Time course of ubiquitin-proteasome and macroautophagy-lysosome pathways in skeletal muscle in rats with heart failure

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ABSTRACT

Patients with heart failure have limited exercise capacity due to not only myocardial dysfunction but also skeletal muscle atrophy. However, the mechanisms and time course of protein degradation in skeletal muscle during heart failure remain unclear, and there is no established standard treatment. The purpose of the present study was to investigate the time course of major protein degradation pathways in skeletal muscle during heart failure. Four-week-old male Wistar rats were randomly assigned to heart failure induced by monocrotaline or control groups. At 14 and 21 days after monocrotaline injection, the lungs, heart, and gastrocnemius and soleus muscles were removed and analyzed. There was no significant difference in body weight between the groups at 14 days after monocrotaline injection. Although there were no morphological changes in the skeletal muscle of the monocrotaline group at this time point, ubiquitin-proteasome and macroautophagy-lysosome pathways were activated in the monocrotaline group. Additionally, the pathways were less strongly activated in the soleus muscle than in the gastrocnemius muscle. These results suggest that physical exercise that shifts to slow muscle characteristics should begin when there is no indication of skeletal muscle atrophy to prevent exercise intolerance with heart failure.

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Abbreviations: atrogin-1, atrogin-1/MAFbx; MuRF-1, muscle RING finger 1; LC3, microtubule-associated protein 1 light chain 3; p62, p62/SQSTM1; MCT group, heart failure induced by monocrotaline group; Cont group, age-matched control group; ATPase, adenosine triphosphatase; SDH, succinate dehydrogenase; qPCR, quantitative polymerase chain reaction; TNF-α, tumor necrosis factor-alpha; ROS, reactive oxygen species
skeletal muscle (14). In this pathway, proteins ubiquitinated by muscle-specific ubiquitin ligases, atrogin-1/MAFbx (atrogin-1) and muscle RING finger 1 (MuRF-1), are digested by the 26S proteasome (15). However, the 26S proteasome cannot digest aggregated proteins (26). The macroautophagy-lysosome pathway is implicated as a compensatory pathway for proteasomal protein degradation (20) and can remove aggregated proteins. In this pathway, autophagosomes labeled with microtubule-associated protein 1 light chain 3 (LC3) are delivered to the lysosome for protein degradation (12). Labeled autophagosomes are tagged by p62/SQSTM1 (p62) with both LC3- and ubiquitin-binding domains (3); therefore, p62 is known as a mediator for targeting ubiquitin-tagged proteins to the autophagy system. Activation of the ubiquitin-proteasome and macroautophagy-lysosome pathways has been reported in atrophied skeletal muscle induced by heart failure (28). However, the mechanisms of protein degradation in skeletal muscle during heart failure remain unclear, and there is no established standard treatment.

Physical exercise is an effective therapeutic intervention to prevent and ameliorate skeletal muscle atrophy induced by diverse conditions. Exercise attenuates enhanced protein degradation in atrophied skeletal muscle (1), and regular training prevents atrophy (13). However, exercises to combat skeletal muscle atrophy induced by heart failure can worsen myocardial dysfunction. Handoko et al. (16) reported that regular exercise after heart failure induced myocardial inflammation and increased the survival rate. In this study (16), exercise was found to be beneficial in the stable stage but detrimental in the progressive stage, suggesting that the effects of exercise on skeletal muscle atrophy could depend on the stage in heart failure. Therefore, determination of the time course of protein degradation affecting skeletal muscle atrophy in heart failure might allow identification of a stage with low risk for progression of myocardial dysfunction and high skeletal muscle adaptability to exercise. The purpose of the present study was to investigate the time course of major protein degradation pathway activities in skeletal muscle during heart failure.

MATERIALS AND METHODS

Experimental design. This study was approved by the Institutional Animal Care and Use Committee of Hiroshima University (A13-30) and was carried out according to the Hiroshima University Regulations for Animal Experimentation. All experiments were conducted in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (National Research Council, 1996).

Four-week-old male Wistar rats (n = 28) weighing 79–99 g were used in the study. All the animals were randomly assigned to heart failure induced by monocrotaline (MCT, n = 14) or control (Cont, n = 14) groups. Animals in the MCT group were given a single intraperitoneal injection of monocrotaline (30 mg/kg) to induce pulmonary arterial hypertension and subsequent right ventricular hypertrophy and failure; animals in the Cont group received an equivalent volume of saline. It is known that monocrotaline is metabolized in the liver and changed to the active form, which injures vascular endothelium of pulmonary vessels and causes pulmonary arterial hypertension (5–8). All animals were housed in a controlled environment with a fixed 12-h light:dark cycle at a constant temperature of 22 ± 2°C. Food and water were provided ad libitum. The animals in the MCT group were sacrificed with an overdose of sodium pentobarbital at 14 days after monocrotaline injection (n = 7) or on the day that the body weight decreased >9% from its peak (21 ± 0.3 days after monocrotaline injection, n = 7). At 21 days after monocrotaline injection, animals in the MCT group expressed clinical signs of heart failure including weight loss, tachypnea, lethargy, cold extremities, and piloerection. These have been shown to correlate with decompensated right ventricular dysfunction in this model (2, 16). The animals in the Cont group were sacrificed at the same time points as the MCT group (n = 7 at each time point). The lungs, heart, and gastrocnemius and soleus muscles were removed immediately, weighed, frozen in liquid nitrogen, and stored at -80°C until further analysis.

Histological analysis. Serial transverse sections of thickness 10 μm were obtained using a cryostat from the middle part of the skeletal muscle sample and were mounted on glass slides. The sections were stained for myofibrillar adenosine triphosphatase (ATPase) and succinate dehydrogenase (SDH). For ATPase staining, the sections were preincubated in barbital acetate buffer (pH 4.2) for 5 min at room temperature. Following washing with 0.1 M barbital buffer containing 0.18 M CaCl₂ (pH 9.4) for 30 s, the sections were incubated in 0.1 M barbital buffer containing 0.18 M CaCl₂ and 4 mM ATP (pH 9.4) for 45 min at room temperature. The sections were then washed in 1% CaCl₂ and 2% CoCl₂ every 3 min and, finally, in 0.01 M sodium barbital. Following washing with distilled water, the sections...
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Quantitative polymerase chain reaction (qPCR) analysis. Total RNA was isolated from ~20 mg of each skeletal muscle using a lysis buffer containing 0.5 mL TRIZol® reagent (15596-026; Invitrogen, Tokyo, Japan). Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (4374966; Applied Biosystems, CA, USA), and the resultant cDNA samples were stored at −20°C. The mRNA expression levels of atrogin-1 (Rn00591730_m1), MuRF-1 (Rn00590197_m1), LC3 (Rn00563181_m1), and p62 (Rn00677357_g1) were quantified using TaqMan® Gene Expression Assays (Applied Biosystems). The TaqMan® probe and primer set were validated by performing qPCR with a series of cDNA template dilutions to obtain standard curves of threshold cycle time against relative concentration using the normalization gene 18S (Rn03928990_g1). qPCR was performed using the PCR Fast Advanced Master Mix (Applied Biosystems) in a 96-well reaction plate. Each well contained 1 μL cDNA template, 5 μL PCR Fast Advanced Master Mix, 3.5 μL RNase-free water, and 0.5 μL TaqMan® Gene Expression Assays in a reaction volume of 10 μL. All sample and nontemplate control reactions were performed in a CFX96™ Real-Time PCR Detection System (BioRad) under the following conditions: 50°C for 2 min, 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Statistical analysis. Data are expressed as mean ± standard error. The significance of differences between the groups was evaluated using one-way analysis of variance followed by Tukey’s HSD post hoc test. Statistical significance was set at P < 0.05.

RESULTS

Body weight

Body weight in the Cont group increased every day during the experimental period (Fig. 1A). In contrast, body weight in the MCT group peaked at 15 days after monocrotaline injection, flattened, and then gradually decreased after 17 days. At 21 days after monocrotaline injection, body weight in the MCT group decreased by an average of 31% from the value in the Cont group.
monocrotaline injection, the wet weights of the gastrocnemius and soleus muscles in the MCT group decreased by 33% and 25%, respectively, from the values in the Cont group. The ratio of wet weight to body weight of the gastrocnemius muscle was significantly lower in the MCT group than in the Cont group at 21 days after monocrotaline injection. In contrast, there was no significant difference at 21 days in the value for the soleus muscle between the Cont and MCT groups.

Skeletal muscle fiber cross-sectional area
ATPase and SDH staining revealed that the gastrocnemius muscles were composed of type I, IIA, and IIB fibers (Fig. 4A–H), and the soleus muscles were composed of type I and IIA fibers (Fig. 4L–S). More than 90% of the fibers in the gastrocnemius muscle were type IIA and IIB, whereas the soleus muscle was mostly type I fiber.

In both the gastrocnemius (Fig. 4I, 4J, 4K) and soleus (Fig. 4T, 4U) muscles, no fiber types showed significant differences in cross-sectional area between the Cont and MCT groups at 14 days after monocrotaline injection, the wet weights of the gastrocnemius and soleus muscles in the MCT group decreased by 33% and 25%, respectively, from the values in the Cont group. The ratio of wet weight to body weight of the gastrocnemius muscle was significantly lower in the MCT group than in the Cont group at 21 days after monocrotaline injection. In contrast, there was no significant difference at 21 days in the value for the soleus muscle between the Cont and MCT groups.

Wet weight of skeletal muscles
There were no significant differences in the wet weights or ratios of wet weight to body weight of the gastrocnemius (Fig. 3A, 3B) and soleus (Fig. 3C, 3D) muscles between the Cont and MCT groups at 14 days after monocrotaline injection. At 21 days after
monocrotaline injection. However, in both the gastrocnemius and soleus muscles, the values were significantly lower in the MCT group than in the Cont group at 21 days. At this time point, the mean cross-sectional areas of the type I, IIA, and IIB fibers in the gastrocnemius muscle decreased by 17%, 21%, and 20%, respectively, from the values in the Cont group. In the soleus muscle, the mean cross-sectional areas of the type I and IIA fibers decreased by 28% and 33%, respectively, from the value in the Cont group.

Expression levels of mRNA for the ubiquitin-proteasome pathway
At 14 days after monocrotaline injection, although statistically insignificant, the mRNA expression levels of atrogin-1 (Fig. 5A) and MuRF-1 (Fig. 5B) in the gastrocnemius muscle in the MCT group were 2.1- and 2.4-fold higher than in the Cont group, respectively. In contrast, these expression levels in the soleus muscle were almost identical between the Cont and MCT groups at this time point (Fig. 5C, 5D). At 21 days after monocrotaline injection, the mRNA expression levels of atrogin-1 and MuRF-1 in the gastrocnemius and soleus muscles were significantly higher in the MCT group than in the Cont group.

Expression levels of mRNA for the macroautophagy-lysosome pathway
At 14 days after monocrotaline injection, although statistically insignificant, the mRNA expression levels of LC3 (Fig. 6A) and p62 (Fig. 6B) in the gastrocnemius muscle in the MCT group were 1.7- and 3.1-fold higher than in the Cont group, respectively. In contrast, these expression levels in the soleus muscle were almost identical between the Cont and MCT groups at this time point (Fig. 6C, 6D). At 21 days after monocrotaline injection, the mRNA expression levels of LC3 and p62 in the gastrocnemius and soleus muscles were significantly higher in the MCT group than in the Cont group.

DISCUSSION
Although monocrotaline injection increased the ratio of lung wet weight to body weight after 14 days,
Fig. 4  Transverse sections of the gastrocnemius (A–H) and soleus (I–S) muscles stained with myofibrillar adenosine triphosphatase (A–D, L–O) and succinate dehydrogenase (E–H, P–S) in the Cont (A, C, E, G, L, N, P, R) and MCT (B, D, F, H, M, O, Q, S) groups at 14 (A, B, E, F, L, M, P, Q) and 21 (C, D, G, H, N, O, R, S) days after monocrotaline injection. 1: type I fiber; 2: type IIA fiber; 3: type IIB fiber. Scale bar = 100 μm. Cross-sectional area of type I (I, T), IIA (J, U), and IIB (K) fibers in the gastrocnemius (I–K) and soleus (T, U) muscles. Two sections per animal and five randomly chosen fields per section were evaluated. For each muscle fiber type, over 100 muscle fibers were measured. Values represent mean ± SE. * and † are significantly different (P < 0.05) from the Cont group at the same time point and within the same group at 14 days, respectively.
there was no concurrent effect on the skeletal muscle wet weight or the fiber cross-sectional area. In contrast, monocrotaline injection increased the ratio of wet weight to body weight for both lungs and heart and decreased the skeletal muscle wet weight and fiber cross-sectional area after 21 days. These results suggest that skeletal muscle atrophy occurs only in the stage of right ventricular failure, not in the stage of pulmonary arterial hypertension. Steffen et al. (24) reported that treatment with anti-tumor necrosis factor-alpha (TNF-α) from the onset of body weight decrease attenuated skeletal muscle atrophy in monocrotaline-induced heart failure but could not prevent it entirely, suggesting that treatment must begin before body weight loss. Together with our results, this indicates that skeletal muscle atrophy is already in progress at the stage of pulmonary arterial hypertension.

Although skeletal muscle atrophy was not detected by morphological methods at 14 days after monocrotaline injection, the mRNA expression levels for the ubiquitin-proteasome (Cont vs. MCT, atrogin-1: $P = 0.08$, MuRF-1: $P = 0.09$) and macroautophagy-lysosome (Cont vs. MCT, LC3: $P = 0.11$, p62: $P = 0.15$) pathways in the gastrocnemius muscles were marginally increased. Monocrotaline injection can cause physical inactivity (23), decrease food intake (10), and cause deconditioning with hypoxia (10), which activates the ubiquitin-proteasome pathway (14), the macroautophagy-lysosome pathway (20), or both (9) in the skeletal muscle, respectively. However, the Cont and MCT groups showed no differences in physical activity as measured by infrared sensor, food intake (Cont, 22 ± 0.4 g vs. MCT, 20 ± 1.5 g), or oxygen saturation of arterial blood (Cont, 97 ± 0.3% vs. MCT, 97 ± 0.1 g) for at least 14 days after monocrotaline injection in preliminary experiments. Overexpression of TNF-α was previously observed 2 weeks after monocrotaline injection (22), and increased circulating levels of TNF-α trigger apoptosis in skeletal muscle (5, 6). Additionally, overexpression of cytokines such as TNF-α has been reported in vascular inflammation of the lungs induced by monocrotaline injection (25). Therefore, we speculate
that vascular inflammation in the lungs could release upstream factors such as TNF-α and activate the ubiquitin-proteasome and macroautophagy-lysosome pathways at 14 days after monocrotaline injection, with subsequent skeletal muscle atrophy at 21 days.

The skeletal muscle atrophied less in the soleus muscle than in the gastrocnemius muscle after 21 days, but there was no significant difference in the ratio of soleus muscle wet weight to body weight between the Cont and MCT groups. These results suggested that atrophy in soleus muscle is inadequate to explain body weight loss with heart failure. Additionally, there was no significant difference in mRNA expression level for the ubiquitin-proteasome and macroautophagy-lysosome pathways in the soleus muscle at 14 days after monocrotaline injection between the Cont and MCT groups. In both the gastrocnemius and soleus muscles, the type I fiber cross-sectional area decreased less than those of type IIA and IIB fibers. These results suggest that type I fiber considered as oxidative slow-twitch fiber has high tolerance to protein degradation with heart failure. Congestive heart failure is associated with apoptosis in skeletal muscles (5, 6) induced by elevated pro-inflammatory cytokines and reactive oxygen species (ROS) production (7). However, the magnitude of apoptosis is lower in slow muscle (19) and the region that composed mainly of type I fibers in fast muscle (29) than in fast muscle and the region that composed mainly of type II fibers in slow muscle due to the high anti-oxidative capacity of type I fibers (30). Therefore, because pro-inflammatory cytokines and ROS also activate the ubiquitin-proteasome and macroautophagy-lysosome pathways (28), the characteristics of slow-twitch fiber would inhibit protein degradation with heart failure.

In conclusion, although skeletal muscle atrophy did not occur before heart failure, the protein degradation pathways were activated at an early stage. Additionally, protein degradation pathways were activated less in slow muscle than in fast muscle. This study demonstrates that physical exercises that shift to slow muscle such as endurance training (11, 18) should begin before any indication of skeletal mus-
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cle atrophy to prevent exercise intolerance with heart failure. However, this study has some limitations. Although we speculate that vascular inflammation in the lungs was induced by monocrotaline injection and the proinflammatory molecules activate protein degradation pathways, we did not analyze the upstream molecules of the ubiquitin-proteasome and macroautophagy-lysosome pathways such as TNF-α and ROS. Additionally, although exercise at the stage of pulmonary arterial hypertension in the monocrotaline model might worsen myocardial dysfunction, we did not investigate the effects herein. Therefore, further research is required to determine the stage at which exercise has low risk and high efficacy for heart failure.

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