SUPPLEMENTAL MATERIALS

MECHANISMS UNDERLYING SKELETAL MUSCLE WEAKNESS IN HUMAN HEART FAILURE: ALTERATIONS IN SINGLE FIBER MYOSIN PROTEIN CONTENT AND KINETICS

Mark S. Miller, Peter VanBuren, Martin M. LeWinter, Stewart H. Lecker, Donald E. Selby, Bradley M. Palmer, David W. Maughan, Philip A. Ades, Michael J. Toth

Supplemental Methods:

Solutions. For solutions used for *Muscle tissue processing*, dissection solution contained (in mM) 20 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 5 EGTA, 5 MgATP, 1 free Mg^{2+}, 1 DTT and 0.25 phosphate (Pi) with an ionic strength of 175 mEq, pH 7.0, and at pCa 8 (pCa = $-\log_{10}[Ca^{2+}]$), skinning solution contained (in mM): 170 potassium propionate, 10 imidazole, 5 EGTA, 2.5 $MgCl_2$, 2.5 ATP-Na_2H_2, 0.05 leupeptin and 0.05 antipain at pH 7.0, and storage solution was identical to skinning solution, but without leupeptin and antipain. For solutions used for *Single fiber mechanical measurements*, calculations were performed using the equations of Fabiato and Fabiato[1] and stability constants of Godt and Lindley.[2] Relaxing solution was identical to dissecting solution with 15 mM creatine phosphate (CP) and 300 units/ml of creatine phosphokinase (CPK), pre-activating solution was identical to relaxing solution, except at an EGTA concentration of 0.5 mM, activating solution was the same as relaxing solution, except at pCa 4.5 and rigor solution was the same as activating solution, except that it lacked MgATP, CP and CPK. All solutions used for mechanical experiments were adjusted to proper ionic strength (175 mEq) using sodium methane sulfate. Finally,

loading buffer used for various gel electrophoresis applications was 2% SDS, 62.5 mM Tris, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, pH 6.8.

Tissue homogenate MHC protein content and isoform distribution. Muscle tissue (~20 mg) was homogenized (in mM: 250 sucrose, 2 EDTA, 10 Tris, pH 7.4), the homogenate was centrifuged (10,000 g at 4°C) for 10 min and the supernatant, which contains soluble (ie, non-myofibrillar) proteins, was decanted and the pellet resuspended in three volumes of cold (4°C) extraction buffer (in mM: 100 $Na_4P_2O_7$, 5 EDTA, 1 DTT, pH 8.5), incubated on ice for 30 min and then centrifuged (10,000 g at 4°C for 10 min). The supernatant, which contains myofibrillar proteins, was analyzed for protein content (BioRad; Hercules, CA). An aliquot of the supernatant was added to loading buffer (described above in *Solutions* section), heated for 2 min at 65°C and analyzed by SDS-PAGE.

For MHC protein content, 2 µg protein was loaded onto gels (stacking: 4% acrylamide-*N,N'*-methylene-bis-acrylamide (bis) and the resolving 7.5% acrylamide-bis) and run at 70V for 3 hr, which permits visualization of MHC isoforms as a single band. Gels were stained with coomassie blue, scanned and MHC band intensity determined by densitometry (Quantity One; BioRad; Hercules, CA). MHC protein content data are expressed as densitometric units per µg of protein loaded and reflect the average of duplicate measures. MHC content measurements were not performed on one patient because of lack of tissue.

The relative distribution of MHC isoforms (MHC I, IIA, IIX) was determined according to standard methods, as described previously,[3] with minor modifications. Briefly, 0.2 µg protein was loaded. The stacking gel contained 4% acrylamide-bis/5% glycerol (w/v) and the resolving gel 7% acrylamide-bis/30% glycerol (w/v). Gels were run at 70V for 3.5 hr, followed by 200V for 20 hr at 9°C. This permits separation of the three

isoforms of MHC into three distinct bands. The gel was silver stained (Silver Snap; Pierce, Rockford, IL), scanned, the MHC isoforms quantified by densitometry (Quantity One; BioRad; Hercules, CA) and data expressed as a percentage of total MHC densitometric units.

Single muscle fiber morphology and MHC protein content. Fibers had T-clips were placed at either end, were mounted in dissecting solution (20°C) under a compound microscope, pulled taught and clip-to-clip length measured using a manual micrometer. Using a right-angled, mirrored prism, top and side fiber diameters were measured to the nearest tenth of a μm at 250 μm intervals along the length of the fiber at 100X using a digital filar eyepiece micrometer (Lasico, Los Angeles, CA). Following measurements, the fiber was cut adjacent to each T-clip, placed in loading buffer (75 μl), sonicated at 40°C for 1 hr, heated for 5 min at 65°C and stored at -80°C until analysis. Top and side diameters and fiber length were used to calculate the volume of the fiber, assuming an elliptical cross-section. The volume of the fiber per μl of loading buffer was then used to standardize gel loads per unit fiber volume (ie, μl required to obtain 1.5 μm^3 of fiber volume). Because of this requirement for the loading volume (ie, 1.5 μm^3 of fiber volume), we selected a lower bound for fiber diameters of 60 μm to insure that there would be enough sample to run triplicate analysis on each fiber. Thus, the fibers used in these analyses do not reflect a random sample from each group.

For MHC protein measurements, samples were run on 4% acrylamide stacking/7.5% acrylamide resolving gels at 70V for 3 hr and silver stained (Silver Snap; Pierce, Rockford, IL). For actin protein measurements, samples from a sub-set of patients (n=4/group) were run on 4-15% acrylaminde/bis gradient gels at 150 V for 1.5 hr and processed similar to MHC gels. Gels were scanned and the background-adjusted brightness area product (BAP) of each MHC or actin band, run in triplicate, was

quantified (Quantity One; BioRad; Hercules, CA), as described.[4] The BAP for each sample was then adjusted to the running internal standard. Samples in which no MHC band was apparent (density ≤ background) were given a value of zero.

Single fiber mechanical measurements. Aluminum t-clips were placed at both ends of the fiber and the fiber segment was mounted on hooks in dissecting solution at 20°C. Fibers were fixed at two points approximately 1 mm apart with glutaraldehyde, as described,[5-7] with modifications. Briefly, fibers were placed in rigor solution (in mM: 134 potassium propionate, 10 imidazole, 7.5 EDTA and 2.5 EGTA; 20 2,3-butanedione monoxime at pH 6.8) and glutaraldehyde fixative (6% toluidine blue (w/v), 30% glycerol (v/v), 2% glutaraldehyde(v/v)) was applied (15 s/end) using the gravity feed method.[6] The fiber was then placed in dissecting solution with 1% bovine serum albumin to absorb any remaining glutaraldehyde.[5] Fibers were removed from the hooks, t-clipped in the fixed region, and the fiber material beyond the new t-clips removed.

Ultrastructural measurements. Shortly after obtaining tissue from the muscle biopsy, a bundle of muscle fibers was tied to a glass rod at slightly stretch length (~20% of initial bundle length) and was fixed in 1.5% glutaraldehyde, post-fixed with osmium tetroxide, stained with uranyl acetate and embedded in epoxy resin. The muscle bundle was cut in cross-section (~100 nm) and contrasted with lead and uranyl acetate prior to transmission electron microscopy. The number of thick and thin filaments per unit fiber cross-sectional area was assessed at 60,000X in 8 fibers per patient (Sterio Investigator v8.0; MBF Bioscience; Chicago, IL). Briefly, a measurement area was manually selected on the muscle fiber that contained clearly discernable thick and thin filaments. Counting frames (1 μm^2) were automatically overlaid in a grid pattern and thick and thin filaments counted manually using standard rules.[8] Data were expressed as the ratio of thick to thin

filaments. The muscle bundle was then cut 90° to the cross-sectional cut (~100 nm) and processed as above for electron microscopy (JEOL 1210 Transmission Electron Microscope; JEOL, Inc.; Peabody, MA). A-band (25 measurements/volunteer) and sarcomere length (50 measurements/volunteer) measurements were performed at 5,000X, while the percentage of fiber area occupied by myofibrils (3 images for 275 um^2/volunteer) was performed at 8,000X, as described.[9] All cross-sectional area, A-band length and sarcomere length measurements were made using NIH Image (Image J, National Institutes of Health, Bethesda, MD).

Protein and gene expression. Myofibrillar proteins were isolated from muscle tissue (~20 mg), as described above for tissue homogenates. All buffers were ATP free and contained 2 mM *N*-ethylmaleimide to inhibit proteasomal degradation and deubiquitinating isopeptidases.[10] After protein content determination, samples were diluted in loading buffer and subjected to 4-12% acrylamide-bis gradient SDS-PAGE and routine Western blotting using specific monoclonal antibodies (slow MHC #MAB1628, 1:10,000; Millipore, Temecula, CA; ubiquitin #sc-8017, 1:2000; Santa Cruz Biotech, Santa Cruz, CA). After washing, blots were incubated with sheep anti-mouse IgG (#NA931, 1:2000, Amersham, Piscataway, NJ) conjugated to horseradish peroxidase for chemiluminescent detection (Pierce, Carlsbad, CA). We chose to assess MHC breakdown fragments using an antibody directed at the MHC I isoform because this is the most prevalent MHC isoform in human vastus lateralis muscle (Figure 1B). The pattern of MHC I breakdown fragments (Figure 5A) is similar to what has been previously noted in chymotrypsin digests of rat soleus muscle using this antibody,[11] which likely reflects high molecular weight degradation fragments of myosin.[12] Bands/portions of lanes were quantified by densitometry (Quantity One; BioRad; Hercules, CA). For MHC degradation fragments, the primary MHC band and the MHC

fragments were quantified separately. The densitometric signal for MHC degradation fragments was expressed relative to the primary immunoreactive MHC band to control for any variation in MHC protein content and isoform distribution among groups. To quantify the amount of ubiqutinated MHC, the ubiquitin signal corresponding to the MHC band was quantified and expressed relative to the total MHC band density determined from Simple Blue (Invitrogen, Carlsbad, CA) stained gels run concurrently.

For MHC and actin mRNA measurements, RNA was extracted from muscle tissue (≤8 mg) using the MELT Total Nucleic Acid Isolation System (Ambion, Austin TX). Multiplexed amplification reactions were performed using GAPDH as an endogenous control (Applied Biosystems, Assay ID: Hs99999905_m1) using the Quanta Perfecta QPCR Super Master Mix (Quanta Biosciences, Gaithersburg, MD). The following settings were used: Stage 1 (reverse transcription): 45°C for 5 min; Stage 2 (denaturation): 95°C for 3 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 45 s for 40 cycles. The MHC I, IIA, IIX and actin oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs01110632_m1; Hs00430042_m1; Hs00428600_m1 and Hs00559403_m1, respectively). For atrogene mRNA measurements, total RNA was extracted from muscle tissue (~25 mg) using Triazol reagent. Multiplexed amplification reactions were performed using 18S rRNA as an endogenous control (Applied Biosystems; Foster City, CA) using the TaqMan One step PCR Master Mix reagents kit (Applied Biosystems). The following settings were used: Stage 1 (reverse transcription): 48°C for 30 min; Stage 2 (denaturation): 95°C for 10 min and Stage 3 (PCR): 95°C for 15 s and 60°C for 60 s for 40 cycles. The MuRF-1 oligonucleotides were purchased from Applied Biosystems (Assay ID: Hs00261590). The sequences of the forward, reverse and double-labeled oligonucleotides for atrogin-1 were: forward 5'-CTT TCA ACA GAC TGG ACT TCT CGA -3'; reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'; TaqMan probe: 5'- FAM-TGC CAT CCT GGA TTC CAG AAG ATT CAA C-TAMRA-3'.

MHC and actin samples were run in duplicate and atrogene samples in triplicate. All fluorescence data were analyzed by SDS software (Applied Biosystems) and the threshold cycle (Ct) values for each reaction were used to calculate gene expression relative to controls, according to published algorithms (Applied Biosystems).

Supplemental results:

Missing data. Several data points are missing because of logistical and technical problems. For total body composition measurements, one heart failure patient was not tested because he exceeded the weight limit of the dual energy x-ray absorptiometry scanner. Because of this, peak VO_2 data for this subject are also excluded because they could not be corrected for fat-free mass. For computed tomography scans, one patient and one control did not have data because the scan files were corrupted upon transfer from the scanner computer to the storage database and in one control because logistical problems prevented the measurement from being completed. Data for peak VO_2 and accelerometry are not available on one subject because logistical problems prevented completion of these tests. For biochemical assessments (ie, protein and gene expression), variable sample sizes was due to limitations in tissue availability.

Non-parametric statistical analysis. Comparisons between heart failure patient and control groups using non-parametric statistical procedures (Mann-Whitney U test) did not alter the statistical significance of any of the differences noted within the body of the manuscript, as defined by parametric statistical procedures.

Single fiber MHC and actin protein content. In fibers used for MHC protein content measurements, actin bands were evident in all fibers studied, including those with MHC band density less than background. As the actin bands were not quantifiable on gels

used for MHC protein measurements, in a sub-set of patients (n=4/group) for which there were sufficient fibers for triplicate analysis, single fiber actin protein content was evaluated in the same fibers. We did not find differences in actin protein content between controls and patients (157 ± 7 vs. 149 ± 5 BAP X 10^3; n=51 and n=65, respectively).

MHC degradation and gene expression. MHC I breakdown fragments, the amount of ubiquitinated protein corresponding to MHC and expression of E3 ubiquitin ligases in a sub-sample of patients (n=4) and controls (n=4) are shown in Figure 5. We found no differences between heart failure patients and controls in the amount of MHC I breakdown fragments (Figure 5A,C) when assessing the densitometric signal of the fragments (C: 1671 ± 426 vs. HF: 1709 ± 362 arbitrary units (AU)) or when this signal was expressed relative to the primary immunoreactive MHC I band to account for MHC protein depletion in heart failure patients (C: 1.00 ± 0.21 vs. HF: 1.05 ± 0.24 AU). We also found no difference in breakdown fragment signal when expressed relative to total MHC protein determined from Simple Blue stained gels (C: 1.17 ± 0.26 vs. HF: 1.29 ± 0.34 AU). Similarly, there was no difference in the ubiquitin signal corresponding to intact MHC (Figure 5B,D; C: 1414 ± 44 vs. HF: 1301 ± 44 AU) or when the ubiquitin signal was expressed relative to total MHC protein determined from Simple Blue stained gels (C: 0.97 ± 0.30 vs. HF: 1.13 ± 0.07 AU). Parenethetically, in the non-myofibrillar protein fraction, there was no evidence for increased protein ubiquitination on an absolute basis (C: 1236 ± 71 vs. HF: 1268 ± 92 AU; representative gel in Supplemental Figure 1) or when expressed relative to the total MHC protein content determined from concurrently run Simple blue stained gels (C: 0.45 ± 0.01 vs. HF: 0.47 ± 0.03 AU), suggesting that there is no evidence for a general up-regulation of protein breakdown in heart failure patients. Finally, in a sub-set of volunteers (n=4 heart failure; n=6 controls; Figure 5E,F),

no differences were observed in the expression of the E3 ubiquitin ligases: MuRF-1 (C: 1.00 ± 0.20 vs. HF: 1.05 ± 0.20) or atrogin-1 (C: 1.00 ± 0.22 vs. HF: 1.00 ± 0.21).

Using 18S as a housekeeping gene, there was no difference in MHC I (C: 1.00 ± 0.12 vs. HF: 1.07 ± 0.20), MHC IIA (C: 1.00 ± 0.11 vs. HF: 1.02 ± 0.21), MHC IIX (C: 1.00 ± 0.43 vs. HF: 2.00 ± 0.75) or actin mRNA abundance (C: 1.00 ± 0.08 vs. HF: 1.03 ± 0.08).

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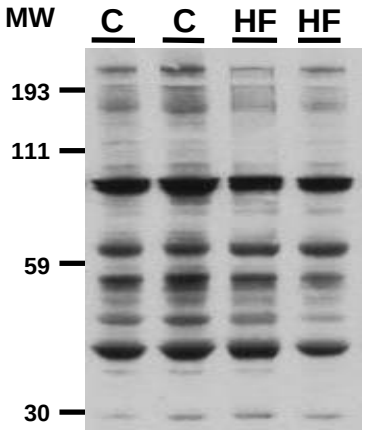

Supplemental Figure 1. Representative blot of ubiquitinated proteins in the non-myofibrillar fraction of skeletal muscle tissue homogenates in controls (C; n=4) and heart failure patients (HF; n=4).