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 μm²; 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 were 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.

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Follistatin protein expression changes. Changes are related to myogenic regulatory factors and myostatin/hypertension and left ventricular hypertrophy which evolves to heart failure by evaluating 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), and right ventricular enlargement. All animals were weighed before and after euthanasia.

2.2. Echocardiographic study

Echocardiographic evaluation was performed using a commercially available echocardiograph (General Electric Medical Systems, Vivid 5, Tirit 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 LVDs, 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 taken 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 diethiothreitol (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/mL in a solution containing 65% (vol/vol) glycerol, 2.5% (vol/vol) 2-mercaptoethanol, 1.15% (wt/vol) SDS, and 0.45% (vol/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 blocking, 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 pleuroperticardial 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 thickness.
Table 1
Blood pressure and anatomic data.

<table>
<thead>
<tr>
<th></th>
<th>WKY (n = 9)</th>
<th>SHR (n = 8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (mmHg)</td>
<td>118±10</td>
<td>221±35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BW (g)</td>
<td>414±57</td>
<td>295±29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV weight (g)</td>
<td>0.80±0.14</td>
<td>1.18±0.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV weight (g)</td>
<td>0.26±0.04</td>
<td>0.50±0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Atria (g)</td>
<td>0.097±0.018</td>
<td>0.243±0.078</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV weight/BW (mg/g)</td>
<td>1.94±0.24</td>
<td>3.97±0.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV weight/BW (mg/g)</td>
<td>0.62±0.08</td>
<td>1.79±0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Atria/BW (mg/g)</td>
<td>0.27±0.02</td>
<td>0.82±0.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver wet/dry (g)</td>
<td>3.28±0.07</td>
<td>5.33±0.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lung (g)</td>
<td>2.32±0.09</td>
<td>3.65±1.00</td>
<td>0.002</td>
</tr>
<tr>
<td>Lung/BW (mg/g)</td>
<td>5.67±1.13</td>
<td>12.39±3.35</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

WKY: normotensive rats; SHR: spontaneously hypertensive rats; n: number of animals; BW: body weight; LV: left ventricle; RV: right ventricle. Data are expressed as the mean± standard deviation. Student’s t test.

Table 2
Echocardiographic data.

<table>
<thead>
<tr>
<th></th>
<th>WKY (n = 9)</th>
<th>SHR (n = 6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD (mm)</td>
<td>8.01±0.95</td>
<td>8.64±1.46</td>
<td>0.330</td>
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<tr>
<td>LVDD/BW (mm/kg)</td>
<td>19.6±3.1</td>
<td>27.7±4.7</td>
<td>0.001</td>
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<tr>
<td>LVSD (mm)</td>
<td>3.88±0.52</td>
<td>5.08±1.51</td>
<td>0.043</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>1.47±0.06</td>
<td>1.82±0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SWT (mm)</td>
<td>1.49±0.05</td>
<td>1.82±0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AO (mm)</td>
<td>4.49±0.38</td>
<td>4.98±0.34</td>
<td>0.024</td>
</tr>
<tr>
<td>LA (mm)</td>
<td>5.73±0.59</td>
<td>7.28±1.17</td>
<td>0.004</td>
</tr>
<tr>
<td>LA/AO</td>
<td>1.29±0.19</td>
<td>1.47±0.26</td>
<td>0.139</td>
</tr>
<tr>
<td>LA/BW (mm/kg)</td>
<td>13.9±0.89</td>
<td>23.3±3.43</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

WKY: normotensive rats; SHR: spontaneously hypertensive rats; n: number of animals; LVDD and LVSD: left ventricle (LV) diastolic and systolic diameters, respectively; BW: body weight; PWT: LV diastolic posterior wall thickness; SWT: diastolic septal wall thickness; AO: aortic diameter; LA: left atrium diameter. Data are expressed as the mean± standard deviation. Student’s t test.

3.3. Morphologic study

Fiber cross sectional areas were lower (WKY 3.615±412; SHR 2.035±224 μm²; P<0.001; Fig. 1), and collagen fractional area was higher (WKY 2.61±0.39; SHR 4.88±0.98%; P<0.001) in SHR.

3.4. Myosin heavy chain (MyHC) isoforms

The relative amount of the MyHC I isoform was higher in SHR than WKY (WKY 80.4±3.33%; SHR 91.2±3.33%; P=0.003; Fig. 2).

3.5. Western blotting analysis

Myogenin protein levels were lower and MRF4 levels were higher in SHR (myogenin: WKY 1.00±0.16; SHR 0.64±0.35 arbitrary units; P=0.02; MRF4: WKY 1.00±0.27; SHR 1.53±0.47 arbitrary units; P=0.014; Fig. 3). MyoD was not statistically different between groups (WKY 1.00±0.16; SHR 0.89±0.22 arbitrary units; P=0.29; Fig. 3). Myostatin and follistatin protein levels were lower in SHR (myostatin: WKY 1.00±0.18; SHR 0.69±0.24 arbitrary units; P=0.01; follistatin: WKY 1.00±0.17; SHR 0.65±0.16 arbitrary units; P=0.001; Fig. 4). The myostatin-to-follistatin expression ratio did not differ between groups (P>0.05). Myogenin levels positively correlated with myocyte cross sectional area (r=0.55; P=0.033). MRF4 levels were positively correlated with relative amount of MyHC isofrom 1 (r=0.622; P=0.013); there was a trend for MRF4 levels to negatively correlate with myocyte cross sectional area (r=−0.45; P=0.08).

4. Discussion

In this study, we showed that heart failure-induced skeletal myopathy in spontaneously hypertensive rats is characterized by atrophy, fibrosis, and changed myosin heavy chain isoforms. Phenotypical muscle alterations are combined with myogenic regulatory factors and myostatin and follistatin protein expression changes.

To the best of our knowledge, this is the first study to evaluate skeletal muscle in SHR with heart failure. The spontaneously hypertensive rat was introduced by Okamoto and Aoki as a genetic model of arterial hypertension[35]. Beginning at 18 months of age, rats develop heart failure features and evolve to death usually within...
In this study, heart failure diagnosis was based on the presence of tachypnea and labored respiration and confirmed by pathological post mortem findings such as right ventricular hypertrophy, pleuropulmonary congestion, left atrial thrombus, and ascites. Systemic congestion was confirmed by the increased liver wet weight-to-dry weight ratio in the SHR group. Transthoracic echocardiography showed that SHR presented dilation of the left cardiac chambers, LV wall hypertrophy, and LV systolic dysfunction.

Skeletal muscle evaluation was performed in the soleus muscle, a muscle susceptible to acute and chronic heart failure conditions [7,36,37]. SHR presented significant muscle atrophy characterized by a 43.7% decrease in average muscle fiber cross sectional area compared to WKY. Decreased muscle fiber cross sectional area or diameter has been commonly observed in experimental heart failure, reducing between 9.7% and 16.5% from control values [6,7,16,38]. The significant degree of atrophy observed in SHR makes it particularly useful in studies for identifying the mechanisms and intracellular signaling pathways involved in heart failure-induced muscle atrophy.

Changes of skeletal MyHC isoform distribution have frequently been described during cardiac failure. In this study we found a relative increase of MyHC I isoform in SHR, suggesting a shift of muscle phenotype toward type I slow fibers, which have a predominantly oxidative metabolism [39]. During heart failure, other studies have reported both preserved MyHCs distribution [4,40] and increased MyHC II isoform [7,36,41,42], which contrast with our results. Six month-old SHRs have shown decreased MyHC I compared to age-matched WKY rats [43]. The variety of MyHC isoform distribution values seen during heart failure suggests that the molecular mechanisms involved in MyHC isoform modulation differ according to the heart failure model used [5,7,44].

We evaluated myogenic regulatory factors and myostatin/follistatin protein expression as potential mechanisms responsible for muscle atrophy and MyHC isoform changes. We observed decreased myogenin, myostatin, and follistatin; increased MRF4; and unchanged MyoD protein levels in the SHR group.

Myogenin is involved in satellite cells differentiation and myotube formation [13,45]. Decreased myogenin expression impairs fiber regeneration and may thus contribute to muscle atrophy [46]. In our study, decreased myogenin expression in SHR and a positive correlation between fiber cross-sectional area and myogenin levels suggest that this myogenic regulatory factor is involved in muscle atrophy. A similar result has been observed in the soleus muscle of rats with myocardial infarction-induced heart failure [5]. Myogenin is predominantly related to oxidative metabolism; one would therefore expect its reduced expression to have induced a decrease in type I MyHC isoform. However, we found increased type I MyHC isoform in SHR. As we evaluated the proportion of MyHCs isoforms in relation to total MyHC, we cannot rule out the possibility of a reduction in absolute amount of type I isoform. The mechanisms involved in myogenin reduction during heart failure are not completely understood. In cell culture, myogenin expression is...
suppressed in differentiating myocytes treated with tumor necrosis factor-α, a cytokine which commonly increases during heart failure [46].

Myogenic regulatory factor MR4 is predominantly expressed in slow oxidative fibers, and appears to play a role in modulating muscle phenotype. In this study, increased MR4 protein expression in SHR was probably involved in the increased percentage of predominantly oxidative type I MyHC isoforms. Reinforcing this hypothesis, we observed a positive correlation between MR4 protein expression and the relative amount of MyHC isoform I. The mechanisms responsible for changes in MR4 expression are not clear. Myostatin-deficient double-muscled Japanese Shorthorn cattle exhibit increased MR4 gene expression [47], suggesting that the decreased myostatin levels in our study may have played a role in increased MR4 expression.

Increased myostatin expression has often been associated with reduced muscle mass in chronic diseases [48,49] and experimental muscle atrophy models [17,50]. Therefore, it was surprising to find decreased myostatin expression combined with significant muscle atrophy in our SHR group. Myostatin activity can be modulated by decreased myostatin expression in cell culture preparations [53], its decreased levels to muscle mass [52]. It has also been suggested that follistatin decreases skeletal muscle atrophy. Few authors have investigated the myostatin pathway in heart failure [14]. Expression of the myostatin gene is markedly decreased in the myocardium and skeletal muscle in a chronic heart failure model. Eur J Heart Fail 2009;11:342–8.

Increased myostatin expression can modulate myosin heavy chain expression in cell culture preparations [53], its decreased levels to muscle mass [52]. It has also been suggested that follistatin decreases skeletal muscle fibrosis and improves muscle healing after injury and disease [53,54]. Reduced follistatin levels in our study may have contributed to muscle fibrosis and atrophy despite reduced myostatin expression in the SHR group. Additionally, as myostatin can increase myogenin expression in cell culture preparations [53], its decreased levels could be involved in down-regulating myogenin in the SHR group. Few authors have investigated the myostatin pathway in cardiac failure [6,24]. Lenk et al. [24] observed that following experimental myocardial infarction, myostatin was up-regulated in gastrocnemius muscle, with values returning to baseline levels after exercise training. We previously observed that rats with long-term myocardial infarction–induced heart failure present muscle atrophy in combination with unchanged myostatin levels and decreased follistatin protein expression [6]. Additional studies are needed to better elucidate the relationship between myogenic regulatory factors, myostatin and the follistatin pathway, and muscle phenotype changes during heart failure.

In conclusion, heart failure–induced skeletal myopathy of spontaneously hypertensive rats is characterized by atrophy, fibrosis, and changed myosin heavy chain isoforms. Reduced myogenin and follistatin protein expression seems to participate in muscle atrophy while increased MR4 protein expression can modulate myosin heavy chain isoform shift in the soleus muscle.

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