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Heart failure-induced skeletal myopathy in spontaneously hypertensive rats ☆

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ABSTRACT

Background: Although skeletal muscle atrophy and changes in myosin heavy chain (MyHC) isoforms have often been observed during heart failure, their pathophysiological mechanisms are not completely defined. In this study we tested the hypothesis that skeletal muscle phenotype changes are related to myogenic regulatory factors and myostatin/follistatin expression in spontaneously hypertensive rats (SHR) with heart failure.
Methods: After developing tachypnea, SHR were subjected to transthoracic echocardiogram. Pathological evidence of heart failure was assessed during euthanasia. Age-matched Wistar–Kyoto (WKY) rats were used as controls. Soleus muscle morphometry was analyzed in histological sections, and MyHC isoforms evaluated by electrophoresis. Protein levels were assessed by Western blotting. Statistical analysis: Student's *t* test and Pearson correlation.
Results: All SHR presented right ventricular hypertrophy and seven had pleuropericardial effusion. Echocardiographic evaluation showed dilation in the left chambers and left ventricular hypertrophy with systolic and diastolic dysfunction in SHR. Soleus weight and fiber cross sectional areas were lower (WKY 3615±412; SHR 2035±224 μm^2; $P<0.001$), and collagen fractional volume was higher in SHR. The relative amount of type I MyHC isoform was increased in SHR. Myogenin, myostatin, and follistatin expression was lower and MRF4 levels higher in SHR. Myogenin and follistatin expression positively correlated with fiber cross sectional areas and MRF4 levels positively correlated with I MyHC isoform.
Conclusion: Reduced myogenin and follistatin expression seems to participate in muscle atrophy while increased MRF4 protein levels can modulate myosin heavy chain isoform shift in skeletal muscle of spontaneously hypertensive rats with heart failure.

1. Introduction

Heart failure is characterized by reduced exercise tolerance with early occurrence of fatigue and dyspnea. In addition to cardiac dysfunction and pulmonary abnormalities, intrinsic skeletal muscle changes can be involved in reduced physical capacity [1–4]. Several skeletal muscle abnormalities have been described in patients and animals with heart failure; these include atrophy, fibrosis, altered myosin heavy chain (MyHC) composition, and decreased oxidative capacity [1,2,5–9].

The mechanisms and intracellular signaling pathways involved in heart failure-induced skeletal muscle abnormalities are not completely understood. There is substantial evidence that myogenic regulatory factors (MRF) MyoD, myogenin, Myf5, and MRF4 act as important regulators of muscle protein expression [10–12]. MRFs are transcriptional factors that activate the expression of muscle specific genes. Myogenin is preferentially expressed in slow twitch fibers and modulates the expression of oxidative enzymes [13]. MyoD, which is preferentially expressed in fast muscle fibers, participates in muscle regeneration [11,14]. MRF4 may play a role in preserving differentiated muscle state [15]. Few studies have evaluated MRFs during heart failure. Our laboratory has observed decreased mRNA levels for MyoD and MRF4 in soleus muscle of rats with rapid onset heart failure [16] while rats with chronic heart failure presented decreased myogenin protein levels [5].

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Myostatin, a member of the transforming growth factor-beta superfamily, modulates muscle growth, acting as a negative regulator of skeletal muscle mass [17,18]. Studies on its physiological role have shown a negative correlation between myostatin gene expression and muscle mass, suggesting a potential to induce skeletal muscle atrophy [17,19,20]. Myostatin activity can be modulated by various proteins. Follistatin has emerged as a potent myostatin antagonist which acts by modifying its *in vivo* activity [21–23]. Few authors have assessed the myostatin/follistatin pathway in heart failure [6,24]. In our laboratory, rats with myocardial infarction-induced heart failure presented muscle atrophy in combination with unchanged myostatin levels and decreased follistatin protein expression [6].

One widely used experimental model for studying heart failure is the spontaneously hypertensive rat (SHR). It presents early arterial hypertension and left ventricular hypertrophy which evolves to heart failure during maturity and senescence. As cardiac failure development is slow, SHRs are considered a useful model to mimic clinical heart failure settings. In this study we characterized skeletal muscle changes of SHR with heart failure by evaluating muscle trophism, fibrosis, and myosin heavy chain isoforms. We tested the hypothesis that heart failure-induced skeletal muscle phenotype changes are related to myogenic regulatory factors and myostatin/follistatin protein expression changes.

2. Materials and methods

2.1. Experimental groups

Male spontaneously hypertensive rats (SHR) and non-hypertensive Wistar–Kyoto (WKY) rats were purchased from the Central Animal House at Botucatu Medical School, UNESP. All animals were housed in a room under temperature control at 23 °C and kept on a 12-hour light/dark cycle. Food and water were supplied *ad libitum*. All experiments and procedures were approved by the Ethics Committee of Botucatu Medical School, UNESP, Botucatu, SP, Brazil.

Systolic arterial pressure was measured by the tail-cuff method at fifteen months of age. Beginning at 18 months old, all rats were observed twice weekly to identify clinical heart failure features. Animals were studied after heart failure had been detected, which was characterized by tachypnea and labored respiration. Age matched WKY rats were studied at comparable ages. After diagnosing heart failure, rats were subjected to transthoracic echocardiography and euthanized two days after. During euthanasia, we evaluated pathological evidence of heart failure such as pleuropericardial effusion, left atrial thrombi, ascites, pulmonary congestion (lung weight-to-body weight ratio > 2 standard deviations above the mean for the WKY group) [25], and right ventricular hypertrophy (right ventricle weight-to-body weight ratio > 0.8 mg/g) [26–29].

At the time of euthanasia, the animals were weighed, anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and decapitated. The heart was removed by thoracotomy and the atria and ventricles were separated and weighed. Both right and left soleus muscles were excised, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C. Fragments of liver were weighed before and after drying sessions (65 °C for 72 h) to evaluate wet/dry weight ratio.

2.2. Echocardiographic study

Echocardiographic evaluation was performed using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5–11.5 MHz multifrequency transducer. Rats were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). A two-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral-valve leaflets, and at the level of the aortic valve and left atrium [29,30]. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV structures were manually measured by the same observer according to the leading-edge method of the American Society of Echocardiography [31]. The measurements obtained were the mean of at least five cardiac cycles on the M-mode tracings. The following structural variables were measured: left atrium (LA) diameter, LV diastolic and systolic dimensions (LVDD and LVSD, respectively), LV diastolic posterior wall thickness (PWT), LV diastolic septal wall thickness (SWT), and aortic diameter (AO). Left ventricular function was assessed by the following parameters: heart rate (HR), endocardial fractional shortening (FS), LV ejection fraction (EF), posterior wall shortening velocity (PWSV), early-to-late diastolic mitral inflow ratio (E/A ratio), E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT).

2.3. Morphologic study

Serial transverse sections of the soleus muscles were cut at 8 μm thickness in a cryostat cooled to −20 °C and stained with hematoxylin and eosin. At least 150 cross-sectional fiber areas were measured from each soleus muscle. Other slides were stained with Sirius Red F3BA and used to quantify interstitial collagen fraction [32]. On average, 20 microscopic fields were analyzed with a 40X lens. Perivascular collagen was excluded from this analysis. Measurements were taking using a compound microscope (Leica DM LS; Nussloch, Germany) attached to a computerized imaging analysis system (Media Cybernetics, Silver Spring, MD, USA).

2.4. Myosin heavy chain (MyHC) isoforms

MyHC isoform analysis was performed in duplicate by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Frozen samples of soleus muscle (100 mg) were mechanically homogenized on ice in 0.8 mL of protein extraction solution containing 50 mM phosphate potassium buffer (pH 7.0), 0.3 M sucrose, 0.5 mM dithiotreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 mM phenylmetylsulphonyl fluoride (PMSF), 10 mM sodium fluoride and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Homogenates were centrifuged at 12,000 × *g* at 4 °C for 20 min to remove insoluble tissue. Total protein quantification was performed in supernatant aliquots by the Bradford method. Samples were then diluted to a final concentration of 1 μg of protein/μL in a solution containing 65% (vol/vol) glycerol, 2.5% (vol/vol) 2-mercaptoethanol, 1.15% (wt/vol) SDS, and 0.45% (wt/vol) Tris–HCl (pH 6.8). Small amounts of the diluted extracts (15 μL) were loaded onto a 7–10% SDS-PAGE separating gel with a 4% stacking gel, run overnight (24–30 h) at 120 V, and stained with Coomassie blue. Two MyHC isoforms, MyHC I and MyHC IIa, were identified according to the molecular mass and quantified by densitometry. Their relative amounts were expressed as the percentage of the total amount of myosin heavy chain.

2.5. Western blotting analysis

Protein levels of soleus muscle were analyzed by Western blotting according to a previously described method [33,34] with specific anti-myogenin (M-225, sc-576), anti-MyoD (M-318, sc-760), anti-MRF4 (C-19, sc-301), anti-myostatin (N-19-R sc-6885-R) or anti-follistatin (H-114 sc-30194) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein levels were normalized to those of GAPDH (6 C5, sc-32233, Santa Cruz Biotechnology). Muscle protein was extracted using Tris-Triton buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate). Supernatant protein content was quantified by the Bradford method. Samples were separated on a polyacrylamide gel and then transferred to a nitrocellulose membrane. After blockage, membrane was incubated with the primary antibody. Membrane was washed with TBS and Tween 20 and incubated with secondary peroxidase-conjugated antibody. Super Signal® West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, USA) was used to detect bound antibodies.

2.6. Statistical analysis

Data are expressed as mean ± standard deviation. Comparisons between the groups were performed by Student's-*t* test. Associations between variables were assessed with Pearson's correlation coefficient. The level of significance was set at 5%.

3. Results

3.1. Group characterization and anatomic parameters

In SHR ($n = 8$), all rats presented right ventricular hypertrophy, 7 had pleuropericadial effusion, 5 pulmonary congestion, 4 atrial thrombi, and 3 ascites. There was no clinical or pathological evidence of heart failure in WKY ($n = 9$). Blood pressure and anatomical data are shown in Table 1. Blood pressure was higher and body weight lower in SHR. LV, right ventricle, and atria weight, in both absolute or body weight normalized values, were greater in SHR than WKY. Liver wet weight-to-dry weight, lung weight, and lung-to-body weight ratio were higher in SHR. Soleus weight was lower in SHR (WKY 0.157 ± 0.029; SHR 0.129 ± 0.021 g; $P = 0.044$).

3.2. Echocardiographic evaluation

Structural cardiac parameters are shown in Table 2. LV diastolic diameter (LVDD) and left atrium diameter-to-aortic diameter ratio were similar between groups. LVDD-to-body weight ratio, LV systolic diameter, LV diastolic posterior wall thickness, LV diastolic septal wall