatal development or exercise training (16, 57, 129, 195). Growth factors such as IGF-1 and insulin bind to their membrane-bound tyrosine kinase receptors, activating PI3K (p110alpha), which phosphorylates phosphatidylinositol bisphosphate to create phosphatidylinositol triphosphate (57, 127, 262). Phosphatidylinositol triphosphate activates Akt through its recruitment to the cell membrane and its phosphorylation at Thr^{308} and Ser^{473} by phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), respectively (36, 57). Akt then stimulates protein synthesis by activating mTOR and inhibiting glycogen synthase kinase (GSK) (36, 57). Activation or restoration of this pathway has been associated with enhanced contractile function and improved calcium kinetic (35, 76, 129, 176), enhanced angiogenesis, glucose uptake, proliferation and anti-apoptotic effect (17, 22, 36) and expression of genes such as GATA4, cardiac troponin I, and alpha-myosin heavy chain (22). Apparently, these benefits are only present when this pathway is transiently activated since long-term activation of IGF-1 or Akt lead to extensive cardiac hypertrophy, increased expression of atrial natriuretic peptide (ANP) and beta-myosin heavy chain, interstitial fibrosis and cardiac dysfunction (36, 50, 226).

On its turn, the binding of hormones/vasoactive factors such as angiotensin (Ang)
endothelin- (ET) 1 and catecholamines to their receptors on the cardiomyocytes [G protein-coupled receptors (GPCR)], and sustained activation of the downstream intracellular signaling pathways, has been classically linked with maladaptive growth [reviewed by references (16, 57, 74, 104)]. Interestingly, exercise transiently increases cardiac expression of ET-1 (111) and circulating levels of catecholamines (84) but this does not result in maladaptation, further suggesting that sustained (but not intermittent) exposure may be a key factor. Of these pathways, the calcium–calmodulin–dependent phosphatase calcineurin has been identified as a potent hypertrophic promoter as it seems to be sufficient, and in many cases necessary, to induce maladaptive growth and dysfunction (180). Data from human studies also support the involvement of calcineurin in the development of HF (155, 219). Calcineurin is triggered by the sustained elevation of intracellular calcium, for instance resultant from inositol triphosphate-mediated calcium release or from sarcoplasmic reticulum calcium-ATPase (SERCA2a) failure (57, 229, 262). Once activated, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT). NFAT is normally hyperphosphorylated and sequestered in the cytoplasm, but is rapidly translocated to the nucleus after calcineurin-mediated dephosphorylation (16, 95, 127). In the nucleus, it is thought to trigger the expression of pro-hypertrophic genes usually associated with maladaptive remodeling. Genes specifically regulated by NFAT in cardiomyocytes are still under investigation (41) but NFAT3c, one of the five NFAT isoforms, was recently shown to directly increase the expression of the miR-23a, a pro-hypertrophic microRNA (156). This microRNA favors cardiomyocyte growth by suppressing the translation of the muscle specific ring finger protein 1 (MuRF1), which is an anti-hypertrophic factor (156). NFAT can also increase gene expression of ET-1 and BNP by interacting with other transcription
factors such as GATA4 (37, 183). Contrasting to pathological settings of HF, the calcineurin/NFAT pathway is not activated in response to growth hormone (GH)-IGF-1 or exercise training-induced cardiac remodeling (262). Moreover, its inhibition was shown to be paralleled with improved cardiac function, supporting its involvement in maladaptation (127, 197).

The reason why certain pathways seem to be preferentially activated in opposition to others in response to a stimulus remains intriguing. One possible explanation may be related with the magnitude of the overloading stimuli and to its impact on the overall population of cardiomyocytes, independently of the overloading cause. Cardiomyocytes are heterogeneous (194, 223), have distinct injury thresholds and thus their response may be conditioned by their ability to tolerate the overload, independently of the cause. Those cardiomyocytes who experience the greater homeostatic imbalances, lesions and damage to proteins, DNA, and membranes will probably die or exhibit greater and/or prolonged activation of certain signaling mediators in comparison to those with greater tolerance. For instance, those cardiomyocytes experiencing greater calcium kinetic deregulation (e.g. due to oxidative damage or energetic failure) will exhibit marked activation of calcium-induced calcineurin pathway (180). If significant amounts of cardiomyocytes are lost for example by necrosis, the surrounding cardiomyocytes and extracellular matrix will be more susceptible to the influences of inflammatory cytokines (102, 111). If the overloading stimulus results in prolonged activation of the sympathetic system, the sustained elevated levels of circulating catecholamines may favor the chronic activation of the cardiac beta-1-adrenergic receptors, and consequently, apoptosis (84, 165). Therefore, the signaling pathways that are detected to be more up-regulated in a certain biochemical assay may not be stimuli-specific per se, but rather a reflection of
the severity of the stimuli and of the different susceptibility of the overall cardiomyocyte population to the stimuli. Such understanding has been difficult by the fact that the great majority of studies use homogenates of the entire, or parts, of the cardiac muscle, which does not allow the subtlest changes to be detected. Moreover, this methodological approach compromises the understanding of important aspects such as the origin of the transcripts or proteins (relative contribution from cardiomyocytes and non-cardiomyocytes), how many cardiomyocytes participate in the response to stress (generalized response or specific to certain regions of the heart), what type of cardiomyocytes express a certain transcript (e.g. young and/or old, mononucleated and/or multinucleated, more or less damaged cardiomyocytes), or even if the same cardiomyocyte co-express multiple genes at the same time.

1.1.2.1.2. Myosin heavy chain isoforms

The ability of the heart to eject blood is highly dependent on myocardial shortening velocity, a propriety largely determined by its myosin heavy chain (MHC) isoforms composition (88). Two distinct isoforms, alpha and beta, are expressed in the mammalian heart. The rodent adult heart expresses predominantly alpha-MHC (>90%), whilst humans express mainly beta-MHC (>95%) (164, 178). While alpha-MHC is associated with a higher adenosine triphosphatase (ATPase) activity and enhanced shortening velocity, beta-MHC is slower but capable to generate the same cross-bridge force at a lower energetic cost (135, 178, 191). Developmental stage, thyroid status and exercise training or chronic work overload induced by disease settings, all alter MHC composition. For example, exercise training generally induces an up-regulation of alpha-MHC in rats (118, 214), though increased beta-MHC has also been reported without any compromise to cardiac function (109). On its turn,
data from human and animals studies suggest that cardiac hypertrophy induced by pathological settings such as long-term hypertension or myocardial infarction is accompanied by increased expression of the slower MHC isoform (88, 97, 110, 118, 164, 178, 190, 191). Recently, it was shown that only a minority of cardiomyocytes, located in specific regions such as in the base of the LV and tips of the papillary muscle from mice, express beta-MHC in response to chronic pressure overload (162). Interestingly, these cardiomyocytes were smaller than those not expressing the beta isoform, challenging the current view of beta-MHC as a marker of maladaptive cardiomyocyte hypertrophy (162).

It has been proposed that a shift from alpha- to beta-MHC might be an adaptive response as this isoform is energetically more efficient and thus preserves energy (97, 106). However, overexpression of beta-MHC in transgenic mice failed to prevent cardiac dysfunction under chronic isoproterenol challenge or in a post-infarction failure model (134). Remarkably, these mice tolerated exercise training without any sign of maladaptation (134). On its turn, transgenic rabbits expressing alpha-MHC were protected from tachycardia-induced cardiomyopathy (114) but not from myocardial infarction or LV pressure overload-induced HF (113). While the impact on cardiac function of MHC isoforms manipulation remains poorly understood, improvement of cardiac function has been constantly associated with a coordinate increase in alpha- and a decrease in beta-MHC in the rat heart (96, 110, 134). Therefore, it remains to be demonstrated if the change in MHC isoform is a cause or a consequence of HF, or if it merely results from the commitment of newly formed cardiomyocytes (152) and is only transitorily maintained while the new cardiomyocyte matures. This interpretation is reasonably sustained by the fact that: i) beta-MHC is increased in maladaptive remodeling (88, 97, 110, 118, 164, 178, 190,
191), ii) differentiation of cardiac stem cells (CSC) into cardiomyocytes is also increased (3, 11, 121, 122, 152, 170, 252), iii) smaller cardiomyocytes, and apparently not larger, express beta-MHC (162) and iv) smaller cardiomyocytes are though to be younger than larger cardiomyocytes (223).

1.1.2.1.3. Excitation-contraction coupling disturbances

Calcium homeostasis has a major role in the process of contraction and relaxation (excitation-contraction coupling). Depolarization of the cardiomyocyte membrane leads to entrance of calcium to the cytosol through the opening of L-type calcium channels (LTCC), triggering further calcium release from the SR via ryanodine receptor (RyR). Intracellular calcium then binds to troponin C in the myofilaments and initiates contraction (19, 126, 264). Subsequent relaxation is dependent of calcium detachment from troponin C, which is recaptured into the SR by SERCA2a or extruded from the cell by the sarcolemmal sodium/calcium exchanger (NCX). Exercise training results in improved cardiac function, which has been associated with enhanced calcium handling. Exercise was shown to increase the expression and activity of SERCA2a, but not total phospholamban (PLN) (128, 264). This up-regulates the SERCA2a/PLN ratio and therefore allows SERCA2a to increase the rate of calcium uptake. Increased phosphorylation status of PLN at Thr\textsuperscript{17} residue mediated by exercise-induced activation of calcium calmodulin-dependent protein kinase (CaMK) II and by Akt was shown to contribute to increase SERCA activity (64, 130). Akt also seems to regulate LTCC stability, thus influencing cardiomyocyte calcium entry, handling and contractility (64). Moreover, exercise seems to increase inotropism by increasing myofilament responsiveness to calcium (265). In contrast, important disturbances were detected in cardiomyocytes from failing hearts (78, 126,
HF has been associated with a sustained increase in intracellular calcium concentration, thus interfering with normal excitation-contraction coupling and relaxation (19, 126). Accumulation of calcium in the cytosol has been implicated in cardiac dysfunction by impairing mitochondrial activity (238), promoting cellular death and proteolysis (71, 72), and by triggering maladaptive hypertrophic pathways (16, 127, 176, 180). Reduced SERCA2a expression and activity has been pointed as a major cause of this calcium homeostatic disruption (29, 72, 177, 220, 229), and consequently in the pathogenesis of the contractile defects observed in HF (78, 126). Of note, its manipulation was shown to improve cardiac function (78, 126, 128, 158, 177, 187, 229) and gene transfer of SERCA2a is currently being tested in clinical trials (90).

1.1.2.1.4. **Energy and metabolism**

In order to maintain proper functioning, the heart needs to have a constant and efficient energetic resource (108, 112). In the healthy heart, oxidative phosphorylation is capable to maintain normal concentrations of ATP, and guarantee adequate supply even when its work output increases 3-to 5 fold in comparison to basal conditions (112). Fatty acid oxidation is the major source of energy, accounting for 60-90% of ATP production, with the remaining 40-10% coming from glucose oxidation (193). Cardiac remodeling induced by exercise training is associated by optimized fatty acid and glucose oxidation machinery (30, 89, 120, 240), enhanced mitochondrial respiration and ATP production (195). In opposition, the failing heart is recognized as an energy-starved engine running out of fuel (193). As HF progresses to the more advanced stages, there is a gradual decline in the activity of mitochondrial respiratory pathways, compromising ATP production (160, 230). Alterations in the substrate utilization, oxidative phosphorylation and high-energy phosphate metabolism, have
all been pointed as possible causes of ATP deficiency (28, 116, 193, 230, 237, 255). These changes seem to be explained, at least partially, by mitochondrial structural abnormalities and reduced activity of electron transport chain complexes (28, 33, 80, 108, 250), which can be impaired for instance by oxidative and nitrative damage (159, 188, 202). The consequent lack of energy dramatically compromises cellular functions such as ion transport (e.g. sodium/potassium pump activity), sarcomeric function and intracellular calcium homeostasis (e.g. SERCA2a functioning), therefore contributing to cardiac dysfunction and HF.

1.1.2.1.5. **Death and renewal**

It is widely accepted that the heart is a postmitotic organ, without the capacity to regenerate (6, 62, 143). Additionally, cardiomyocyte death has been considered a relatively rare event in the healthy normal (55, 84, 167) or exercised myocardium (118, 132, 234), but to be exacerbated in both human and animal settings of HF, thus contributing to the progression of the disease (87, 181, 198, 258). In failing human heart, apoptosis was estimated to account for an annual rate of cardiomyocyte loss of 2-4% while necrosis contributes with 11% and autophagy also with 11% (170). Accordingly, in the absence of cardiac disease, the heart was supposed to have a constant number of cardiomyocytes throughout the life, with the same age of the individual. In line with these assumptions, any increase in cardiac mass in response to workload was attributed mainly to cardiomyocyte hypertrophy and any loss of cardiomyocytes was considered irremediable, but partially compensated by the hypertrophy of the surviving cardiomyocytes (6, 62). However, there is now strong evidence to support a more dynamic view of the heart, where cardiomyocyte growth, death and renewal co-exist and contribute to the normal homeostasis of the heart (15,
Indeed, it seems that cardiac cellular losses are not so rare as initially thought. Evidence shows that it occurs continuously during the life span as a consequence of the normal wear and tear, increase as we age and is more significant in man than in women (15, 87, 121, 122). It has been estimated that approximately 3x10^6 cardiomyocytes per day are eliminated in the healthy adult human heart, increasing to 179x10^6 and 97x10^6 in the acute and chronic infarcted heart, respectively (6, 87, 198). Estimates have not been conducted in the exercised heart but some evidence also suggests that cardiac cell death may be increased, at least in response to acute prolonged exercise. For instance, increased pro-apoptotic markers have been reported in the heart from rats submitted to a bout of exercise until exhaustion (107). Also, increased circulating levels of cardiac troponin T (186, 192, 249), structural abnormalities such as myofibrillar disruption (119) and abnormalities of the cardiac interstitium characterized by accumulation of collagen (13, 42, 261, 263) have been detected after strenuous exercise, suggesting that cardiomyocyte death occurred. Theoretically, the rate of cardiomyocyte loss would be a little higher to that presented by the normal sedentary heart since, comparatively, each bout of exercise imposes a greater demand to each cardiomyocyte. Overall, these observations provided some support to the concept of ongoing cardiomyocyte degeneration and loss, which is progressively more evident as the duration and/or severity of the cardiac workload increases.

Such rates of cellular death imply that in few years the heart would completely disappear. However, it seems that the heart contains a population of CSC that are able to differentiate into new cardiomyocytes, as well as into endothelial and smooth muscle cells (11, 62, 121, 122). This allows the heart to compensate the loss of cardiomyocytes and thus, to some extent, maintain cardiac structural and functional
integrity. The magnitude of CSC activation and differentiation into new cardiomyocytes seems to be related with the level of the cardiac workload (6, 248). Indeed, activation of a large proportion of CSC and addition of new cardiomyocytes (hyperplasia) have been reported under cardiac-demanding conditions such as acute and chronic cardiac infarction (3, 12, 121, 224, 252), chronic pressure overload (251), and exercise training (63, 132, 157, 257). Excepting to exercise training, this response fails to normalize the workload or to correct the structural and functional abnormalities of the infarcted or chronically pressure overloaded heart, which ultimately progresses to HF (152). Possible explanations include loss of CSC of the damaged area by apoptosis/necrosis and the difficulty of CSC from spared areas to migrate into the scar (152). A few authors also argue that some cardiomyocytes retain the ability, though limited, to reenter the cell cycle and suffer mitotic division (12, 17, 22, 136, 143, 223). This property seems to be specific from approximately half of the mononucleated cardiomyocytes which were demonstrated to be the only to complete cytokinesis (17). Of note, exercise training, but not chronic pressure overload, induced an increase in cardiomyocyte proliferation in the rat heart (22). Altogether, these data suggest that cardiomyocyte death, together with regeneration, plays a determinant role in the homeostasis of the heart and challenges the dogma that the adult heart is a postmitotic organ, without renewal capacity. Besides cellular hypertrophy, hyperplasia may also underlie the cardioprotective phenotype induced by exercise training. Contrarily to cardiac overloading diseases (and probably strenuous exercise), the workload imposed by moderate exercise training is constantly and fully compensated by the differentiation of CSC and proliferation of cardiomyocytes.
1.1.3. Extracellular Matrix (ECM) Remodeling

The matrix support of the heart is predominantly collagen with relatively small amounts of fibronectin, laminin and elastin (115). Under normal conditions or in a setting of physiological growth, a fine network of collagen fibers provides structural integrity and helps to maintain normal cardiac performance (9, 123, 172). An exception has been recently provided, with data showing that strenuous and prolonged exercise training can increase the fibrotic levels in the RV of rats (13). A few human data also support the idea that elevated volumes of training are associated with the development of fibrosis but need confirmation from longitudinal studies (42, 261, 263). In pathological settings (45, 54, 67, 207, 210, 254), regardless the etiology, fibrillar collagen (fibrosis) can accumulate as reactive (e.g., an adverse accumulation collagen) or as reparative fibrosis (i.e., scar tissue) that replaces the cardiomyocytes that are lost by necrosis (26, 232). Apoptosis does not lead to fibrosis since it is devoid of inflammatory reaction (232). Accumulation of fibrosis adversely affects compliance (increase stiffness), electrical activity (facilitates arrhythmogenesis) and oxygen diffusion (promotes an ischemic environment), increasing the susceptibility for HF development (16, 54, 95, 115, 182, 254). Collagen is synthesized by myofibroblasts, which are thought to result from differentiation of resident fibroblasts or recruitment of microvascular pericytes, endothelial cells and bone marrow-derived circulating progenitor cells (133, 273). TGF-beta is considered the most important activator of myofibroblasts (146) but neurohumoral factors (e.g. ET-1, Ang II and aldosterone), as well as inflammatory mediators [e.g. interleukin-6 (IL-6), tumor necrosis factor (TNF)-alpha] are also involved (85). Of note, osteopontin (OPN), a matricellular protein and cytokine (266), was shown to be determinant in the reorganization of the ECM during cardiac remodeling as it modulates both TGF-beta-
and Ang II-mediated fibrotic response (43, 148, 256). Moreover, OPN favors collagen accumulation by restraining metalloproteinases (MMP) through inhibition of IL-1beta (267). MMP are collagenases responsible for collagen degradation, whose activity is repressed by endogenous tissue inhibitors (TIMP). The interaction and balance of MMP and TIMP determines the maintenance of ECM homeostasis (236). The pro-inflammatory status of HF patients (increased IL-6, TNF-alpha and IL-1beta) favors MMP activation (85). Increased MMP activity and decreased levels of TIMP results in excessive degradation of the ECM and subsequent ventricular dilatation and their modulation seems to provide important ameliorations of cardiac function (23, 117, 154, 208). Of note, it has been recently proposed that increased activity of MMP-9 and -14 are important mediators of CSC invasion to the fibrotic tissue, potentially to repopulate the scarred area (224). Current data suggest that resident CSC do not seem to be able to spontaneously migrate from the viable tissue to fibrotic areas (6) but it seems that activation of growth factors facilitates the infiltration of the scarred tissue and generation, to some extent, of cardiomyocytes and coronary vessels (224).

1.1.4. Cardiac vascularity

Several evidences indicate a strong relation between cardiac capillary density, cardiomyocyte hypertrophy and cardiac function (48, 103, 225, 231, 247). Adequate perfusion is fundamental for myocardial homeostasis. As the heart remodels in response to exercise training, concomitant capillary growth is thought to guarantee that capillary density and perfusion remains normal (145, 260). This adaptation contrast with what happens in response to sustained or progressive workloads induced by pathological settings, where a mismatch between cardiac capillaries and the size of the cardiomyocytes occurs (225). Reduced capillary density has been observed in
both humans and animal settings with HF (1, 20, 76, 176, 207, 225). Vascular rarefaction compromises oxygen delivery favoring an hypoxic environment, with subsequent loss and degeneration of cardiomyocytes, atrophy, and interstitial fibrosis, contributing to HF progression (32, 81, 102, 231). Angiogenesis, the growth of new vessels from existing ones, is determinant for normal organ growth and wound healing. Under physiologic conditions, growth factors such as vascular endothelial growth factor (VEGF) and Angiopoietin 1 provide a tight control between angiogenesis and organ growth (19). Late stages of HF are associated with decreased expression of angiogenic factors, coincident with the progressive loss of capillaries and cardiac function (1, 32, 59, 176, 205). Treatment with VEGF or Angiopoietin 1 was shown to prevent the loss of capillaries and rescue cardiac function (225, 275). The use of vascular growth for therapeutic purposes is currently under exploration in clinical trials (244).

1.2. Exercise training-induced cardiac protection

1.2.1. Brief historical perspective

The notion that exercise training can provide a protective phenotype to the myocardium seems to be out of any dispute and the recognition of its potentialities as a non-pharmacological option to prevent cardiovascular diseases (CVD) is not from these days. We had opportunity to access some papers from the late 1880s, early 1890s and 1900s were it was already possible to find a serious concern regarding to the use of exercise training with both therapeutic (8, 65, 161, 227, 245) and preventive purposes (8, 65). From these, we would like to highlight two papers published in Transactions of the American Climatological Association journal. The first one is from 1895 and was written by a physician named Robert Babcock (8),
were the preconditioning effects of exercise against subsequent angina pectoris is reported:

“Improved arterial circulation is so manifest a result of these exercises that Dr. Schott has known them to lessen the frequency, nay, even the severity of attacks of angina pectoris in individuals with arteriosclerosis who had been unable to indulge in even very moderate physical exercise taken in the ordinary ways of walking, etc.; permanent amelioration of the sufferer's condition has been achieved in some of these cases.”

(Babcock, 1895, p304)

The second report is from another physician, Henry Elsner (65), who in 1910 was, apparently, aware of the beneficial effects of exercise in preserving cardiovascular health:

“Therefore to the busy brain-worker, whether he has hypertension or not, we are forced to recommend periods of quiet, prolonged rest, change of scene, proper exercise, and temperance in all things.”

(Elsner, 1910, p150)

Although these early evidences mainly based on empiric observation, the link between exercise and health was still looked with much skepticism by the medical community. It was only in the middle of 20th century that physical exercise started to be generally recognized as an important way to promote cardiovascular health, and accepted as an important preventive measure (203). The first steps are attributed to Professor Jeremy Morris and his associates, who showed for the first time an association between vigorous exercise and protection against coronary heart disease, by comparing active conductors with sedentary drivers of the London double-decker buses. They concluded that vigorous exercise was a natural defense of the body, providing protection to the ageing heart against ischemia and its deleterious consequences (185). In the following years, numerous epidemiological studies were
performed, supporting the reduced incidence of cardiovascular events (91, 168, 173) and all-cause mortality (173) in individuals engaged in regular physical exercise.

1.2.2. Exercise training and cardiac tolerance to pressure overload

The above-mentioned epidemiological studies suggest that individuals engaged in regular physical exercise develop a resistant heart to different harmful stimuli (91, 168, 173). As initially reviewed, exercise training induces a series of compensatory adaptations that translate into improved cardiac function. These adaptations are believed to allow the heart to respond more efficiently to the daily hemodynamic demands, without significant disturbances of cellular homeostasis (increased tolerance). For instance, increased activity of MAPK (ERK, JNK and p38) and gene expression [c-myc, c-fos, c-jun, ET-1, brain natriuretic peptide (BNP) and IGF-1] was observed in the heart of sedentary rats after a single bout of exercise (111, 233). When trained animals performed the same bout of exercise, this effect was lost, indicating that the heart from these animals was more tolerant to that exercise’s intensity. Increased tolerance provided by exercise was also observed against more demanding and injurious insults such as in experimental ischemia-reperfusion (I-R) (25, 38, 71, 105, 109, 150, 270), myocardial infarction (MI) (47, 49, 56, 70) or doxorubicin cardiotoxicity (7). Of note, cardiac protection to I-R was shown to be promoted by short (i.e., 1-5 day) and long-term (i.e., weeks to months) exercise training (51, 211), and seems to extend to both male and female (51, 92, 151), in the young and aged hearts (239), and, importantly, to be present several days after cessation of exercise training (150). While the mechanisms underlying such improved response are still poorly comprehended [reviewed in references (83, 212)], evidence points for elevated myocardial levels of antioxidants (71, 270), increased expression of sarcolemmal (24,
38) and, potentially, of mitochondrial (213) ATP-sensitive potassium as important mediators of exercise-induced cardioprotection against I-R.

The question that follows is if the increased tolerance in the acute phase persist and translate in subsequent less remodeling of the myocardium in the healing phase. Remodeling of the left ventricle (LV) after I-R or MI injury is associated with changes in LV geometry, function, and histologic characteristics that lead to increased risk of HF (47, 49, 69, 70, 82). Apparently, because prior exercise results in reduced infarcted area, less workload is imposed to each cardiomyocyte and less activation of the signaling pathways involved in cardiac remodeling are expected to occur. Moreover, because cardiomyocytes from exercised hearts are characterized by several intrinsic beneficial adaptations that improve contractility, they are supposed to tolerate better the resultant pressure overload, and thus cardiac function should be improved (58). A few number of studies give support to these ideas by showing that exercise training prior to permanent coronary artery ligation protected cardiac function, decreased maladaptive remodeling and improved survival, several weeks after myocardial infarction induction (47, 49, 69, 70). Improvements were related with increased arteriolar density, lower ECM remodelling and pro-apoptotic markers, decreased mRNA expression of ANP and improved energetic status (decreased aldolase and increased cytochrome c-oxidase and fatty acid binding protein mRNA expression) (49, 69, 70). Overall, these findings suggest that even when regular exercise fails to prevent a major cardiovascular event, it can still act to prevent cardiac dysfunction and improve survival (49). Therefore, it is important to assess if the long-lasting benefits of prior exercise can indeed be extended to other relevant cardiac insults, namely to pressure overload conditions. Cardiac diseases such as pulmonary and systemic hypertension or aortic stenosis impose significant pressure overload to
the heart. As initially described, the heart has the ability to adapt and develop short-term compensatory responses, but ultimately maladaptation ensues and HF occurs (60, 73, 172, 184, 241). Increasing the tolerance of the heart to pressure overload could eventually prevent or delay cardiac dysfunction and HF.
2. AIMS

With these concepts in mind, our major purpose in this work was to address if prior exercise training could increase the tolerance of the heart to both acute and chronic cardiac pressure overload and to provide some insights about potential underlying mechanism. We also intended to verify if the cardioprotective phenotype of exercise training could be mimicked by a stimulus of different nature, designed to simulate the duration and magnitude of the exercise-induced cardiac overload.

This goal is sustained by the specific aims presented in each paper that resulted from this entire work, namely:

a) Paper I:
   • to test if moderate exercise training increases tolerance to acute pressure overload stimulus, protecting from cardiac dysfunction;
   • to test if exercise training prevents the activation of mechanisms implicated in cardiac remodeling.

b) Paper II:
   • to investigate if the exercise-induced protective cardiac phenotype could be mimicked by chronic intermittent cardiac overload (designed to mimic the duration and magnitude of exercise induced overload) induced by beta-adrenergic stimulation with dobutamine;
   • to investigate if the cardiac phenotype induced by chronic intermittent beta-adrenergic stimulation could mimic the protection conferred by exercise training against left ventricular acute pressure overload.
c) Paper III:

- to assess the impact of exercise training performed at different time points of RV chronic pressure overload secondary to experimental PAH induced by monocrotaline (MCT) on cardiac function;
- to assess if exercise training could modulate important markers of cardiac maladaptation, namely calcium handling disturbances, alpha to beta-MHC shift, neurohumoral activation, collagen deposition, inflammation, oxidative phosphorylation impairment and oxidative damage.
3. RESULTS
Moderate exercise training provides left ventricular tolerance to acute pressure overload

First published December 24, 2010; doi:10.1152/ajpheart.01008.2010.

Daniel Moreira-Gonçalves, Tiago Henriques-Coelho, Hélder Fonseca, Rita Maria Ferreira, Francisco Amado, Adelino Leite-Moreira, and José Alberto Duarte
Moderate exercise training provides left ventricular tolerance to acute pressure overload

Daniel Moreira-Gonzalves,

Francisco Amado,

Adelino Leite-Moreira,

José Alberto Duarte

1 Faculty of Medicine, Department of Physiology and 2Faculty of Sport, Department of Sport Biology, Research Center in Physical Activity and Health, University of Porto, Porto, Portugal, and 3Department of Chemistry, Organic Chemistry Division and Agricultural Products, Department of Chemistry/University of Aveiro, Aveiro, Portugal

Submitted 6 October 2010; revised 18 November 2010; accepted 16 December 2010

The heart has a remarkable adaptive ability, allowing it to continuously adjust its structure and function to variable demands and stimuli (38, 43). However, like most physiological systems (15), the heart's ability to adapt and maintain its performance in response to different challenges is limited by restricted boundaries, which, when surpassed, result in a maladaptive phenotype. This can be illustrated by the elevation in workload imposed to the heart either by exercise training, which is transmural and morphological, or by hypertension or cardiac valve disease, which is persistent and pathological. In response to increased load, the heart will develop hypertrophy to normalize wall stress and maintain cardiac output (42). Hypertrophy can be reconditioned by an enhancement of cardiac performance and a shift in a compensatory cardiac phenotype, as in exercise training, or by chamber dilation, impaired ventricular relaxation and filing, and eventually cardiac failure when the cause has a pathological nature (14, 37, 43).

Several pharmacological options are available to improve cardiac function by reducing overload and/or modulating maladaptive remodeling. However, recent studies using exercise training in different models of chronic pressure overload suggest that the heart is capable of maintaining its baseline function in the presence of constant overload. These studies showed that moderate exercise training avoided heart failure (HF) development, resulting in increased survival (9, 33) and enhanced cardiac function (3, 13, 23, 35, 41). The underlying mechanisms seem to be related to a more favorable hypertrophic phenotype (9, 21, 22), including reduction of myocardial fibrosis and apoptosis (15, 22, 23) and increase in capillary density (15). However, it must be highlighted that these exercise-induced improvements were independent of any significant effect on the leading state of the heart. In other words, exercise training did not result in any reduction of cardiac overload, but maladaptive remodeling and functional deterioration were prevented. In light of these data, it might be suggested that it is not the presence of persistent overload per se that determines the activation of the cellular responses involved in maladaptive remodeling and exhaustion of the heart. Rather, the maladaptation is probably related to the intolerability of the heart to sustain the relative overload magnitude, with exercise training being capable of shifting cardiac performance to a level, where working chronically under these conditions would be better tolerated. This hypothesis needs, however, to be supported by data showing that exercise training can increase cardiac tolerance to an acute pressure overload, which favors homeostasis and attenuates the magnitude of the cellular responses to stress. Therefore, the objective of this study was to test if moderate exercise training increases tolerance to acute pressure overload stimuli, protecting cardiac function and thus resulting in less activation of the mechanisms involved in cardiac remodeling (23, 28, 43).

MATERIALS AND METHODS

Animals and experimental design. Animals experiments were performed according to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and approved by the Ethical Committee from the University of Porto. Male Wistar rats weighing 240 g were randomly separated into the following two groups: 1) trained to TL, submititng to treadmill exercise training, and 2) sedentary (n = 8, n = 8).
with movement confined to the cage space. Animals were maintained on a 12:12 h light-dark cycle and received food and water ad libitum.

For the learning group, animals that had been trained were adapted to treadmill exercise for five consecutive days. This adaptation period involved a gradual increase in running time, beginning with 10 min/day and ending with 60 min/day for 15 min. After 2 days of rest, the animals were trained 5 days per week (Monday to Friday) for 14 wk. Training duration and treadmill speed were gradually increased over the course of the first 5 wk of training until 90 min/day at 50% grade (estimated heart rate at 70% maximum oxygen consumption) was maintained for the last 7 wk. Then, constant training.

A similar exercise program to the one used in this study was previously shown to decrease cardiovascular disease and mortality. Exercise training significantly improves various cardiovascular risk factors.
RESULTS

Cerebral effects of exercise training protocol. Exercise training did not result in any significant alterations concerning body weight, height, LV, RV or diastolic oxygenation (data not shown). However, histopathological analysis revealed that exercise training induced an increase in CSA from both LV (38±7 vs. 35.9±6 mm², p < 0.001) and RV (25.8±2 vs. 25.2±1 mm², p < 0.001), suggesting that important structural adaptations occurred in cerebral tissue (Fig 1A). Regarding the hemodynamic profile at baseline, despite a tendency to enhanced relaxation and contractile function, none of the parameters was significantly altered by training (p > 0.05). Our exercise training protocol, however, resulted in a significant 30% increase in the ESV of the LV and RV groups (p = 0.03 vs. SED), an important antioxidant effect (Fig 1B).

Effects of UV acute pressure overload on cardiac hemodynamics. Regarding the SED – Shunt group, all parameters remained unaltered throughout the experimental protocol, which documents the stability of the experimental separation and is in accordance with previous reports from our group using the same approach (10, 16, 27, 32). Pressure overload by decending thoric aortic banding resulted in several alterations in LV function from the SED – Band group that were prevented by exercise (Fig 2A). Importantly, there were no alterations in RV hemodynamics among bands groups (Fig 2B and C), sug-

Identification of SED PAGE. Results by mass spectrometry. Protein bands, matching to labeled bands for each gel at each condition, were excised manually from 12.5% SDS-PAGE stained with Coomassie Brilliant Blue. The gel pieces were washed three times with 25 mM ammonium bicarbonate/50% acetonitrile and further dried in a Speed Vac (Thermo Savant). Trypsin peptides were reconstituted in sodium phosphate/sodium chloride solution and mixed (1:1) with a matrix consisting of a matrix – 9-amino-9H-fluorescein/acetic acid. Aliquots of samples were spotted on the MALDI-TOF sample plate. Peptide mass spectra were obtained on a VERSATOF-TOF mass spectrometer (FAB + ESI Phenomex Analyzers, Applied Biosystems, Foster City, CA) in the positive ion reflection mode. Spectra were obtained in the mass range between 500 and 4,500 Da with c. 2,010 laser shots. For each sample, a data-dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin elastase, or acetonitrile peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a protein mass accuracy of better than 50 ppm. Spectra were processed and analyzed by the Xcalibur Protein Sequence Search (Applied Biosystems), which uses internal search, (Matrix Science) software for searching the peptide mass fingerprints and MASCOT database. Searches were performed against the SwissProt 1020210 and modi-cate the Mascot search as the taxonomic category and the following parameters: 1) two missed cleavages by trypsin; 2) mass tolerance of precursors ions 25 ppm, product ions 0.5 Da; 3) trypsin by basic (modified) arginine; 1 the modification of arginine and a variable modifier.
suggesting that this overload was selective for LV. After aortic coarctation, a 60% increase in LV cavity overload was achieved, as represented by the rise in LVP_{max} (Fig. 2A). This increase in LVP_{max} was successfully sustained until 60 min by both groups. After that, SED - Band animals showed a progressive decline, despite a progressive additional narrowing of the descending aorta, reaching a minimum at 120 min that was significantly lower than at baseline (at baseline: 70.6 ± 21.7 vs. 92.6 ± 24.4 mmHg; P = 0.002) and than in LX - Band (70.6 ± 21.7 vs. 154.6 ± 22.1 mmHg; P = 0.000). Note that EX + Band remained stable along the entire protocol, with no apparent decompenatory response, indicating that these hearts were able to better sustain the increased afterload. Regarding dp/dt_{max},

we observed a similar response in both groups until 60 min of banding. After this point, while EX - Band showed a nonsignificant increase in contractility as illustrated by the slight rise in dp/dt_{max} at 90 min (P = 0.107 vs. 0 min) and 120 min (P = 0.09 vs. 110 min), SUD + Band decreased to deteriorate until the end of the protocol (Fig. 7B). This deterioration only reached significance at 120 min compared with their baseline values (2,144 ± 1,164 vs. 6,077 ± 1,317 mmHg/s; P = 0.01) and with LX - Band (2,144 ± 1,164 vs. 8,610 ± 2,860 mmHg/s; P = 0.002).

Regarding diastolic function, after pressure overload-induced important disturbances in SUD + Band, Figure 7C shows that, while EX + Band did not suffer any alteration in

![Figure 1: Graphical representation of dp/dt_{max} response](image)

![Figure 2: Graphical representation of dp/dt_{max} response](image)

![Figure 3: Graphical representation of dp/dt_{max} response](image)

![Figure 4: Graphical representation of dp/dt_{max} response](image)

![Figure 5: Graphical representation of dp/dt_{max} response](image)

![Figure 6: Graphical representation of dp/dt_{max} response](image)

![Figure 7: Graphical representation of dp/dt_{max} response](image)
alb%

SED+Sham SED+Band s EX+Band s

Cardiac Damage Score

SED+Sham SED+Band EX+Band s

LV RV

Fig. 3. Representative electron micrographs from LV and RV of sedentary and exercised rats submitted to heart surgery (SED = Sham, a and b, respectively; SED = Band, c and d, respectively; and EX = Band e and f, respectively). Results are expressed as mean ± S.D. *P < 0.05 and vs. Sham (a) and vs. EX + Band (b).

18% of SED+Sham, SED+Band, and EX+Band saw their velocity of pressure fall, being significantly impaired at 120 min compared with their baseline values (2.22 ± 1.87 vs. 4.70 ± 2.74 mmHg, P = 0.049) and with EX+Band (2.22 ± 1.87 vs. 7.85 ± 0.24 mmHg, P = 0.009). Compared with baseline values, SED+Band presented a significant increase in heart rate at 120 min, indicating a slower relaxation (144 ± 3.2 vs. 244 ± 8.3, P = 0.007). In EX+Band no significant alterations were registered (118 ± 2.1 vs. 128 ± 6.8, P = 0.435) (Fig. 3).

No significant alterations were observed in heart rate, and diastolic pressure, or end-systolic pressure during the protocol (data not shown).

Effects of LV acute pressure overload on cardiac morphology. Myocardial damage is shown in Fig. 4. The lowest values observed were the intracellular edema and mitochondrial swelling, with the SED+Band group being the most affected (P < 0.01). Significant alterations were detected in both LV and RV from both overworked groups, although more severely in SED+Band (P < 0.01). The SED+Sham group exhibited normal structure, with preserved mitochondrial morphology and without any evidence of cellular or interstitial edema.

Effects of LV acute pressure overload on protein expression of the active form of caspase-3 and NF-κB. Immunohistochemistry analysis revealed positive staining for both active caspase-3 and NF-κB after only 120 min of acute pressure overload in the SED+Band group. As shown in Fig. 4, exercise training prevented activation of caspase-3 (P < 0.01). Although no signs of active caspase-3 were found in

Fig. 4. Representative electron micrographs from myocardial sections (100X). a: positive control; b: positive control; c: negative control (b) and D and E: non-stained nuclei (black arrow), d: positive control; e: negative control (arrow); f: positive control (black arrow).
SED + Sham, 4% positive cardiomyocyte nuclei were observed in SED + Band and only 0.5% in EX + Band. Significance was only found in SED + Band compared with SED + Sham \( P < 0.001 \) and EX + Band \( P = 0.002 \). Regarding mononuclear cells, 0.2% positive nuclei were found in SED + Sham, 0.5% in SED + Band, and 0.6% in EX + Band. Although significance was obtained in SED + Band and EX + Band compared with SED + Sham \( P < 0.01 \), protein expression of active caspase-3 observed in LV from EX + Band was significantly lower in relationship to SED + Band \( P = 0.007 \). No differences were found in RV in any of the groups. Concerning NF-kB staining, differences were only observed in LV cardiomyocytes from SED + Band compared with SED + Sham \( P = 0.002 \) and EX + Band \( P = 0.001 \), with 6.3, 0, and 0.1% of positive cardiomyocyte nuclei, respectively.

Effects of LV acute pressure overload on total protein carbonylation and tyrosine nitration. Total protein carbonylation and 3-nitrotyrosine contents were analyzed and are shown in Fig. 5. Acute pressure overload resulted in increased levels of protein carbonylation (Fig. 5, a and b) in both ventricles of SED + Band, although only significant in LV, where a 30% increase was observed \( P = 0.026 \) vs. SED + Sham. No differences were noted between EX + Band and SED + Sham groups \( P = 0.747 \). Regarding 3-nitrotyrosine protein formation, the overloaded groups presented increased levels in the LV (Fig. 5c, SED + Band) compared with SED + Sham \( P = 0.002 \). Exercise training did not completely prevent 3-nitrotyrosine protein formation (28% increase, \( P = 0.017 \) vs. SED + Sham), but its levels were attenuated compared with SED + Band \( P = 0.012 \) vs. SED + Sham.

Identification of protein more susceptible to oxidative stress by SDS PAGE MS/MS. As shown in Fig. 6, B and C, two DNPH-positive bands and one 3-nitrotyrosine-positive band of different molecular weights were detected by Western blot, suggesting that these are proteins more prone to oxidation with respect to nitration. The results also suggest that there are some basal levels of protein oxidation and nitration in proteins of the normal healthy heart. To identify proteins more susceptible to oxidative/nitrosative damage, MALDI-TOF/TOF analysis was performed (Table 1). A concurrent decrease in calcium-ATPase activity (SERCA2a) was also detected, although not significant (Table 1). One possible mechanism for this is calcium-mediated Ca^2+ current inhibition through SERCA2a. Calcium overload, which has been shown to increase SERCA2a expression, induced a decrease in SERCA2a activity. Calcium overload, which has been shown to increase SERCA2a expression, induced a decrease in SERCA2a activity.
Hand, it’s known that alterations in the activation pattern of kinases and phosphatases induced by oxidative stress modify the phosphorylation status of myocardial proteins altering its affinity to calcium (48). However, because the net contribution of myocardial calcium sensitivity to the relaxation rate is very controversial (38, 48), it seems more reasonable to assume the failure of SR/RE2 as mainly responsible for the altered DP/DP0 and its absence in SED Band animals (7, 36). The resulting calcium overload would also explain the increased mitochondrial swelling, as well as the expression of active caspase 3 found in sedentary overloaded animals. In fact, as suggested by our immunohistochemical results, the decreased hemodynamic profile of sedentary overloaded animals was also accompanied by significant expression of active caspase 3 and NF-κB. Importantly, this response was prevented in EX 1 Band animals, highlighting the positive role of exercise training in modulating the expression of both caspase 3 and NF-κB, which are known to play an essential role in the transition between compensatory hypertrophy and III. Indeed, the inhibition of both apoptosis (17, 44, 49) and NF-κB (20, 38, 45) has been shown to prevent cardiac remodeling and dysfunction.

An interesting point highlighted by our morphological data was the fact that cardiomyocytes do not seem to respond equally to acute pressure overload. There was a great intra- and intergroup heterogeneity in terms of cellular response. In fact, while some cells presented serious ultrastructural damage, apoptosis, and NF-κB activation, others had a normal structure and ultrastructure. This heterogeneity was even more evident in SHED Band animals than in EX Band animals. A potential explanation for the heterogeneous cellular phenotype observed in EX Band animals might result from the functional demand imposed by exercise training, favoring the autocrine response and death of the most dysfunctional and susceptible cells to injury. This idea, although speculative, is supported by reports showing that endurance exercise training is accompanied by terminal deoxynucleotidyl transferase (TdT) and SAPK inflammatory response and activation of apoptotic mediators (IκB, caspase 3, cleaved caspase 3, and cleaved PARP) in LV cardiomyocytes (18) as well as enhanced abundance of cardiac progenitor cells (21). In addition, cardiac autophagy has been recently associated with exercise training, with some data pointing to its involvement in exercise-induced cardioprotection (4, 40). However, the role of apoptosis and autophagy in the exercised heart is still under debate, and further data are needed to clarify this issue.

The heterogeneous susceptibility of cells to damage can also be linked with the different levels of reactive oxygen and

Table 1. Proteins Identified in the DPH and 2-nitrotyrosine-positive bands by SDS-PAGE-MS/MS

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein Name</th>
<th>Accession No</th>
<th>M.W.</th>
<th>KDa</th>
<th>pI</th>
<th>Peptide Score</th>
<th>Matched Site Score</th>
<th>Coverage (%)</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>α-actinin 1</td>
<td>A07001.1</td>
<td>85.30</td>
<td>11</td>
<td>2.87</td>
<td>11</td>
<td>191</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>II</td>
<td>ATP synthase</td>
<td>A59728.1</td>
<td>59.72</td>
<td>17</td>
<td>2.22</td>
<td>17</td>
<td>451</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>ATP synthase</td>
<td>A59728.1</td>
<td>56.32</td>
<td>14</td>
<td>1.97</td>
<td>14</td>
<td>197</td>
<td>100</td>
<td>26</td>
</tr>
</tbody>
</table>

identifies that at cardiac tissue more susceptibility to cardiomyocyte contraction, non-contractile, S-ANPAGE-MS/MS/MS. The following information is presented, mode of cardiac function, protein expression, a, b, and c, protein nature, and reactive protein (p). The protein, protein content, protein score, and protein confidence index values (kDa) are % of protein average, and protein biological function DPH, 2D ionophore digestion.
nitrogen species (RONS) production and scavenging efficiency. We found that 2 h of LV pressure overload were enough to result in increased protein oxidation and tyrosine nitration in sedentary overloaded animals, whereas, in exercise-trained animals those changes were attenuated (Fig. 5t). Importantly, it seems that while some proteins were more prone to oxidative damage, others seem to be more susceptible to nitrosative damage. This is in agreement with previous findings following ischemia-reperfusion, where different susceptibility of proteins to oxidation and tyrosine nitration was also described (31). Our results also suggest that mitochondria are a novel target of acute pressure overload-induced oxidant damage. We found increased levels of oxidative damage in sedentary overloaded animals with the absence of ATP synthesis in the prone to nitration and the β-subunit of ATP synthase and aconitase hydrolase, the most susceptible to carbamylation (Fig. 6). Oxidative damage to proteins is generally related to alterations in their biochemical characteristics, such as enzymatic activities, structural functions, or susceptibility to proteolysis (5, 6). Thus the increased oxidation of aconitase hydrolase, which is a rate-limiting enzyme of the tricarboxylic acid cycle (29, 33), and ATP synthase, responsible for the use of electron-donated H⁺ and energy from the mitochondrial respiratory chain to produce ATP (30), might impair energy production in sedentary overloaded animals. For instance, the limited availability of ATP can interfere with myosin detachment from actin, calcium disassociation from TnC, and active excretion of calcium by the SR (26) and, consequently, affect systolic and diastolic function. In addition, it is important to note that the enhanced oxidative stress induced by acute pressure overload can further directly damage calcium-handling proteins, favoring the accumulation of this ion in the cytosol (12), with a consequent interference in relaxation rate, as suggested by our hemodynamic results. Altogether, these data suggest that mitochondrial dysfunction might be involved in the early deterioration of cardiac function. Activation of oxidative damage in cardiac proteins from the exercise-trained overloaded heart might be partially justified by the increased expression of MnSOD and other in mitochondrial adaptations induced by training. Indeed, recent evidence suggests that improvements in both antioxidant defense mechanisms and mitochondria might at least partially underlie the exercise-induced cardiac protection (1, 12, 19). MnSOD was shown to protect against oxidative damage in ischemia-reperfusion (12) and pressure overload-induced heart failure (47). Our exercise training protocol resulted in a 50% increase in MnSOD protein (Fig. 1b), and our laboratory has previously shown increased activity of this antioxidant enzyme using a similar exercise training protocol (11). In addition, exercise training seems to promote the development of an improved mitochondrial phenotype that is resistant to reactive oxygen species-induced cytochrome c release and caspase-mediated cardiotoxicity (1, 19). Additionally, these data suggest that acute pressure overload-induced RONS can lead to mitochondrial dysfunction, and exercise training can prevent it.

In conclusion, our results highlight the vulnerability of the normal healthy heart to severe acute pressure overload that might occur in pathological conditions and as an initial trigger of early cardiac damage. Exercise training seems to induce a cardioprotective phenotype that is clearly advantageous by decreasing the tolerance to acute cardiac overload.

Protection against this acute stimuli might modulate cumulative deleterious adaptations in sedentary hearts that will be manifested later in life (34).

ACKNOWLEDGMENTS

We are thankful to Celeste Serrade for technical support with animal care, tracing protocol, and tissue processing for immunochemical evaluation.

GRANTS

This study was supported by Portugal Foundation for Science and Technology Grants PTDC/BIOMED/6581/2003 and SFRH/BPD/81374/2012, respectively.

DISCLOSURES

None.

REFERENCES


Intermittent beta-adrenergic stimulation mimics exercise-induced cardiac phenotype and protects against left ventricular acute pressure overload insult

Am J Physiol Heart Circ Physiol, 2012 (submitted)

Daniel Moreira-Gonçalves, Tiago Henrieques-Coelho, Hélber Fonseca, Rita Ferreira, Ana Isabel Padrão, Cátia Santa, Sara Vieira, Francisco Amado, Adelino Leite-Moreira and José Alberto Duarte
ABSTRACT

Cardiac overload imposed by exercise training promotes a unique cardioprotective phenotype. In the present study we tested whether chronic intermittent cardiac overload induced by beta-adrenergic stimulation, designed to mimic the duration and magnitude of exercise induced overload, could provide similar benefits. Male Wistar rats were submitted to treadmill running (Ex,n=20), dobutamine (Dob; 2mg/kg,s.c.,n=20) or placebo administration (Cont,n=20) for 5 days/week during 8 weeks. Next, animals were sacrificed for histological and biochemical analysis or submitted to left ventricular (LV) hemodynamic evaluation in baseline conditions, in response to isovolumetric contractions and to sustained LV acute pressure overload (35% increase in peak systolic pressure maintained for 2 hours). Baseline cardiac function was enhanced in Ex and the response to isovolumetric heartbeats was improved in both Dob and Ex. Increased tolerance to sustained acute pressure overload was also observed in Dob and Ex, in contrast to Cont that presented diastolic dysfunction. Cardiac hypertrophy was present in Dob and Ex without an increase of collagen and osteopontin-1. Their hypertrophic phenotype was identical as they exhibited similar MHC isoforms composition, similar increase in phospho Akt/mTOR and SERCA2a and normal levels of calcineurin. In-gel assessment of oxidative phosphorylation showed increased activity of mitochondrial complex IV and V in both Dob and Ex. Chronic submission to intermittent cardiac overload by beta-adrenergic stimulation provides a cardioprotective phenotype resembling several features of exercise training. These data suggest that the duration and magnitude of the stimuli may play a role in the development of an adaptive or maladaptive phenotype.

Keywords: exercise; intermittent cardiac overload; hypertrophy; cardioprotection
INTRODUCTION

Cardiac overload represents one of the most important modulators of cardiac phenotype. Under chronic loading conditions induced by disease states such as hypertension or aortic stenosis, the heart may develop heart failure. In opposition, chronic workload elevations elicited by exercise training provide an adaptive phenotype (5), which confers cardiac protection against subsequent cardiac insults (2, 17, 18, 39) and can even correct cardiac functional, structural and molecular abnormalities caused by previous pathological overloading states (22, 35, 37). Moreover, distinct features at the cellular and molecular level have been identified that differentiate these two phenotypes. Exercise training is associated with cardiac hypertrophy in the absence of collagen deposition, normal or increased alpha-MHC isoform, activation of the IGF-1/PI3K/Akt/mTOR pathway and mitochondrial improvements (5, 24, 27, 37). In opposition, cardiac hypertrophy induced by pathologic overloading states is accompanied by increased collagen levels, a shift to the slower beta-MHC isoform, activation of the calcineurin/NFAT pathway and mitochondrial dysfunction (1, 5, 45).

The reason of such a divergent response remains unknown but the features of the stimuli, namely its duration and intensity, may be determinant (21, 29, 30). If the stress is too severe or if it is too prolonged, the cell might not have sufficient time to recover, its integrity can be compromised and cellular death pathways might be favored, progressively contributing to maladaptation (10, 34). On its turn, if there is a perfect match between the stress demands and the cellular response, pro-survival pathways are activated and an improved homeostatic capacity is attained (10). In this sense, the protective adaptations induced by exercise training would result from the
cumulative effects of transient changes in gene transcription induced by each acute bout of exercise (48, 52). Increased activity of MAPK and enhanced gene expression of c-myc, c-fos, c-jun, endothelin-1, BNP and IGF-1 were detected after an acute bout of exercise (25, 26). Moreover, Atf3, Fos, Apold1 and Pxdn gene expression were also shown to be up-regulated in response to acute exercise (48). Of note, these gene expression modifications tended to be attenuated after a period of training, suggesting the acquisition of an improved homeostatic state. In contrast, prolonged overloads of pathological origin are paralleled with chronic elevations of some of these mediators, indicating that their sustained up-regulation may underlie the development of a maladaptive phenotype (5, 15, 20, 25, 40, 45). Thus, the cell’s lack of an appropriate recovery period, together with a progressive or sustained elevated functional demand, may be the reason why stimuli like hypertension or aortic stenosis lead to cardiac dysfunction, whereas intermittent cardiac overloads during repeated bouts of exercise develops a cardioprotective phenotype. Further substantiating this hypothesis, is the observation that contrarily to chronic pressure overload, intermittent transverse aortic constriction (iTAC) was able to induce a mild hypertrophic phenotype with preserved systolic function and fetal gene expression that resembled the exercised group (45). iTAC animals also developed diastolic and beta-adrenergic dysfunction, cardiomyocyte apoptosis and vascular rarefaction (45) but these disturbances may fairly be attributed to the severity of the overload that was not controlled. Consequently, the time that mediated between each iTAC application was not adequate to allow the cellular recovery, and possibly the capacity of the cell to maintain genomic and macromolecular integrity was progressively lost.

Thus, it is possible that the regular submission to different intermittent and tolerable amounts of stresses may produce beneficial adaptations similar to exercise.
Therefore, we hypothesized that a stimuli of different nature but with comparable cardiac overloads in terms of magnitude, applied during the same period of time, would resemble the acute hemodynamic demands induced by exercise training, and thus, when repeated over time, would result in comparable cardioprotective phenotype. To test this hypothesis, we submitted rats to exercise training or to similar chronic controlled intermittent cardiac overload induced by beta-adrenergic stimulation with dobutamine, and compared their phonotypical adaptations and tolerance against acute pressure overload.
MATERIAL AND METHODS

Animal experiments were performed according to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, Revised 1996). The ethical committee of the University of Porto, Portugal approved all studies.

Preliminary hemodynamic experiments were performed in order to determine the dose of dobutamine that could reproduce some aspects of our exercise training protocol. Specifically, we were looking for a dosage that could induce a similar hemodynamic demand (~40% increase heart rate and ~15% increase in peak systolic pressure) (36), that could be maintained for the same period (90 minutes), and that could be applied daily for several weeks (5 days/weeks during 8 weeks). To perform this first task, male Wistar rats (n=10; age=5-6 weeks, Charles River Laboratories, Barcelona) were anaesthetised by inhalation of a mixture of sevoflurane (4%) and oxygen, intubated for mechanical ventilation (60 cpm, tidal volume set at 1 ml/100g; Harvard Small Animal Ventilator, Model 683) and placed over a heating pad (body temperature is maintained at 37°C). One pressure-volume catheter (model-FTM-1912B-8018, 1.9F, Scisense) was introduced in the left ventricle through the right carotid artery as previously described in detail (42). After stabilize, dobutamine (Mayne Pharma, Portugal) was administered subcutaneously (s.c.) and hemodynamic parameters were recorded every 10 min for at least 100 min. Considering previous data from literature, different doses of this drug were tested in order to define the most suitable (8, 9, 12, 33, 51), namely 4, 2 and 1 mg/Kg. Data was stored and analyzed with PVAN 3.5 software (Millar). The results that best fitted our criteria were obtained with the administration of 2 mg/Kg of dobutamine. Results from 3
independent experiments with acute dobutamine were averaged and are shown in Figure 1. Dobutamine induced an increase of ~15% in peak systolic pressure, ~30% in HR and ~190% in dP/dtmax (Figure 1-A, B and C, respectively), which resembles previous published data using the same concentration (9).

**Study design**

Male Wistar rats (n=60; age=5 weeks; Charles River laboratories, Barcelona) were housed in groups of 5 rats/cage, in a controlled environment at a room temperature of 22°C, with inverted 12:12-h light-dark cycle, in order to match animals handling and training with their most active period. All animals had free access to food and water. After 1 week of quarantine, they were randomly attributed to one of the following protocols: 1) treadmill exercise training (Ex; n=20), 2) dobutamine administration (Dob; n=20) and 3) placebo administration (Cont; n=20). Animals assigned to the Ex group trained for 8 weeks, 5 days/week. Exercise duration and treadmill speed was gradually increased over the course of the first 3 weeks of training until animals achieved 90 min/day at 25 m/min. After that, both intensity and exercise duration were maintained constant. Animals from Dob group were injected (s.c) with 2 mg/kg of dobutamine (Mayne Pharma, Portugal) for 8 weeks, once a day, 5 days/week. Animals from Cont group and Ex group received an equal volume of sodium chloride (NaCl) 0.9% (s.c.). Dosages were adjusted weekly according to the body weight and dilutions were performed with 0.9% NaCl.

**Hemodynamic evaluation**

Twenty-four hours after ending the protocols, half of the animals from each group were anaesthetised by inhalation of a mixture of sevoflurane (4%) and oxygen, and
were euthanized by exsanguination. Tissue samples were collected and prepared for
histological analysis and biochemical studies as will be explained latter. The other
half of the animals was also anaesthetised by inhalation of a mixture of sevoflurane
(4%) and oxygen, intubated for mechanical ventilation (60 cpm, tidal volume set at 1
ml/100g; Harvard Small Animal Ventilator, Model 683) and placed over a heating
pad (body temperature is maintained at 37°C). Under binocular surgical microscopy
(Wild M651.MS-D, Leica;Herbrugg, Switzerland), the right jugular vein was
cannulated for fluid administration (prewarmed Lactated Ringer's solution) to
compensate for perioperative fluid losses. The heart was exposed by a median
sternotomry and the pericardium was widely opened. Descending thoracic aorta was
dissected and a silk suture 2/0 was placed around it and passed through a plastic tube
in order to allow aortic constriction during the experimental protocol. LV
hemodynamic function was measured with conductance catheters (model-FTM-
1912B-8018, 1.9F, Scisense), connected to MVP-300 conductance system (Millar
Instruments; Houston, USA) through an interface cable (PCU-2000 MPVS, FC-MR-
4, Scisense), coupled to PowerLab16/30 converter (ADInstruments) and to a personal
computer for data acquisitions. Parameters from conductance catheter were recorded
at a sampling rate of 1000Hz, in order to accurately capture all of the features of the
pressure-volume waveforms produced by the fast beating hearts of rats.

Experimental Protocol
After complete instrumentation, the animal preparation was allowed to stabilize for 15
min. Next, hemodynamic recordings were performed in baseline conditions and under
inferior vena cava or ascending aortic occlusions, the latter producing isovolumetric
heartbeats. Sustained and selective acute pressure overload to the LV was obtained by
controlled banding of the thoracic descending aorta, just above the diaphragm, during 120 minutes (min). Briefly, this was performed by gently pulling a silk suture, previously placed around the descending thoracic aorta, against a plastic tube, until an elevation of ~35% of left ventricular peak systolic pressure (LVPmax) was obtained. At that time, the constriction was fixed with the help of a clamp and the imposed overload was continuously monitored. Adjustment of the constriction was provided in order to maintain the same cardiac overload during the entire protocol. Hemodynamic measurements were made in baseline steady-state conditions (immediately before banding), at 60 and 120 minutes of banding. All recordings were obtained with the ventilation suspended. Data were stored and analyzed with PVAN3.5 software (Millar).

**Measured parameters**

Heart rate (HR), peak systolic pressure (Pmax), end-systolic pressure (ESP), end-diastolic pressure (EDP), peak rate of pressure rise (dP/dtmax), peak rate of pressure fall (dP/dtmin), constant time of isovolumetric pressure decay (Tau), maximum volume (Vmax), minimum volume (Vmin), end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), ejection fraction (EF), cardiac output (CO), stroke work (SW) and maximal elastance (Emax), were obtained using PVAN3.5 (Millar Instruments). To assess intrinsic myocardial function, end-systolic pressure–volume relation (ESPVR), preload-recrutable stroke work (PRSW), end-diastolic pressure–volume relation (EDPVR) and arterial elastance (Ea) were determined from pressure–volume loops recorded during transient preload reduction by gently pulling the inferior vena cava with a silk suture previously placed around it. An estimate of myocardial oxygen consumption was made from the double product obtained by
multiplying heart rate with LVPmax, and fractional shortening was calculated as FS (%) = \[((\text{LVEDV} - \text{LVESV})/\text{LVEDV})\] *100 (35).

**Conductance calibration**

Parallel conductance values were obtained by the injection of approximately 100 µl of 10% NaCl into the right atrium. Calibration from relative volume units (RVU) conductance signal to absolute volumes (µl) was undertaken using a previously validated method of comparison to known volumes in Perspex wells (41).

**Tissue Preparation**

The heart and right gastrocnemius muscle from animals that were euthanized at the end of the chronic protocols (not submitted to hemodynamic evaluation), were excised and weighed. Under binocular magnification (x3.5), the LV+septum was dissected from the RV and weighed separately. Heart weight, LV and gastrocnemius were normalized to body weight (BW). Samples from LV were fixed and prepared for light microscopy (LM) following routine procedures, or frozen with liquid nitrogen for protein and enzymatic studies.

**Microscopic evaluation**

Cubic pieces coming from the basal, intermediate, and apical cardiac regions of each LV were fixed [4% (v/v) buffered paraformaldehyde] by diffusion during 24 hours and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xilene was used in the transition between dehydration and impregnation. LV blocks were embedded in the upright position in order to distinguish the endocardium, midwall, and the epicardium of the LV free wall in cross sections. Serial sections (5
µm of thickness) of paraffin blocks were cut by a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohols finishing in phosphate buffered saline solution prepared by dissolving Na₂HPO₄ (1.44 g), KH₂PO₄ (0.24 g), NaCl (8 g), KCl (0.2 g) and adjusting pH to 7.2. Deparaffinised sections from LV were stained for haematoxylin-eosin, performed by immersing slides in Mayer’s haematoxylin solution for 3–4 min followed by immersion in 1% eosin solution for 7 min, dehydration with graded alcohols through xylene, and mounted with DPX. Cardiomyocytes surface area (CSA) was measured and only round to ovoid nucleated myocyte were considered for analysis. Around 1000 cardiomyocytes per group were analyzed. In order to determine the amount of cardiac fibrosis, LV sections were stained with Picrosirius red and quantified as described before (16). In each section, 5 representative images were considered for analysis to compensate for variations within sections. For quantitative comparisons, random microscopic fields (magnification of x400) from each region were considered.

**Left Ventricular Mitochondrial isolation**

Left ventricle mitochondria isolation was performed using the conventional methods of differential centrifugation, as previously described in detail (2). All procedures were performed at 0-4°C. Briefly, after excised, samples from left ventricles (pools of 2 animals) were immediately minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES-KOH (pH 7.4), and 0.1% defatted BSA (catalog. no A6003, Sigma). The minced blood-free tissue was resuspended in isolation medium containing protease subtilopeptidase A type VIII (catalog no. P5380, Sigma; 1 mg/g tissue) and homogenized with tightly fitted Potter-Elvehjen homogenizer and Teflon pestle. The suspension was incubated for 1 minute (4°C) and
rehomogenized. An 0.5 mL aliquot of cardiac muscle homogenate was reserved for western blotting analysis of specific protein targets and the remaining homogenate was centrifuged at 14,500 g during 10 minutes. The supernatant fluid was decanted, and the pellet, essentially devoid of protease, was gently resuspended in isolation medium. The suspension was centrifuged at 750 g for 10 minutes, and the resulting supernatant was centrifuged at 12,000 g for 10 minutes. The pellet was resuspended and repelleted at 12,000 g for 10 minutes. The final pellet, containing the mitochondrial fraction, was gently resuspended in a washing medium containing 250 mM sucrose, 10 mM HEPES-KOH, pH 7.4. Mitochondrial protein concentration was spectrophotometrically estimated with the colorimetric method “RC DC protein assay” (Bio-Rad) using bovine serum albumin (BSA) as standard.

Blue-native PAGE separation of mitochondrial membrane complexes and in-gel activity of respiratory chain complex IV and V

BN-PAGE was performed using the method described by Schagger and von Jagow (47). Briefly, mitochondrial fractions (200 µg of protein) from each experimental group were pelleted by centrifugation at 20000g for 10 minutes and then resuspended in solubilization buffer (50 mM NaCl, 50 mM Imidazole, 2 mM ε-amino n-caproic acid, 1 mM EDTA pH 7.0) with 1 % (w/v) digitonin. After 10 minutes on ice, insoluble material was removed by centrifugation at 20000g for 30 minutes at 4°C. Soluble components were combined with 0.5 % (w/v) Coomassie Blue G250, 50 mM ε-amino n-caproic acid, 4 % (w/v) glycerol and separated on a 4-13 % gradient acrylamide gradient gel with 3.5 % sample gel on top. Anode buffer contained 25 mM Imidazole pH 7.0. Cathode buffer (50 mM tricine and 7.5 mM Imidazole pH 7.0) containing 0.02 % (w/v) Coomassie Blue G250 was used during 1 hour at 70 V, the
time needed for the dye front reach approximately one-third of the gel. Cathode buffer was then replaced with one containing only 0.002 % (w/v) Coomassie Blue G250 and the native complexes were separated at 200 V for 4 h at 4 °C. A native protein standard HMW-native markers (GE Healthcare, Buckinghamshire, UK) was used. The gels were stained with Coomassie Colloidal for protein visualization or incubated at 37 °C with 35 mM Tris, 270 mM glycine buffer, pH 8.3, supplemented with 14 mM MgSO$_4$, 0.2 % (w/v) Pb(NO$_3$)$_2$, and 8 mM ATP for evaluation of the ATP hydrolysis activity of complex V (54). Lead phosphate precipitation that is proportional to the enzymatic ATP hydrolysis activity, was stopped by 50 % (v/v) methanol (30 min), and the gels were then transferred to water. Gels were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA). Band detection and analysis were performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

Spectrophotometric evaluation of respiratory chain complex V was also measured as previously described (43). The phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-colour complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity.

**Western blotting analysis**

Equivalent amounts of total protein from each group were electrophoresed on a 12.5 % SDS-PAGE as described by Laemmli (31). One sample from each of the groups that were studied was applied in the same gel. Gels containing total proteins or mitochondrial proteins (separated by 2-D BN-PAGE) were blotted onto a nitrocellulose membrane (Whatman®, Protan®) and nonspecific binding was blocked with 5 % (w/v) dry non-fat milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 and
0.5 % Tween 20). Membranes were then incubated with primary antibody solution (1:1000 dilution; mouse anti-ATP synthase subunit beta, ab14730, abcam; mouse anti-SERCA2 ATPase, ab2861, abcam; rabbit anti-calcineurin A, ab52761; mouse anti-3-nitrotyrosine, clone 2A8.2, Chemicon; rabbit anti- osteopontin, ab8448; rabbit anti-Akt, #9272, Cell Signalling; rabbit anti-Phospho-Akt, #4058, Cell Signalling; rabbit anti-mTOR, #2983, Cell Signalling; rabbit anti-Phospho-mTOR, #2971, Cell Signalling; rabbit anti-iatrogen-1, #AP2041, ECM Bioscience). After 2 hours incubation, the membrane was washed with TBS-T and incubated with anti-mouse or anti-rabbit IgG peroxidase secondary antibody (1:1000 dilution, Amersham Pharmacia Biotech). Immunoreactive bands were detected with enhanced chemiluminescence reagents (ECL, Amersham Pharmacia Biotech) according to the manufacturer's procedure and images were recorded using X-ray films (Kodak Biomax light Film, Sigma). The films and the gels were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad) and analyzed with QuantityOne software version 4.6.3 (Bio-Rad, Hercules, CA). Four independent experiments were considered for analysis. Equal loading was confirmed by staining the membrane with Ponceau S.

**MHC isoform determination**

Left ventricles were weighed and transferred to a glass homogenizer. A 1:19 ratio of 100 mM phosphate buffer, pH 7.4, containing 0.02% bovine serum albumin was added. Tissue sections were thoroughly homogenized with tightly fitted Potter-Elvehjen homogenizer and Teflon pestle. Total protein concentration was spectrophotometrically assayed with the colorimetric method “RC DC protein assay” (Bio-Rad) using bovine serum albumin (BSA) as standard. Alpha- and beta-isoforms of cardiac myosin heavy chain were separated by gel electrophoresis following the
procedure described by Talmadge and Roy (50). The amount of protein run on the gel was 1 mg per lane. To avoid inter-gel variation, one sample from each of the groups studied was applied in the same gel. The stacking gel consisted of 30% glycerol and 4% acrylamide: $N,N'$-methylene-bis-acrylamide in the ratio of 50:1, 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS). The separating gels were composed of 30% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. Polymerization was initiated with 0.05% N,N,N’,N’-tetramethylethylenediamine and 0.1% ammonium persulfate. The gels were run in a Mini-Protean system (Bio-Rad) at 4°C. The running conditions were 70V (constant voltage) for 30 hours. The gels were stained with Coomassie Colloidal, scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) and optical density analysis of MHC bands was performed using QuantityOne Imaging software (v4.6.3, Bio-Rad). Five independent experiments assayed in duplicate were considered for analysis.

**Statistical Analysis**

Kolmogorov-Smirnov test was performed to check the normality of the data. Kruskal-Wallis test followed by Dunn’s test was used for non-normal data (cross sectional analysis of cardiomyocytes). Between group’s comparisons of baseline hemodynamics, morphometric, fibrosis, Western blot, MHC, BN-PAGE and enzymatic activity data were performed with one-way ANOVA. For comparisons of hemodynamic data during pressure overload, a repeated measures two-way ANOVA test was performed. Significant differences were evaluated with Tukey’s *post hoc* analysis. All statistical analysis was performed with Graph Pad Prism software.
(version 5.0). Data are expressed as mean ± standard deviation (SD). Significance level was set at $P<0.05$. 
RESULTS

General morphometric features of animals submitted to the chronic protocols

Table 1 summarizes the analyzed morphometric parameters. In comparison to Cont group, all other groups presented lower BW (P<0.001). Gastrocnemius weight was reduced in Ex group (P<0.05 vs. Cont) but not when normalized to BW. Only exercise training resulted in increased HW/BW (P<0.001 vs. Cont). Regarding LV mass evaluated by the LV/BW ratio, it was significantly increased in Ex (P<0.01 vs. Cont) and Dob (P<0.05 vs. Cont).

Characterization of cardiac function under baseline steady-state conditions

In vivo contractile function was assessed with a pressure-volume catheter. Full hemodynamic data is summarized in Table 2. Heart rate was significantly increased in Ex in comparison to all other groups (P<0.05). No differences were noted on Pmax, DP, ESP, EDP or dP/dtmin. Peak rate of pressure rise was significantly reduced in Dob (P<0.05 vs. Cont). Relaxation, evaluated by the constant time Tau, was improved in Ex (P<0.05 vs. all other groups). Considering volume-derived parameters, no differences were detected.

Pressure-volume derived parameters obtained from inferior vena cava occlusion, namely ESPVR, PRSW and Emax, were found to be significantly increased in Ex (P<0.05 vs. all other groups). No alterations were observed in EDPVR (P>0.05). Figure 2 shows typical examples of pressure-volume loops under vena cava occlusion from where these parameters were acquired.
Characterization of cardiac function in response to beat-to-beat isovolumetric contractions and to sustained acute pressure elevations

As illustrated in Figure 3, isovolumetric heartbeats presented similar peak systolic pressure and dP/dtmax in all groups, but shorter time constant Tau (faster relaxation) in Ex and Dob (P<0.05 vs. Cont). Pressure overload by descending thoracic aortic banding induced a 35% increase in systolic pressure in all groups, as shown by the rise in LV Pmax (Figure 4-A). All groups were able to maintain the imposed overload for the entire duration of the banding, and no differences in LV Pmax were observed between groups at any moment. Peak rate of pressure rise (Figure 4-B) showed a compensatory increase at 60 min of banding in Ex and Dob, although significant only in the latter (P<0.05 vs. baseline). At 120 min of pressure overload, dP/dtmax further increased in Ex (P<0.05 vs. Cont and Dob). A slower relaxation (prolonged time constant Tau, Figure 4-D, and smaller dP/dtmin, not shown), was observed in Cont group after 60 (P<0.05 vs. baseline, Dob and Ex) and 120 min of banding (P<0.05 vs. baseline and Ex). Only minor changes were observed in HR and EDP (data not shown).

Characterization of the hypertrophic phenotype

As depicted in Figure 5-A and B, cardiomyocyte hypertrophy was found in Ex (22%) and Dob (38%) (P<0.001 vs. Cont), but more marked in Dob (P<0.001 vs. Ex). No alterations were detected in terms of collagen deposition or osteopontin-1 protein expression (Figure 5-C and D). SERCA2a (Figure 6-A) was significantly increased in Dob and Ex (P<0.05 vs. Cont). A modest increase in the beta/alpha-MHC ratio was present in all experimental
groups (Figure 6-B) but without significance (P>0.05). Regarding calcineurin protein expression (Figure 6-C), we observed normal values in all groups. The Akt/mTOR pathway was also assessed. No differences were noted in the expression of total Akt. However, both Dob and Ex groups exhibited a significant increase of Ser\textsuperscript{473} phosphorylation of Akt (Figure 6-D) (P<0.05 vs. Cont). Regarding mTOR, a significant increase was found in the expression of its total levels and of its phosphorylation at Ser\textsuperscript{2448} in Dob and Ex group (P<0.05 vs. Cont).

The BN-PAGE densitometric analysis did not reveal differences in the protein complexes organization, as can be depicted from the representative density traces for complexes’ bands (Figure 7-A). In-gel activity showed elevated complex IV (Figure 7-B) and V (Figure 7-C) activity in Dob (P<0.05 vs. all groups) and Ex (P<0.05 vs Cont). Spectrophotometric quantification of respiratory chain complex V was also performed in order to corroborate the in-gel activity of complex V. As shown in Figure 7-D, elevated activity of this complex was detected in Dob and Ex (P<0.05 vs. Control).
DISCUSSION

The present study addressed the question whether chronic intermittent cardiac overload induced by beta-adrenergic stimulation with dobutamine could provide cross-tolerance to acute sustained pressure overload. We showed that chronic intermittent dobutamine administration, similarly to exercise training, prevents from diastolic dysfunction secondary to acute sustained pressure overload. The improved tolerance evidenced by both dobutamine-treated and trained animals may be related with their similar cardiac hypertrophic phenotype.

In this work, animals were submitted to intermittent pharmacological cardiac overload to test whether they could develop cross-tolerance to acute pressure overload and a phenotype similar to that induced by exercise training. Namely, we used a dosage of dobutamine (2mg/kg) that increased cardiac overload and contractility (9) (Figure 1). Dobutamine, a beta 1- and 2-adrenoreceptor agonist (51), reasonably mimicked the duration and magnitude of an acute cardiac overload imposed by the exercise training protocol (36). This strategy allowed us to have a certain control over the magnitude of the hemodynamic demand that was imposed. This issue is of major importance since if the stimulus is too severe, cells may not have sufficient time for the homeostatic recovery and may fail to activate or maintain a protective response, resulting in the activation of signaling cascades that eventually will favor cellular death pathways (21, 29, 30). The cumulative effects of such imbalance may lead to cardiac dysfunction in the long-term (34). This notion is corroborated by our previous findings (39) that the healthy normal heart develops severe functional disturbances accompanied by the activation of important signaling pathways implicated in maladaptive remodeling in response to an acute pressure overload. Moreover, a link between long-term intensive exercise training, right ventricular dysfunction and
increased susceptibility to arrhythmia was recently shown in a rat model, reinforcing the concept that the beneficial effects of exercise training may be dose-dependent (4). Given that in the study from Perrino et al. (45) the severity of overload was not considered, the maladaptation developed by the iTAC may reflect the cumulative damaging effects of exaggerated intermittent hemodynamic overloads. In our study, the controlled pharmacologically-induced intermittent cardiac overload did not result in any compromise of cardiac function at baseline nor in response to single-beat afterload elevations, which is an intervention that allows the detection of diastolic dysfunction that may not be evident during evaluation at rest, but is revealed during exercise or hemodynamic stress (11, 32).

As evidenced by the hemodynamic results, exercised animals tolerated very well the 35% increase in cardiac overload, while significant diastolic dysfunction was observed in sedentary animals. These observations are in line with our previous findings that contrarily to sedentary animals, the heart from exercised animals is able to work under loading conditions without decompensating (39). It is important to note that the severity of the banding was reduced from 60% (previous study) to 35% (present study) of LV peak systolic pressure, which further highlights the vulnerability of the normal healthy heart to sustained acute increases in afterload. Remarkably, chronic administration of dobutamine conferred protection against cardiac dysfunction, as evidenced by the stability of diastolic parameters, resembling the response of the exercised animals. The enhanced tolerance observed in our study is in line with the preconditioning effects induced by dobutamine (3) and other beta1- and 2-adrenergic agonists (46) in the rat heart against ischemia-reperfusion injury.

The similar performance of Dob and Ex groups in response to the sustained acute pressure overload suggests that their respective conditioning programs may
have promoted similar beneficial adaptations at the cardiac level. In fact, dobutamine has been shown to reproduce some of the typical features of exercise training, namely cardiac hypertrophy (7, 9) without fibrosis (9), cardiovascular and metabolic enhancement (14, 33, 51), increased mitochondrial activity (8), improvement of vascular endothelial function (44), increased cardiac (7) and skeletal (14) muscle capillary density, without developing cardiac adrenergic desensitization (12). In order to provide some insights into the mechanisms underlying the protective effects of intermittent cardiac overload induced by dobutamine, we analyzed some markers of cardiac remodeling. Adaptive hypertrophy is characterized by hypertrophy of cardiomyocytes with little or no fibrosis (37, 53). We found that cardiomyocyte hypertrophy was present in Dob and Ex groups without increased levels of fibrosis. Consistently, we also detected normal levels of osteopontin-1, a matricellular protein that is increased during stress-induced cardiac remodeling, that was shown to mediate cardiac fibrosis and diastolic dysfunction (53). This data, in addition to the unchanged diastolic stiffness (normal EDPVR and EDP at baseline), suggest normal intrinsic myocardial function (23). Therefore, it seems plausible to assume that these factors did not account for the divergent performance of Ex and Dob from Cont.

To further explore the hypertrophic phenotype developed by each of the interventions, we assessed the Akt/mTOR and calcineurin protein expression, two pathways with distinct roles in the promotion of adaptive or maladaptive hypertrophy, respectively (5, 27). Our data shows that the while the former was activated in Dob and Ex groups, the last was not. This may contribute to explain, at least partially, the similar results obtained by Dob and Ex in response to acute pressure overload. Indeed, activation of the Akt/mTOR signal cascade was proposed to be related with improved contractile function and calcium handling (27).
Protein levels of SERCA2a are typically increased in adaptive cardiac hypertrophy. We found increased total protein levels of SERCA2a in Ex and Dob while normal values were present in Cont. Although this does not provide information about its functionality, the increased baseline levels together with the unaffected dP/dtmin and time constant Tau in Ex and Dob after acute pressure overload suggest preserved activity of SERCA2a (13) and a more efficient transport of calcium to the sarcoplasmic reticulum (38). The accumulation of calcium in the cytosol is implicated in mitochondrial swelling (39, 49) and proteolysis (18, 19). Calcium overload also decreases mitochondrial ATP (49) production and thus ultimately may also contribute to diastolic dysfunction by limiting the energy for SERCA2a activity. The greater activity of mitochondrial complexes IV and V found in Dob and Ex suggests that these groups are more prepared to support the energetic cost of an elevated cardiac overload, without compromising the ATP that is needed to maintain intracellular homeostasis.

A slight increase in the beta-to-alpha MHC ratio was observed in Dob and Ex, but this phenomenon is apparently not a marker of failure (6). Indeed, we showed that this small shift to beta-MHC isoform did not compromise the ability to tolerate the increased overload. Our data is corroborated by findings from Hwang and coworkers who reported increased beta-MHC in the LV of trained rats who also exhibited an improved cardiac response to a brief period of ischemia and reperfusion (24). Moreover, it was shown that mice expressing predominantly cardiac beta-MHC isoform tolerate exercise training without any sign of maladaptation (28). Beta-MHC can generate cross-bridge force with higher economy of energy consumption than alpha-MHC (28). Therefore, it could be possible that the more economical phenotype from Ex and Dob consumed less energy for contraction to support the elevated
cardiac overload, leaving more ATP to maintain intracellular homeostasis (24), and thus avoiding diastolic dysfunction.

Overall, our data suggest that the magnitude of the initial hemodynamic stimulus may influence the subsequent development of an adaptive or maladaptive cardiac phenotype. Indeed, the chronic submission to intermittent cardiac overload induced by dobutamine provided cross-tolerance to subsequent acute pressure overload. The protection afforded by dobutamine administration seems to be related with a more physiological hypertrophic phenotype resembling some features of exercise induced-hypertrophy. We propose that, besides the type of the initial hemodynamic stimulus (45), its duration, magnitude or severity may be determinant for the subsequent development of an adaptive or maladaptive cardiac phenotype.
ACKNOWLEDGMENTS

We are very thankful to Miss Celeste Resende for her technical support with animal care, training protocol and tissue processing for morphological evaluation.
GRANTS

This study was supported by the Portuguese Foundation for Science and Technology Grant PTDC/DES/104567/2008. Daniel Moreira-Gonçalves and Hélder Fonseca are supported by the Portuguese Foundation for Science and Technology Grants SFRH/BD/33123/2007 and SFRH/BD/38110/2007, respectively.
Disclosures

None
REFERENCES


**Figure Legends**

**FIGURE 1:** Acute effects of subcutaneous administration of 2 mg/Kg of dobutamine on left ventricular (LV) hemodynamics. Black arrows indicate the time when drug was administrated. Error bars are Mean±SD

**FIGURE 2:** Typical examples of PV-loops obtained during inferior vena cava (IVC) occlusion in animals submitted to the chronic protocols

**FIGURE 3:** Effects of total occlusion of the ascending aorta (isovolumetric heartbeats) on LV Pmax, dP/dtmax and tau. Error bars are Mean±SD. *P<0.05 vs. Cont

**FIGURE 4:** Effects of 120 min of acute pressure overload on LV systolic and diastolic function. Baseline values were considered 100% and are represented by the dashed line. Values at 60 and 120 min are represented as percentage of variation relative to baseline. Error bars are Mean±SD. #P<0.05 vs. baseline *P<0.05 vs. Cont; †P<0.05 vs. Dob, ‡P<0.05 vs. Ex

**FIGURE 5:** Effects of the different chronic protocols on cardiomyocyte hypertrophy, fibrosis and osteopontin-1 protein levels. Results illustrated in figures are plotted in graphic bars. Error bars are Mean±SD. *P<0.05 vs. Cont; ‡P<0.05 vs. Ex

**FIGURE 6:** Effects of the different chronic protocols on SERCA2a (A), MHC isoforms (B), Calcineurin-a (C) and Akt/mTOR pathways (D and E). Error bars are Mean±SD. *P<0.05 vs. Cont
FIGURE 7: Effects of the different chronic protocols on oxidative phosphorylation:
A) LV mitochondrial BN-PAGE profile of the experimental groups; B) Representative images of histochemical staining, with semi-quantitative analysis of in-gel activity of complex IV; C) Representative images of histochemical staining, with semi-quantitative analysis of in-gel activity of complex V; D) Activity of complex V assayed by spectrophotometry. Error bars are Mean±SD. *P<0.05 vs. Cont; ‡P<0.05 vs. Ex
Table 1: General morphometric characterization

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Dob</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>437±34</td>
<td>376±49*</td>
<td>365±37*</td>
</tr>
<tr>
<td>Gast (g)</td>
<td>2.54±0.3</td>
<td>2.33±0.2</td>
<td>2.26±0.3*</td>
</tr>
<tr>
<td>Gast/BW (g/Kg)</td>
<td>5.8±0.9</td>
<td>6.4±0.5</td>
<td>6.3±0.7</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.10±0.1</td>
<td>1.05±0.1</td>
<td>1.08±0.2</td>
</tr>
<tr>
<td>HW/BW (g/Kg)</td>
<td>2.5±0.2</td>
<td>2.8±0.2</td>
<td>3.0±0.1*</td>
</tr>
<tr>
<td>LV+S (g)</td>
<td>0.74±0.1</td>
<td>0.71±0.1</td>
<td>0.71±0.1</td>
</tr>
<tr>
<td>LV+S/BW (g/Kg)</td>
<td>1.69±0.2</td>
<td>1.91±0.2*</td>
<td>1.96±0.2*</td>
</tr>
</tbody>
</table>

BW: body weight; Gast: gastrocnemius; Gast/BW: gastrocnemius/body weight; HW: heart weight; HW/BW: heart weight/body weight; LV+S: left ventricle+septum; LV+S/BW: left ventricle+septum/body weight; g: grams; Kg: kilograms. Data are presented as Mean±SD *P<0.05 vs. Cont.
Table 2: Baseline hemodynamic characterization

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Dob</th>
<th>Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>392.1±38.2</td>
<td>399±37</td>
<td>422±24 *†</td>
</tr>
<tr>
<td><strong>Pmax (mmHg)</strong></td>
<td>121.8±14.5</td>
<td>115.4±8.9</td>
<td>120.1±12.4</td>
</tr>
<tr>
<td><strong>DP (mHg/bpm)</strong></td>
<td>47854±8344</td>
<td>46810±6117</td>
<td>50759±7315</td>
</tr>
<tr>
<td><strong>ESP (mmHg)</strong></td>
<td>113.2±16.5</td>
<td>106.0±8.9</td>
<td>111.2±12.5</td>
</tr>
<tr>
<td><strong>EDP (mmHg)</strong></td>
<td>5.8±2.5</td>
<td>4.6±2.1</td>
<td>3.9±0.9</td>
</tr>
<tr>
<td><strong>dP/dtmax (mmHg/sec)</strong></td>
<td>8868.4±1708.3</td>
<td>7154.7±1192.4 *</td>
<td>8788.5±2177.3</td>
</tr>
<tr>
<td><strong>dP/dtmin (mmHg/sec)</strong></td>
<td>-9221.3±1919.1</td>
<td>-8109.6±1780.2</td>
<td>-9607.5±2182.3</td>
</tr>
<tr>
<td><strong>Tau W</strong></td>
<td>9.0±1.0</td>
<td>9.0±1.0</td>
<td>7.7±0.9 *†</td>
</tr>
<tr>
<td><strong>EDV (uL)</strong></td>
<td>146.8±34.3</td>
<td>151.8±51.2</td>
<td>142.8±25.5</td>
</tr>
<tr>
<td><strong>ESV (uL)</strong></td>
<td>56.1±21.5</td>
<td>46.1±25.7</td>
<td>42.4±14.9</td>
</tr>
<tr>
<td><strong>SV (uL)</strong></td>
<td>101.1±24.1</td>
<td>117.8±50.1</td>
<td>106.6±18.6</td>
</tr>
<tr>
<td><strong>EF (%)</strong></td>
<td>67.8±9.1</td>
<td>74.7±11.9</td>
<td>74.3±7.3</td>
</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>62±12</td>
<td>69±14</td>
<td>71±7</td>
</tr>
<tr>
<td><strong>CO (uL/min)</strong></td>
<td>39696.5±10350.0</td>
<td>46740.5±19162.1</td>
<td>44814.8±7362.7</td>
</tr>
<tr>
<td><strong>SW (mmHg*uL)</strong></td>
<td>9978.3±2320.4</td>
<td>11072.9±5052.2</td>
<td>10305.2±2634.2</td>
</tr>
<tr>
<td><strong>Ea (mmHg/uL)</strong></td>
<td>1.2±0.4</td>
<td>1.0±0.4</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td><strong>ESPVR (mmHg/uL)</strong></td>
<td>2.4±1.1</td>
<td>2.0±1.0</td>
<td>3.8±1.5 *†</td>
</tr>
<tr>
<td><strong>PRSW</strong></td>
<td>95.3±25.7</td>
<td>119.9±55.8</td>
<td>169.9±32.9 *†</td>
</tr>
<tr>
<td><strong>Emax</strong></td>
<td>5.8±2.4</td>
<td>6.4±2.7</td>
<td>10.5±4.6 *†</td>
</tr>
<tr>
<td><strong>EDPVR (mmHg/uL)</strong></td>
<td>0.06±0.0</td>
<td>0.05±0.0</td>
<td>0.06±0.0</td>
</tr>
</tbody>
</table>

HR: heart rate; bpm: beats per minute; Pmax: peak systolic pressure; DP: double product; ESP: end-systolic pressure; EDP: end-diastolic pressure; dP/dtmax: peak pressure rise; dP/dtmin: peak pressure fall; Tau: constant time; ESV: end-systolic volume; EDV: end-diastolic volume; SV: stroke volume; EF: ejection fraction; CO: cardiac output; SW: stroke work; Ea: arterial elastance; ESPVR: end-systolic pressure volume relation; PRSW: preload-recrutable stroke work; Emax: maximal elastance; EDPVR: end-diastolic pressure volume relation. Data are presented as Mean±SD. *P<0.05 vs. Cont; †P<0.05 vs. Dob.
Figure 1
Figure 2

Control

Dobutamine

Exercise
Figure 3
Figure 4
Figure 5

A) Cont, Dob, Ex

H&E

Picrosirius

B) Cardiomyocyte Cross Sectional Area (µm²)

C) Fibrosis (% from total area)

D) Osteopontin (% from Cont)

* †
Figure 6

(A) SERCA2a (% from Cont)
(B) MHC-N/MHC-NW
(C) Calcineurin (% from Cont)
(D) Akt (% from Cont)
(E) mTOR (% from Cont)
Cardioprotective effects of exercise training at different time points during the development of experimental pulmonary arterial hypertension

Circulation, 2011 (under review)

Daniel Moreira-Gonçalves, Hélder Fonseca, Rita Ferreira, Ana Isabel Padrão, Francisco Vasques-Nóvoa, Sara Vieira, Francisco Amado, José Alberto Duarte, Adelino Leite-Moreira and Tiago Henriques-Coelho
**Background**- Increasing evidences suggest that right ventricle failure (RVF) in pulmonary arterial hypertension (PAH) is associated with several modifications including inflammation and neurohumoral activation, extracellular matrix remodeling and mitochondrial dysfunction. We investigated whether exercise training at different time points could act as an upstream modulator of multiple signaling pathways involved in RV dysfunction in monocrotaline (MCT) model of PAH.

**Methods and Results**- Male Wistar rats were submitted to normal cage activity (SED+MCT) or to treadmill exercise training before (EXbefore+MCT), during (EXafter+MCT) and after (EXtreat+MCT) the establishment of RV pressure overload induced by MCT (60 mg/kg). Exercise training prevented muscle atrophy (EXbefore+MCT and EXafter+MCT) and attenuated cardiac hypertrophy (lower right ventricle/body weight ratio and right ventricle/left ventricle ratio) in all MCT-trained groups. Cardiac function was improved in MCT-trained groups with normalization of cardiac remodeling (normal SERCA2a protein levels, beta/alpha MHC isoform, ET-1 and VEGF mRNA). Cardiac fibrosis, inflammation (lower TNF-alpha/IL-10 mRNA ratio), and mitochondrial oxidative damage were reduced by exercise. Survival rate was enhanced in all MCT-trained groups.

**Conclusions**- These data highlight the beneficial effects of exercise in an experimental model of PAH and the putative underlying cardioprotective mechanisms.

**Keywords**: exercise training; pulmonary hypertension; cardiac remodeling; right ventricular dysfunction;
INTRODUCTION

Pulmonary arterial hypertension (PAH) has a complex pathophysiology that includes pulmonary vascular remodeling, right ventricle (RV) hypertrophy and failure.\(^1\) Potential mechanisms for adverse cardiac dysfunction leading to RV failure (RVF) include cardiomyocyte remodeling,\(^2\) neurohumoral activation,\(^6\) inflammation,\(^9\) and oxidative stress,\(^10\) among others.\(^11\) Therapies that improve RV function through the modulation of these pathways may be an interesting strategy for PAH, as recently proposed.\(^2,\)\(^10,\)\(^12\)

There are strong evidences that aerobic exercise training can prevent or revert LV maladaptive remodeling in both experimental\(^13\)-\(^17\) and clinical settings.\(^18,\)\(^19\) Whether similar benefits can be extended to RVF remains largely unknown. Recent evidence suggests that exercise is safe in patients with stable PAH.\(^20,\)\(^21\) Exercise training may have the unique potential to represent a unifying therapy, acting in multiple ways, and operating as an upstream modulator of the multiple signaling pathways involved in RV dysfunction in the context of PAH.

In this study, we intend to elucidate whether exercise training performed at different time points, namely, before, during and after RV chronic pressure overload secondary to experimental PAH induced by monocrotaline could prevent cardiac dysfunction and remodeling, and modulate the main signaling pathways activated in PAH.
METHODS

Animal experiments were performed according to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, Revised 1996). The ethical committee of the University of Porto, Portugal approved all studies.

Male Wistar rats (n=110; age=4 weeks; Charles River Laboratories, Barcelona) were housed in groups of 5 rats/cage, in a controlled environment at a room temperature of 22°C, with inverted 12:12-h light-dark cycle, in order to match animals handling and training with their most active period, and had free access to food and water. Animals were randomly submitted to four different experimental protocols (supplementary data; Figure 1S): i) sedentary injected with MCT or vehicle (SED+MCT, n=25 and SED+Control, n=10; respectively), ii) 4 weeks-exercise training before MCT or vehicle injection (EXbefore+MCT, n=15 and EXbefore+Control, n=10), iii) 4 weeks-exercise training after MCT or vehicle injection (EXafter+MCT, n=15 and EXafter+Control, n=10) and iv) 2 weeks-exercise training after 2 weeks of MCT or vehicle injection (EXtreat+MCT, n=15 and EXtreat+Control, n=10), a time point where significant elevation of RV pressure is already present. Exercise groups were designed to study the effects of pre-conditioning (EXbefore+MCT), training after the beginning of PAH (EXafter+MCT) and training after PAH establishment (EXtreat+MCT). All animals received one subcutaneous injection of MCT (60 mg/kg, Sigma, Barcelona, Spain) or an equal volume of vehicle (1 mL/kg of saline) at the 8th week of living. Regarding the exercise training protocol, after 1 week of habituation, animals exercised for 60 minutes/session, with a running speed of 30 meters/minute, no grade, 5 days/week for 4 weeks (estimated work rate of 70% maximum oxygen
consumption). In the last week of training it was necessary to decrease the intensity from 30 m/min to 25 m/min in EXafter+MCT and EXtreat+MCT in order to allow all animals to perform 60 min of running. Ten animals from SED+MCT, four animals from EXbefore+MCT, three animals from EXafter+MCT and three animals from EXtreat+MCT died during the last week of protocol.

For the survival studies, additional 80 animals were randomly divided as follows: SED+Control (n=5), SED+MCT (n=15), EXbefore+Control (n=5), EXbefore+MCT (n=15), EXafter+Control (n=5), EXafter+MCT (n=15), EXtreat+Control (n=5), and EXtreat+MCT (n=15), and submitted to their respective protocols. After that, their movement was confined to the cages’ area from day 28 until day 42 after MCT injection, which was the study endpoint.

**Measurements**

At day 28-29 after MCT or vehicle administration, animals were prepared for bi-ventricular hemodynamic evaluation with pressure-volume catheters. At the end of the experiments, samples from RV were collected and stored accordingly for microscopy (cross sectional area and fibrosis measurements), RT-PCR (GAPDH, ET-1, TNF-alpha, VEGF-A and IL-10) and protein analysis (SERCA2a, myosin heavy chain isoforms, BN-PAGE analysis of oxidative phosphorylation system organization and in-gel activity of complex V, ATPsynthase beta, and 3-nitrotyrosine). For an expanded Material and Methods section, please see the online-only Data Supplement.

**Statistical Analysis**

All data are presented as mean±SEM. Kolmogorov-Smirnov test was performed to check the normality of the data. Kruskal-Wallis test followed by Dunns test was used
for non-normal data while two-way ANOVA with a Students-Newman Keuls post-hoc test was used for normally distributed data. All statistical analysis was performed with Graph Pad Prism software (version 5.0). Kaplan–Meier survival analysis and the Gehan–Breslow test was performed, and pairwise comparisons were made using the Holm–Sidak method. Results were considered significantly different when P<0.05.
RESULTS

Effects of exercise training on morphometric characteristics

Table 1 summarizes all the analyzed parameters. Body weight loss was observed in SED+MCT (-20%) and EXtreat+MCT (-13%) (P<0.001). Exercise training prevented body weight loss in EXbefore+MCT and EXafter+MCT groups. MCT induced RV hypertrophy in sedentary animals (P<0.001 vs. SED+Cont). Exercise training partially reverted this effect in all trained groups that received MCT injection (EXbefore+MCT, EXafter+MCT and EXtreat+MCT) as shown by the RV/BW and RV/LV ratio (P<0.01 vs. SED+MCT). MCT administration induced hypertrophy at the level of the cardiomyocytes in all MCT-treated groups (P<0.001 vs. respective control group). EXbefore+MCT exhibited significantly less hypertrophy when compared to all other MCT-treated groups (P<0.001). EXafter+MCT and EXtreat+MCT presented cardiomyocyte hypertrophy similar to SED+MCT.

MCT treatment did not induce any changes in LV morphometric parameters. There were no differences in LV parameters among control groups.

Lung weight was significantly increased in all MCT-treated groups (P<0.001). Lung to body weight ratio was attenuated in EXbefore+MCT and EXafter+MCT groups (P<0.05 vs. SED+MCT).

Exercise training averts RV diastolic dysfunction in MCT-treated rats

Table 2 summarizes the results from bi-ventricular hemodynamic evaluation. $RVP_{\text{max}}$ increased in SED+MCT (+99%), EXafter+MCT (+71%) and EXtreat+MCT (+73%) groups (P<0.001 vs. respective control group). In EXbefore+MCT, exercise preconditioning prevented $RVP_{\text{max}}$ increase. Heart rate was reduced in SED+MCT (P<0.001 vs. SED+Control) and normalized in all MCT-trained groups. Figure 1
shows typical RV PV-loops representative from all groups during IVC occlusion from which ESPVR, EDPVR and Ea were obtained. Right ventricle Ea increased in SED+MCT group as compared with SED+Cont group (Figure 1-A). Exercise training induced a decrease in Ea but significance was obtained only in EXafter+MCT as compared with SED-MCT group (P<0.05). ESPVR significantly increased in SED-MCT and EXbefore+MCT groups in comparison to their respective controls (P<0.05), whereas in EXafter+MCT and EXtreat+MCT there was a smaller increase in ESPVR (P<0.05 vs. SED+MCT).

Diastolic function was markedly impaired in SED+MCT group, namely there was an increase in end-diastolic pressure and a longer RV time constant tau (P<0.01 vs. all other groups). Exercise training normalized both end-diastolic pressure and tau in all three MCT-trained groups. Peak rate of RV pressure fall was increased in all MCT-treated groups but significance was only present in EXbefore+MCT (P<0.01) and EXafter+MCT (P<0.05) groups in relation to their respective control pairs. Right ventricle EDPVR was increased in SED+MCT, as compared to SED+Control (P<0.001) and to all MCT-trained groups (P<0.001). It was also decreased in all Control-trained groups (P<0.05 vs. SED+Control).

Regarding the LV, SED+MCT presented a significant decrease in P_{max} and increase in tau (P<0.01 vs. SED+Control). Exercise training normalized these alterations in all MCT-trained groups.

**Exercise training prevents pathological remodeling in MCT-treated rats**

Right ventricular SERCA2a protein expression (Figure 2-A) was significantly reduced in SED-MCT (P<0.001 vs. all groups), whereas normal values were observed in all MCT-trained groups.
A significant increase in the beta/alpha-MHC isoform ratio from RV was found in SED+MCT groups (P<0.05 vs. respective control group) (Figure 2-B). All the MCT-trained groups predominantly expressed more alpha-MHC (lower ratio beta/alpha-MHC) and a significant difference was present in both EXbefore+MCT and EXafter+MCT groups (P<0.05 vs. SED+MCT; P<0.01 vs. respective control groups). Exercise training induced an increase in beta/alpha-MHC isoform ratio in control groups and significance was achieved in EXbefore+Control and EXafter+Control (P<0.01 vs. SED+Control).

ET-1 gene expression was quantified in the RV (Figure 2-C) and LV (supplementary data; Figure 2S). A significant increase of ET-1 mRNA was observed in both ventricles from SED+MCT (P<0.001 vs. SED+Control), while in all MCT-trained groups its expression was significantly down-regulated (P<0.05 vs. SED+MCT). Significant down-regulation of VEGF mRNA was observed in SED+MCT in comparison to all groups (P<0.05). Exercise training completely prevented or reverted any alteration in VEGF mRNA on MCT-trained groups (Figure 2-D).

**Exercise training prevents RV fibrosis and RV myocardial inflammation in MCT-treated rats**

Significant amounts of collagen were detected in SED+MCT in comparison to all control and other MCT-treated groups (P<0.001). Exercise training normalized collagen deposition in all MCT-trained groups. Results and representative images are shown in Figure 3-A and B.

An elevated inflammatory state was observed in SED+MCT group as evidenced by the increased TNF-alpha/IL-10 mRNA ratio (P<0.05 vs. all groups). Exercise training
improved the anti-inflammatory state in all MCT-trained groups (P<0.05 vs. SED+MCT).

Exercise training prevents RV mitochondrial oxidative phosphorylation and oxidative damage in MCT-treated rats

The BN-PAGE densitometric analysis revealed no significant differences in the oxidative phosphorylation complexes organization, as can be depicted from the representative density traces for complexes’ bands (Figure 4-A). Complex V in-gel activity (Figure 4-B) was significantly impaired in SED+MCT (P<0.05 vs. SED+Control). Exercise training completely rescued the RV ability to aerobically produce ATP in the MCT-trained groups (P<0.05 vs. SED+MCT). Western blot analysis of ATP synthase subunit beta was performed in order to validate the protein expression profile observed and no differences were detected (Figure 4-C).

In order to investigate if the decreased mitochondrial complex V activity could be related with oxidative damage, membranes containing mitochondrial complexes from SED+Cont and MCT-treated animals separated by BN-PAGE were probed for 3-nitrotyrosine (Figure 4-D). Significant levels of mitochondrial membrane protein nitration were found only in SED-MCT, with the Complex V as the main target of this posttranslational modification (P<0.001 vs. SED+Control). Exercise training prevented nitration in all MCT-trained groups (P<0.001 vs. SED+MCT).

Exercise training improved survival rate in MCT-treated rats

Survival rate 42 days after MCT injection was 73% in EXafter+MCT group, 25% in EXbefore+MCT group, 16% in EXtreat+MCT group and 13% in SED+MCT group. A significant improvement on survival curves was observed in all MCT-trained
animals (P<0.05). Mortality rate was null in Control groups. Results are illustrated in Figure 5.
DISCUSSION

The present study demonstrates that exercise training at different time-points exerts a positive impact in the RV response to chronic pressure overload, protecting from cardiac dysfunction and improving survival in an experimental model of PAH. This suggests that exercise can act as an upstream modulator of several pathological pathways activated in the RV during PAH. The benefits of exercise training may be associated with the prevention of calcium handling disturbances, alpha to beta-MHC shift, decreased neurohumoral activation, collagen deposition and inflammation and preserved oxidative phosphorylation through the reduction of mitochondrial oxidative damage.

The recent recognition that exercise training can be safely performed by PAH patients,\(^\text{20,}\text{21}\) justifies the urgent need to investigate the impact of exercise training on the overloaded RV. In the present study, we demonstrate that exercise training at different time points of MCT-induced PAH improves survival rate and ameliorates RV function. In animals submitted to preconditioning (EXbefore+MCT) the RV was protected from significant afterload elevations as well as from cardiac hypertrophy, highlighting that cardioprotection can be sustained for several weeks after the cessation of exercise training. Those animals that were trained during the development (EXafter+MCT) and after the establishment of RV pressure overload (EXtreat+MCT) experienced levels of pressure overload and cardiomyocyte hypertrophy comparable to SED+MCT, nevertheless their RV diastolic function was completely preserved. These results contrast with those published by Handoko et al.,\(^\text{24}\) who reported worsening of the RV cardiac function by exercise training in rats with PAH induced by the same dosage of MCT that we used. It is known that improvements in cardiovascular function induced by exercise are intensity-dependent.
and require higher intensities of training for maximal benefit.\textsuperscript{25, 26} In the present work, animals were submitted to a longer and more intense exercise-training program, which may explain the beneficial effects of exercise obtained in our study.

Right ventricular function is widely accepted as the main prognostic factor in PAH.\textsuperscript{1} The signaling pathways activated in the RV during the progression from hypertrophy to failure secondary to PAH\textsuperscript{2-10, 12, 27} show some similarities to those activated in LV failure.\textsuperscript{11, 28} ET-1 activation is an important player in PAH pathophysiology and its blockade is part of the therapeutic options currently used in PAH.\textsuperscript{8, 29-31} We found that ET-1 mRNA levels were increased in the RV of SED+MCT group, but they were normalized in all MCT-trained groups. Exercise-induced inhibition of ET-1 might explain the improvement of RV function, as well as, the preservation of LV function.\textsuperscript{8} Deregulation of the extracellular matrix with collagen deposition and fibrosis is another hallmark of RV dysfunction.\textsuperscript{2} The SED+MCT animals presented increased levels of RV fibrosis that was accompanied by a pro-inflammatory state, with an imbalance between TNF-alpha and IL-10,\textsuperscript{32, 33} favoring the formation of cardiac fibrosis.\textsuperscript{33} Exercise training completely prevented the development of RV fibrosis and promoted an anti-inflammatory status (decreased TNF-alpha/IL10 ratio). The switch from alpha- to beta-MHC is widely used as an indicator of maladaptive cardiac remodeling. In accordance with previous reports,\textsuperscript{4} we found an increase in the slower beta-isoform in the RV of SED+MCT. In contrast, all MCT-trained groups expressed more alpha-MHC isoform, which is in line with the beneficial effects of exercise training previously reported in LV failure.\textsuperscript{34, 35} Paradoxically, in our control trained animals there was also an up-regulation of the beta–MHC. Our observation corroborates previous findings from Hwang and coworkers who also described increased beta-MHC in the RV and LV of healthy rats.
submitted to treadmill running. There are some evidences that an increase in beta/alpha-MHC ratio has no deleterious impact on cardiac structure or contractile function under basal conditions or in response to exercise. In sedentary animals treated with MCT there was also a decrease in SERCA2a, another important feature of heart failure. In opposition to SED+MCT group, we found normal SERCA2a protein levels in all MCT-trained animals, which are in line with their preserved relaxation rate. Exercise training was suggested to protect cardiac function in different models of cardiac failure by enhancing cardiac capillarization. We evaluated mRNA expression of VEGF, which was shown to reflect cardiac capillary density, and found that exercise training prevented its down-regulation. Moreover, a similar pattern of increase in cardiac hypertrophy and VEGF mRNA was observed in all trained groups, which is congruent with the concept that physiological cardiac growth is associated with enhanced angiogenesis.

Impaired oxidative phosphorylation can affect cardiac function by compromising the energetic supply of ATP to the cardiomyocytes. Mitochondrial complex V activity revealed decreased mitochondrial energy-producing ability in the RV from MCT-treated sedentary animals. Our observation corroborates previous findings reporting low ATP levels in the RV of MCT-treated rats. Limited availability of ATP can interfere with the contractile apparatus and calcium kinetics and negatively affect diastolic function. Importantly, exercise training rescued mitochondrial oxidative phosphorylation capacity in all MCT-trained groups. As major sources of reactive oxygen and nitrogen species, mitochondria themselves, and particularly oxidative phosphorylation complexes, are highly susceptible to functional impairment due to oxidative and nitrative damage. We found increased levels of 3-nitrotyrosine in the mitochondrial complex V from SED-MCT animals, which may
account for the decreased oxidative phosphorylation, as previously demonstrated in neuronal mitochondria.\textsuperscript{46} Importantly, exercise training prevented protein nitration in all MCT-trained groups, which may reflect its buffering capacity due to improved anti-oxidant mechanisms. This is corroborated by the results from Redout et al\textsuperscript{10} who showed prevention of protein nitration in the RV of MCT-treated rats with an anti-oxidant mimetic.

Our data strengths the hypothesis that RV dysfunction is not entirely dependent of cardiac overload.\textsuperscript{2,31} Exercise training seems to provides a cardioprotective phenotype that allows the RV to work under overloading conditions with better tolerance. Similar observations were previously reported in the LV, where exercise training prevented cardiac dysfunction in different animal models of chronic pressure overload, independently of any hypotensive effect.\textsuperscript{13,15,17} Thus, therapeutic approaches aimed to specifically improve the RV performance in the presence of persistent overload, as occurs in PAH, may potentially be beneficial.

**CONCLUSIONS**

The findings from the present study indicate that exercise preconditioning, as well as exercise performed during or after the establishment of RV chronic pressure overload secondary to MCT-induced PAH averts RV dysfunction and improves survival. The putative mechanism for the cardioprotection at the RV level afforded by exercise training may include prevention of calcium handling disturbances, alpha to beta-MHC shift, decreased neurohumoral activation, collagen deposition and inflammation, and preserved mitochondrial function. Interestingly, the majority of these beneficial effects were independent from afterload levels. Altogether, these data highlight that
exercise training can be a new modulator of RV function and can represent an important adjunctive therapeutic option in the management of PAH patients.
ACKNOWLEDGMENTS

We are very thankful to Miss Celeste Resende, Rodney Paixão, Marina Neto and Manuel Pinto for their technical support with animal care, training protocols and tissue processing. We are also very grateful to Nádia Novais for her help with hemodynamic evaluation and to Maria José Mendes and Miss Antónia Teles for their valuable collaboration in the sample’s preparation for molecular biology analysis.
FUNDING SOURCES

This study was supported by the Portuguese Foundation for Science and Technology Grant PTDC/DES/104567/2008. Daniel Moreira-Gonçalves and Hélder Fonseca are supported by the Portuguese Foundation for Science and Technology Grants SFRH/BD/33123/2007 and SFRH/BD/38110/2007, respectively.
DISCLOSURES

None
REFERENCES


44. Leite-Moreira AF. Current perspectives in diastolic dysfunction and diastolic heart failure. *Heart.* 2006;92:712-718


FIGURE LEGENDS

FIGURE 1: Representative examples of RV PV-loops obtained during inferior vena cava (IVC) occlusion (A) and graphic representation of derived parameters (B). Only animals showing volume signal in a range of 5-10 RVU<sup>47</sup> were considered for this analysis (n=5 for SED+Control, n=6 for EXbefore+Control, n=5 for EXafter+Control, n=5 for EXtreat+Control, n=6 for SED+MCT, n=9 for EXbefore+MCT, n=8 for EXafter+MCT and n=6 for EXtreat+MCT). ESPVR: end-systolic pressure volume relation; Ea: arterial elastance; EDPVR: end-diastolic pressure-volume relation. Error bars are mean±SEM. *P<0.05 vs. SED+Control, †P<0.001 vs. respective control group and ‡P<0.05 vs. SED+MCT.

FIGURE 2: Effects of exercise training markers of RV remodeling: A) SERCA2a protein expression; B) alpha/beta-MHC isoform ratio; C) ET-1 mRNA; D) VEGF mRNA. Error bars are mean±SEM (n=8, n=5, n=8, n=7 animals per group for SERCA2a, cross sectional analysis, MHC isoform and mRNA quantification, respectively). †P<0.05 vs. respective control group, ‡P<0.05 vs. SED+MCT.

FIGURE 3. Effects of exercise training on RV fibrosis (A and B) and TNF-alpha/IL-10 mRNA ratio (C). Error bars are mean±SEM (n=5 and n=7 animals per group for fibrosis and mRNA quantification, respectively). †P<0.05 vs. respective control group and ‡P<0.05 vs. SED+MCT.

FIGURE 4: Effects of exercise training on mitochondrial oxidative phosphorylation and oxidative stress: A) RV mitochondrial BN-PAGE profile of the experimental groups; B) Representative images of histochemical staining, with semi-quantitative
analysis of in-gel activity of complex V; C) Validation of the protein expression profile of ATP synthase subunit beta by Western blotting; D) Formation of 3-nitrotyrosine in mitochondrial complex V. Error bars are mean±SEM (n=6; 3 pools of 2 different animals, assayed in duplicate). *P<0.001 vs. SED+Control, †P<0.05 vs. respective control group and ‡ P<0.05 vs. SED+MCT.

**FIGURE 5:** Impact of exercise training on survival: exercise training delayed mortality. All rats from EXbefore+Control, EXafter+Control and EXtreat+Control survived but were omitted here to improve the clarity of the graphic. MCT: monocrotaline pyrrole. †P<0.001 vs. respective control group and ‡ P<0.05 vs. SED+MCT.
# Table 1. General morphometric characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SED (n=10)</td>
<td>EXbefore (n=10)</td>
</tr>
<tr>
<td><strong>BW (kg)</strong></td>
<td>0.343±0.0</td>
<td>0.347±0.0</td>
</tr>
<tr>
<td><strong>HW (g)</strong></td>
<td>0.870±0.0</td>
<td>0.945±0.0</td>
</tr>
<tr>
<td><strong>HW/BW (g)</strong></td>
<td>2.542±0.0</td>
<td>2.676±0.1</td>
</tr>
<tr>
<td><strong>Lung W (g)</strong></td>
<td>1.481±0.1</td>
<td>1.512±0.1</td>
</tr>
<tr>
<td><strong>Lung W/BW (g/kg)</strong></td>
<td>4.278±0.4</td>
<td>4.278±0.2</td>
</tr>
<tr>
<td><strong>Right Ventricle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RV (g)</strong></td>
<td>0.177±0.0</td>
<td>0.187±0.0</td>
</tr>
<tr>
<td><strong>RV/BW (g/kg)</strong></td>
<td>0.518±0.0</td>
<td>0.530±0.0</td>
</tr>
<tr>
<td><strong>RV/LV (g/g)</strong></td>
<td>0.290±0.0</td>
<td>0.282±0.0</td>
</tr>
<tr>
<td><strong>CSA (µm²)</strong></td>
<td>238.5±4.3</td>
<td>231.2±5.0</td>
</tr>
<tr>
<td><strong>Left Ventricle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LV (g)</strong></td>
<td>0.616±0.0</td>
<td>0.666±0.1</td>
</tr>
<tr>
<td><strong>LV/BW (g/kg)</strong></td>
<td>1.801±0.0</td>
<td>1.801±0.0</td>
</tr>
</tbody>
</table>

BW: body weight; HW: heart weight; HW/BW: heart weight/body weight; LW: lung weight; LW/BW: lung weight/body weight; RV: right ventricle weight; RV/LV: right ventricle/left ventricle weight; CSA: cross sectional area; LV: left ventricle weight; LV/BW: left ventricle weight/body weight; g: grams; kg: kilograms; Data is presented as mean±SEM. *P<0.05 vs. SED+Cont, †P<0.05 vs. respective control group and ‡ P<0.05 vs. SED+MCT and §P<0.05 vs. EXafter+MCT and EXtreat+MCT.
Table 2. Hemodynamic evaluation parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SED (n=10)</td>
<td>SED (n=15)</td>
<td>EXbefore (n=10)</td>
</tr>
<tr>
<td></td>
<td>EXafter (n=10)</td>
<td>EXafter (n=10)</td>
</tr>
<tr>
<td></td>
<td>EXtreat (n=10)</td>
<td>EXtreat (n=12)</td>
</tr>
<tr>
<td><strong>Heart Rate</strong></td>
<td>413±7</td>
<td>372±9</td>
</tr>
<tr>
<td><strong>RV Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P_{\text{max}}) (mmHg)</td>
<td>24.3±0.5</td>
<td>27.9±1.1</td>
</tr>
<tr>
<td>(dP/dt_{\text{max}}) (mmHg/sec)</td>
<td>1852±55</td>
<td>2100±113</td>
</tr>
<tr>
<td>(EDP) (mmHg)</td>
<td>3.6±0.4</td>
<td>3.0±0.2</td>
</tr>
<tr>
<td>(dP/dt_{\text{min}}) (mmHg/sec)</td>
<td>-1397±53</td>
<td>-1588±100</td>
</tr>
<tr>
<td>(\text{Tau}) (ms)</td>
<td>10.7±0.6</td>
<td>10.0±1</td>
</tr>
<tr>
<td><strong>LV Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P_{\text{max}}) (mmHg)</td>
<td>121.4±3.7</td>
<td>119.2±4.1</td>
</tr>
<tr>
<td>(dP/dt_{\text{max}}) (mmHg/sec)</td>
<td>8560±501</td>
<td>8806±434</td>
</tr>
<tr>
<td>(EDP) (mmHg)</td>
<td>4.0±0.5</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>(dP/dt_{\text{min}}) (mmHg/sec)</td>
<td>-10352±559</td>
<td>-8722±458</td>
</tr>
<tr>
<td>(\text{Tau}) (ms)</td>
<td>8.5±0.2</td>
<td>9.3±0.3</td>
</tr>
</tbody>
</table>

\(P_{\text{max}}\): maximum pressure; \(dP/dt_{\text{max}}\): peak rate of pressure rise; \(dP/dt_{\text{min}}\): peak rate of pressure fall; EDP: end-diastolic pressure; Tau: time constant of ventricular pressure decay. Data is presented as mean±SEM. *P<0.05 vs. SED+Control, †P<0.05 vs. respective control group and ‡P<0.05 vs. SED+MCT.
FIGURE 2

A. SERC2a (% from SED+Control)

B. beta/alpha-MHC ratio

C. ET-1/GAPDH mRNA (fold change)

D. VEGF/GAPDH mRNA (fold change)
FIGURE 5
SUPPLEMENTAL METHODS

Hemodynamic evaluation

Twenty four hours after ending their respective protocols, rats were anaesthetized by inhalation with a mixture of 4% sevoflurane with oxygen, intubated for mechanical ventilation (respiratory frequency 100 min\(^{-1}\) and weight adjusted tidal volume; Harvard Small Animal Ventilator- Model 683) and placed over a heating pad (37°C). The right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative fluid losses. A median sternotomy was performed to expose the heart and the pericardium was widely opened. Two 1.9F microtip pressure–volume conductance catheters (FTS-1912B-8018, Scisense) were inserted by apical puncture on the RV and LV cavity, along the ventricular long axis. The catheters were connected to MVP-300 conductance system through interface cable (PCU-2000 MPVS, FC-MR-4, Scisense), coupled to PowerLab16/30 converter (AD Instruments) and a personal computer for data acquisitions. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at the end of expiration under steady-state conditions or during preload reductions (inferior vena cava occlusion). Parameters from conductance catheter were recorded at a sampling rate of 1,000 Hz, in order to accurately capture all of the features of the pressure–volume waveforms produced by the fast-beating rat hearts. Data were stored and analyzed with Millar conductance data acquisition and analysis software (PVAN3.5).

Measured parameters

The following parameters were calculated: heart rate (HR), maximum pressure (P\(_{\text{max}}\)), minimum pressure (P\(_{\text{min}}\)), end-systolic pressure (ESP), end-diastolic pressure (EDP),
peak rate of pressure rise (dP/dt\textsubscript{max}), peak rate of pressure fall (dP/dt\textsubscript{min}) and time constant of ventricular pressure decay (Tau). RV end-systolic pressure–volume relation (ESPVR), arterial elastance (E\textsubscript{a}) and end-diastolic pressure–volume relation (EDPVR) were determined from pressure–volume loops recorded during transient occlusion of the inferior vena cava by external compression of the vessel. Because the parallel conductance volume varied widely by the amount and speed of the saline injection, we opted to use relative volume units (RVU) instead of microliters, which has the disadvantage of failing to give precise estimation of volume intercepts of P-V relations but it allows reasonable ESPVR, E\textsubscript{a} and EDPVR assessment once the slope of these indexes are independent of units calibration.\textsuperscript{1}

**Tissue Preparation**

Once hemodynamic data collection was completed, animals were euthanized by exsanguination and the heart, lung and right gastrocnemius muscle were excised and weighed. Under binocular magnification (x3.5), the LV+septum was dissected from the RV and weighed separately. Heart weight was normalized to body weight (BW/BW). RV was normalized to BW (RV/BW) and LV (RV/LV). Samples from RV were fixed and prepared for light microscopy (LM) following routine procedures or frozen with liquid nitrogen for mRNA or protein studies.

**Microscopic evaluation**

RV samples extracted from the basal, intermediate, and apical cardiac regions of each animal were fixed, paraffin-embedded, sectioned and mounted on silane-coated slides. RV blocks were embedded in the upright position in order to distinguish the endocardium, midwall, and the epicardium of the RV free wall in cross sections. For
cardiomyocytes surface area (CSA) measurements deparaffinised sections were stained for haematoxylin-eosin, photographed and analyzed as previously explained. In order to determine the amount of cardiac fibrosis, RV sections were stained with Picrosirius red and quantified as described before.

**Right Ventricular Mitochondrial isolation**

Right ventricle mitochondria isolation was performed using the conventional methods of differential centrifugation, as previously described. All procedures were performed at 0-4°C. Briefly, after excised the samples from right ventricles (4 independent experiments; pools of n=2 different animals) were immediately minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES-KOH (pH 7.4), and 0.1% defatted BSA (catalog. no A6003, Sigma). The minced blood-free tissue was resuspended in isolation medium containing protease subtilopeptidase A type VIII (catalog no. P5380, Sigma; 1 mg/g tissue) and homogenized with tightly fitted Potter-Elvehjen homogenizer and Teflon pestle. The suspension was incubated for 1 minute (4°C) and rehomogenized. A 0.5 mL aliquot of cardiac muscle homogenate was reserved for Western blotting analysis of specific protein targets and the remaining homogenate was centrifuged at 14,500 g during 10 minutes. The supernatant fluid was decanted, and the pellet, essentially devoid of protease, was gently resuspended in isolation medium. The suspension was centrifuged at 750 g for 10 minutes, and the resulting supernatant was centrifuged at 12,000 g for 10 minutes. The pellet was resuspended and repelleted at 12,000 g for 10 minutes. The final pellet, containing the mitochondrial fraction, was gently resuspended in a washing medium containing 250 mM sucrose, 10 mM HEPES-KOH, pH 7.4. Mitochondrial protein concentration was spectrophotometrically estimated.
with the colorimetric method “RC DC protein assay” (Bio-Rad) using bovine serum albumin (BSA) as standard.

**Blue-native PAGE separation of mitochondria membrane complexes and in-gel activity of respiratory chain complex V**

BN-PAGE was performed using the method described by Schagger and von Jagow. Briefly, mitochondrial fractions (200 µg of protein) from each experimental group were pelleted by centrifugation at 20,000g for 10 minutes and then resuspended in solubilization buffer (50 mM NaCl, 50 mM Imidazole, 2 mM ε-amino n-caproic acid, 1 mM EDTA pH 7.0) with 1 % (w/v) digitonin. After 10 minutes on ice, insoluble material was removed by centrifugation at 20,000g for 30 minutes at 4°C. Soluble components were combined with 0.5 % (w/v) Coomassie Blue G250, 50 mM ε-amino n-caproic acid, 4 % (w/v) glycerol and separated on a 4-13 % gradient acrylamide gradient gel with 3.5 % sample gel on top. Anode buffer contained 25 mM Imidazole pH 7.0. Cathode buffer (50 mM tricine and 7.5 mM Imidazole pH 7.0) containing 0.02 % (w/v) Coomassie Blue G250 was used during 1 hour at 70 V, the time needed for the dye front reach approximately one-third of the gel. Cathode buffer was then replaced with one containing only 0.002 % (w/v) Coomassie Blue G250 and the native complexes were separated at 200 V for 4 h at 4°C. A native protein standard HMW-native markers (GE Healthcare, Buckinghamshire, UK) was used. The gels were stained with Coomassie Colloidal for protein visualization or incubated at 37 ºC with 35 mM Tris, 270 mM glycine buffer, pH 8.3, supplemented with 14 mM MgSO₄, 0.2 % (w/v) Pb(NO₃)₂, and 8 mM ATP for evaluation of the ATP hydrolysis activity of complex V. Lead phosphate precipitation that is proportional to the enzymatic ATP hydrolysis activity, was stopped by 50 % (v/v) methanol (30 min), and the gels were then transferred to water. Gels were scanned in Molecular Imager Gel Doc XR+.
System (Bio-Rad, Hercules, CA, USA). Band detection and analysis were performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

**Western blotting analysis**

Equivalent amounts of total protein from each group were electrophoresed on a 12.5 % SDS-PAGE as described by Laemmli. Gels containing total proteins or mitochondrial proteins (separated by 2-D BN-PAGE) were blotted onto a nitrocellulose membrane (Whatman®, Protan®) and nonspecific binding was blocked with 5 % (w/v) dry non-fat milk in TBS-T (100 mM Tris, 1.5 mM NaCl, pH 8.0 and 0.5 % Tween 20). Membranes were then incubated with primary antibody solution (1:1000 dilution; GAPDH, Santa Cruz, sc-47724; ATP synthase subunit beta, Abcam, ab-14730; mouse anti-SERCA2 ATPase, Abcam, ab2861; 3-nitrotyrosine, Chemicon, Clone 2A8.2). After 2 hours incubation, the membrane was washed with TBS-T and incubated with anti-mouse or anti-rabbit IgG peroxidase secondary antibody (1:1000 dilution, Amersham Pharmacia Biotech). Immunoreactive bands were detected with enhanced chemiluminescence reagents (ECL, Amersham Pharmacia Biotech) according to the manufacturer's procedure and images were recorded using X-ray films (Kodak Biomax light Film, Sigma). The films and the gels were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad) and analyzed with QuantityOne software version 4.6.3 (Bio-Rad, Hercules, CA). Equal loading of membranes was confirmed by staining the membranes with Ponceau S or GAPDH immunoblotting.

**MHC isoform determination**

Right ventricle samples were weighed and transferred to a glass homogenizer. A 1:19 ratio of 100 mM phosphate buffer, pH 7.4, containing 0.02% bovine serum albumin was added. Tissue sections were thoroughly homogenized with tightly fitted Potter-
Elvehjen homogenizer and Teflon pestle. Total protein concentration was spectrophotometrically assayed with the colorimetric method “RC DC protein assay” (Bio-Rad) using bovine serum albumin (BSA) as standard. Alpha- and beta-isofoms of cardiac myosin heavy chain were separated by gel electrophoresis following the procedure described by Talmadge and Roy. The amount of protein run on the gel was 1 mg per lane. To avoid inter-gel variation, one sample from each of the groups studied was applied in the same gel. The stacking gel consisted of 30% glycerol and 4% acrylamide: \( N,N' \)-methylene-bis-acrylamide in the ratio of 50:1, 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS). The separating gels were composed of 30% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. Polymerization was initiated with 0.05% \( N,N,N',N' \)-tetramethylethylenediamine and 0.1% ammonium persulfate. The gels were run in a Mini-Protean system (Bio-Rad) at 4°C. The running conditions were 70V (constant voltage) for 30 hours. The gels were stained with Coomassie Colloidal, scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) and optical density analysis of MHC bands was performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

**Relative quantification of mRNA**

Two-step real-time RT-PCR was performed as previously described. Briefly, after total mRNA extraction (no. 74124; Qiagen), standard curves were obtained for each gene correlating (R ≥ 0.98) the mRNA quantities in graded dilutions of a rat cardiac tissue sample with the respective threshold cycles (second derivative maximum method). Equal amounts of mRNA from every sample underwent three separate two-step realtime RT-PCR experiments for each gene, using SYBR green as marker (no.
GAPDH was used as internal control and results are relative to the mean obtained for the SED+Control group and normalized for GAPDH (fold increase). All the analysis was performed in duplicates. Specific PCR primer pairs for the studied genes are presented in Table S1.
### Table S1: Primers used in mRNA quantification by real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>fw: TGG CCT TCC GTG TTC CTA CCC</td>
</tr>
<tr>
<td></td>
<td>rev: CCG CCT GCT TCA CCA CCT TCT</td>
</tr>
<tr>
<td><strong>ET-1</strong></td>
<td>fw: CGG GGC TCT GTA GTC AAT GTG</td>
</tr>
<tr>
<td></td>
<td>rev: CCA TGC AGA AAG GCG TAA AAG</td>
</tr>
<tr>
<td><strong>TNF-alpha</strong></td>
<td>fw: TGG GCT ACG GGC TTG TCA CTC</td>
</tr>
<tr>
<td></td>
<td>rev: GGG GGC CAC CAC GCT CTT C</td>
</tr>
<tr>
<td><strong>VEGF-A</strong></td>
<td>fw: GTA CCT CCA CCA TGC CAA GT</td>
</tr>
<tr>
<td></td>
<td>rev: GCA TTA GGG GCA CAC AGG AC</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>fw: GAA GGA CCA GCT GGA CAA CAT</td>
</tr>
<tr>
<td></td>
<td>rev: CCT GGG GCA TCA CTT CTA CC</td>
</tr>
</tbody>
</table>

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ET-1: endothelin-1; TNF-alpha; tumor necrosis factor-alpha; VEGF-A: vascular endothelial growth factor-A; IL-10: interleukin-10; fw: forward; rev: reverse.
**Figure S1**: Illustration of the study design. The effects of exercise training were assessed at different time points of the disease. Animals were randomly divided as follows: i) sedentary animals injected with MCT or vehicle (SED+Control and SED+MCT, respectively), ii) 4 weeks-exercise training before MCT or vehicle injection (EXbefore+MCT and EXbefore+Control, respectively), iii) 4 weeks-exercise training after MCT or vehicle injection (EXafter+MCT and EXafter+Control, respectively) and iv) 2 weeks-exercise training after 2 weeks of MCT or vehicle injection (EXtreat+MCT and EXtreat+Control, respectively). The experimental design was programmed in order that all animals could receive one subcutaneous injection of MCT or vehicle solution at the age of 8 weeks. White square represents movement confined to the cage’s dimensions while grey squares represent the period of exercise training. After ending their respective protocols, all animals were submitted to hemodynamic evaluation (H.E.).
**Figure S2:** Effects of exercise training in LV ET-1 mRNA. Error bars are mean±SEM (n=7 animals per group). †P<0.001 vs. respective control group and ‡ P<0.01 vs. SED+MCT.
SUPPLEMENTAL REFERENCES


4. GENERAL DISCUSSION

In the present work, it was hypothesized that enhancing the ability of the heart to support pressure overload would prevent cardiac dysfunction and failure, and decrease the magnitude of activation of signaling pathways associated with cardiac maladaptation. Our data clearly shows that moderate exercise training induces a cardioprotective phenotype that improves the cardiac response to acute and chronic cardiac pressure overload. Exercise prevented cardiac dysfunction and attenuated the activation of several mediators related with the development of maladaptation. Moreover, intermittent chronic overload induced by beta-adrenergic stimulation with dobutamine promoted several cardiac adaptations that resembled those induced by exercise training and conferred protection to acute pressure overload.

In the first and second studies, we show that two hours of sustained constriction of the descending thoracic aorta results in severe functional impairment of the heart of sedentary animals. Our observation corroborates previous conclusions from both the RV (44, 86, 221) and LV (147, 179), that the normal healthy heart has a limited ability to tolerate acute workload demands. On its turn, exercised animals tolerated the two hours of pressure overload, without notorious deterioration of cardiac function, which is conform to the cardioprotective effects of exercise training against other cardiac insults (25, 38, 47, 49, 69-71, 105, 109, 150, 270). Reduced cardiac performance of sedentary animals was associated with disturbed calcium homeostasis (suggested by altered dP/dt_{min}, dP/dt_{max}, and increased mitochondrial swelling), increased apoptosis, NF-κB activation, and oxidative damage (especially of mitochondrial proteins), all of which have been implicated in the process of maladaptive remodeling (39, 68, 101, 125, 126, 158, 199, 243, 246, 269, 274). Morphological analysis revealed greater inter- and intra-group variability in terms of
cardiomyocytes’ injury threshold to the impact of the acute pressure overload. In the same microscopic field, it was possible to observe that some cardiomyocytes exhibited more damage (e.g. intracellular edema, mitochondrial swelling and apoptosis), and reactivity to the overload (e.g. increased Nf-KB expression), than others. These observations are in accordance with the notion that cardiomyocytes are characterized by structural and functional heterogeneity, which becomes more obvious when challenged by demanding situations (170, 194, 204, 223), where the most susceptible are injured, die and eventually are replaced (11, 62, 121, 122). Of note, although submitted to the same magnitude of overload, these alterations were scarcer in the heart from exercised animals, supporting the notion that exercise training provides chronic cardiac adaptations that translate into improved homeostasis (increased tolerance) and, consequently, less activation of signaling pathways implicated in the maladaptive remodeling of the heart. Exercise training induced cardiomyocyte growth (and eventually hyperplasia), which, according to the Laplace law of the heart, result in a relatively smaller increase in wall tension per unit volume of myocardium (5). Preserved dP/dt\text{\textsubscript{min}} (study I and II) after acute pressure overload and increased SERCA2a expression (study II), indirectly suggest improved calcium handling (51, 177) and thus, lower cytosolic calcium accumulation, explaining the reduced mitochondrial swelling and lower levels of apoptosis found in study I (71, 72, 238). Also, exercise induced an increase in phospho-Akt, which is known to modulate SERCA2a activity and LTCC stability, thus improving calcium kinetic and cardiomyocyte contractility (35, 76, 129, 176). Reduced apoptosis, as well as lower levels of total oxidative damage may be related with increased anti-oxidant defenses, namely MnSOD (71, 253). The lower damage to mitochondrial proteins (study I) together with increased mitochondrial ATP production (study II) in exercised animals
suggest increased mitochondrial functionality (28, 33, 80, 108, 159, 202, 250), thus contributing to the higher cardiac performance during pressure overload. A schematic representation of these findings is presented in Figure 2 and 3.

Figure 2- Exercise training increases tolerance to left ventricular acute pressure overload. Exercise training induced several chronic adaptations that translated into an enhanced cardiac performance against pressure overload and its deleterious effects such as structural derangements and biochemical alterations. Our main findings are reported in the yellow rectangles. Detailed information is provided in the text. Ca\(^{2+}\): calcium; K\(^{+}\): potassium; Na\(^{+}\): sodium; LTCC: L-type calcium channels; RyR: ryanodine receptor; SERCA: sarcoplasmic reticulum calcium-ATPase; PLN: phospholamban; RONS: reactive oxygen and nitrogen species; MnSOD: manganese superoxide dismutase; Nf-KB: nuclear factor kappa B; Cyt C: cytochrome C.

In the second study, we also evaluated whether the development of a cardioprotective phenotype is uniquely provided by exercise training, or if it could be induced by other stimuli. We hypothesized that using a stimulus of different nature that mimicked the duration and magnitude of the overload induced by exercise training could result in an adaptive phenotype. To test our hypothesis, we used dobutamine, a beta 1- and 2-adrenoreceptor agonist in the concentration of 2 mg/kg.
s.c.). By performing a series of acute hemodynamic studies, we found that this dosage reasonably mimics the duration and magnitude of an acute cardiac overload imposed by the exercise training protocol (~40% increase in heart rate and ~15% increase in peak systolic pressure) (175). This strategy allowed us to have a certain control over the magnitude and duration of the hemodynamic demand that was imposed. Animals chronically treated with 2 mg/kg of dobutamine (5 days/week during 8 weeks) developed an overall cardiac phenotype that resembled several features of adaptive remodeling. Namely, they developed hypertrophy, with normal levels of osteopontin-1, collagen and calcineurin, which are typically elevated in maladaptive remodeling (16, 176, 271). Also, similar MHC isoforms composition as well as a similar increase in phospho-Akt/mTOR, total SERCA2a and oxidative phosphorylation was observed in both exercised and dobutamine-treated animals. In order to test whether the cardiac phenotype induced by dobutamine was cardioprotective, we submitted dobutamine-treated animals to sustained acute pressure overload for two hours. Remarkably, both exercised and dobutamine-treated animals exhibited a similar performance in response to the overload, preventing cardiac dysfunction. Although our data does not allow to make any cause-effect assumption, it is possible that the above-mentioned adaptations (cardiomyocyte hypertrophy, increased SERCA2a, phospho-Akt/mTOR and mitochondrial activity) may partially explain the increased tolerance to pressure overload (Figure 3). These data suggest that the cardiac overload induced by chronic intermittent beta-adrenergic stimulation resulted in an adaptive phenotype, favoring the notion that the duration of overload may indeed be a determinant factor for the development of an adaptive or maladaptive phenotype (152). Indeed, even the exercise benefits seem to be time-dependent since prolonged bouts of exercise performed for long periods have been
associated with the development of several features of maladaptive phenotypes such as cardiac dysfunction, fibrosis and cellular death (4, 5, 13, 42, 109, 186, 192, 201, 261, 263). Altogether, these data suggest that cardiac adaptation or maladaptation can be a consequence of the severity and/or duration of the stimuli together with improper recovery, independently of the stimuli’s nature. When the imposed stress is too severe or prolonged, the cells might not be able to recover homeostasis, their integrity can be compromised and cellular death pathways might be favored, progressively

Figure 3- Chronic intermittent workload induced by dobutamine, promoted cardiac adaptations (yellow rectangles) that resembled exercise training. These alterations, together with cardiomyocyte hypertrophy, could underlie the increased tolerance to left ventricular acute pressure overload. Detailed information is provided in the text. MMP: metalloproteinase; Ca\(^{2+}\): calcium; LTCC: L-type calcium channels; RyR: ryanodine receptor; SERCA: sarcoplasmic reticulum calcium-ATPase; PLN: phospholamban; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; GSK-3: glycogen synthase kinase; mTOR: mammalian target of rapamycin; p-NFAT: phosphorylated nuclear factor of activated T cells; NFAT: dephosphorylated nuclear factor of activated T cells; MHC: myosin heavy chain.

contributing to maladaptation (40, 75, 137, 138, 171). On its turn, if there is a perfect match between the stress demands and the cellular responses, pro-survival pathways
are preferentially activated, an improved homeostatic capacity (increased tolerance) can be attained (40), and an adaptive phenotype takes place.

After showing that exercise training increased tolerance to acute pressure overload, we wanted to know if the same would be true against chronic pressure overload. To accomplish this aim we changed our focus to the RV and used a model of RV chronic pressure overload induced by monocrotaline (MCT). MCT is a pyrrolizidine alkaloid found in the plant *Crotolaria spectabilis*. After being bioactivated in the liver, its bioactive metabolite selectively injures the vascular endothelium of the lung and induces an increase in vascular resistance and pulmonary arterial pressure, with subsequent RV hypertrophy (27, 124, 228). With a dosage of 60 mg/kg, RV pressure overload is observed around 14 days after its administration (228), and RV hypertrophy progresses to failure and death around day 28 (45, 66, 94, 99, 100). Therefore, we trained rats at different time points of RV pressure overload (before, during and after its establishment) and evaluated the preventive and therapeutic roles of exercise training. We hypothesized that exercise training would act as an upstream modulator of the multiple signaling pathways implicated in RV dysfunction and failure (20, 31, 45, 66, 98, 131, 163, 209, 216). Our results show that exercise training performed before (preconditioning), during or after RV chronic pressure overload establishment prevents from cardiac dysfunction and improves survival. The underlying mechanisms may be associated with the prevention of calcium handling abnormalities (128, 220, 265) and alpha to beta MHC shift (96, 200), capillary density preservation (76, 93, 94), decreased neurohumoral activation (14, 46, 79), collagen deposition and inflammation (2, 139, 142, 176, 222, 268), preserved mitochondrial function and reduced oxidative damage (2, 217, 235, 253) that was found in all exercised groups. A schematic overview of these findings is provided in Figure 4. Our data, together with previous work from other groups (20, 59, 216) suggest that therapies that improve RV function through the modulation of these pathways may be an interesting strategy for PAH prevention and treatment. The overall improvements exhibited by those animals that were exercised during or after RV overload were independent of any pulmonary hypotensive effect of exercise training. Indeed, animals from these groups showed RV peak systolic pressure values comparable to their sedentary overloaded counterparts, but without compromise of cardiac function. From here it is possible to conclude that exercise training provides a
cardioprotective phenotype that allows the RV to work under overloading conditions with better tolerance. Similar observations were previously reported in the LV, where exercise training was demonstrated to prevent cardiac dysfunction in different animal models of chronic pressure overload, independently of any hypotensive effect (21, 76, 176). Exercise preconditioning (exercise before RV overload) prevented from significant afterload elevation which, together with previous studies (47, 49, 69, 70), support the notion that the exercise benefits can be sustained for several weeks after cessation. Although our data does not provide any explanation for the lower RV overload observed in this group, it is possible that the four weeks of training that anticipated MCT administration were sufficient to provide a more resistant vascular

---

**Figure 4**- Exercise training prevents against right ventricular chronic pressure overload damage. Exercise training induced a series of improvements (yellow rectangles) that collectively were associated with improved cardiac function. VEGF: vascular endothelial growth factor; ET-1: endothelin-1; TNF-alpha: tumor necrosis factor-alpha; IL-10: interleukin-10; Ca²⁺: calcium; K⁺: potassium; Na⁺: sodium; LTCC: L-type calcium channels; RyR: ryanodine receptor; SERCA: sarcoplasmic reticulum calcium-ATPase; PLN: phospholamban;
endothelial phenotype, and thus decreased the overload imposed by the vascular remodeling of the lungs. Indeed, increased endothelial progenitor cells induced by exercise training were associated with enhanced endothelial regenerative capacity (144, 149, 218), and inhibition of the formation of neointima after carotid artery injury (144).
5. MAIN CONCLUSIONS

Considering the overall findings supported by our studies, the main conclusions that derive from them and that we would like to highlight are:

1. Exercise training improved cardiac tolerance to sustained acute pressure overload and prevented from cardiac dysfunction observed in sedentary animals.

2. The improved hemodynamic response of exercised animals was associated with less ultra-structural damage of the cardiomyocytes, lower expression of NF-kB and active form of caspase-3 and decreased oxidative damage to cardiac proteins.

3. Mitochondria were found to be an early and preferential target of oxidative damage induced by acute pressure overload, with aconitate hydratase and ATP synthase alpha subunit identified as the proteins more susceptible to carbonilation and ATP synthase beta as the more prone to nitration.

4. Tolerance to acute pressure overload reflected by improved functional, structural and molecular integrity may be related with the exercise-induced adaptive phenotype.

5. Chronic intermittent cardiac overload induced by beta-adrenergic stimulation induced a cardiac phenotype that resembled several features the one induced by exercise training, namely similar MHC isoforms composition, Akt/mTOR activation, increased SERCA2a expression and mitochondrial activity. Like in exercise training, cardiomyocyte hypertrophy was not accompanied by fibrosis or osteopontin-1 and calcineurin up-regulation.
6. Similarly to exercise training, chronic intermittent cardiac overload induced by beta-adrenergic stimulation increased the cardiac tolerance to an acute sustained pressure overload, preventing cardiac dysfunction.

7. Besides the nature, the duration and the magnitude of the stimuli may be determinant for the development of an adaptive or maladaptive cardiac phenotype.

8. Exercise preconditioning, as well as exercise performed during or after the establishment of RV chronic pressure overload secondary to MCT-induced PAH averts RV dysfunction and improves survival. Exercise training seems to provide a cardioprotective phenotype that allows the RV to work under overloading conditions with better tolerance.

9. The cardioprotective effects of exercise training seem to persist for several weeks after exercise cessation.

10. Exercise can act as an upstream modulator of several pathways activated in the RV during PAH. The benefits of exercise training may be associated with the prevention of calcium handling disturbances, alpha to beta-MHC shift, decreased neurohumoral activation, collagen deposition and inflammation and preserved oxidative phosphorylation through the reduction of mitochondrial oxidative damage.
6. REFERENCES


