

Overcoming Muscle Atrophy in a Hibernating Mammal Despite Prolonged Disuse in Dormancy: Proteomic and Molecular Assessment

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Abstract Prolonged disuse of skeletal muscle causes significant loss of myofibrillar contents, muscle tension, and locomotory capacity. However, hibernating mammals like bats appear to deviate from this trend. Although low functional demands during winter dormancy has been implicated as a factor contributing to reduced muscle loss, the precise mechanism that actively prevents muscle atrophy remains unclear. We explored proteomic and molecular assessments of bat muscle to test a hypothesis that expression levels of major myofibrillar proteins are retained during hibernation, with periodic arousals utilized as a potential mechanism to prevent disuse atrophy. We examined changes in myofibrillar contents and contractile properties of the pectoral or biceps brachii muscles of the bat *Murina leucogaster* in summer active (SA), hibernation (HB) and early phase of arousal (AR) states. We found the bat muscles did not show any sign of atrophy or tension reduction over the 3-month winter dormancy. Levels of most sarcomeric and metabolic proteins examined were maintained through hibernation, with some proteins (e.g., actin and voltage dependent anion channel 1) 1.6- to 1.8-fold upregulated in HB and AR compared to SA. Moreover, expression levels of six heat shock proteins (HSPs) including glucose-regulated protein 75 precursor were similar among groups, while the level of HSP70 was even 1.7-fold higher in HB and AR than in SA. Thus, considering the nature of arousal with strenuous muscle shivering and heat stress, upregulation or at least balanced regulation of the chaperones (HSPs) would contribute to retaining muscle properties during prolonged disuse of the bat. *J. Cell. Biochem.* 104: 642–656, 2008. © 2008 Wiley-Liss, Inc.

Key words: interbout arousal; bat; heat shock protein; hibernation; *Murina leucogaster ognevi*; muscle atrophy; proteomics

From the viewpoint of loading stress versus atrophy, skeletal muscle of mammalian hibernators offer an interesting question to gravitational biologists. In most nonhibernators, including humans, prolonged disuse of skeletal muscle, as seen in bed rest, hindlimb suspension

or spaceflight, causes significant loss of myofibrillar contents [Fitts et al., 2000]. This loss occurs mainly due to unbalanced regulation of gene expression (i.e., high degradation and low synthesis of the proteins). The extent of the wasting differs among the proteins (e.g., actin vs. myosin), which leads to serious degeneration of myofibrillar integrity and further reduction in muscle tone and locomotor capacity [Fitts et al., 2000; Seo et al., 2006].

The skeletal muscle of hibernators appears to deviate from this general trend. Among small-sized hibernators, bats in temperate regions spend 4–6 months of deep dormancy with low body temperature (T_b) for overwintering, while they resume daily normothermic activities like breeding, feeding and flying for the rest of the year. Mechanical stress imposed to

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skeletal muscle varies accordingly, following the circannual cycle of metabolic and muscular workloads [Altringham, 1996; Choi et al., 1998]. Thus, in considering the trend of nonhibernators, these animals are expected to experience significant muscle atrophy from extended disuse. However, previous evidence illustrates that hibernators show little or only marginal changes in muscle mass and contractile properties during winter dormancy. In support of this phenomenon, Hudson and Franklin [2002] demonstrated a positive correlation of muscle atrophy with metabolic rates in various mammals with hibernator muscles being virtually non-atrophic. How do hibernators overcome muscle atrophy and functional depression for long periods of inactivity?

In hibernators, several conflicting processes may exist intrinsically that affect muscle properties: (1) the atrophic process that is ensued by the long-term disuse of the muscle as seen in nonhibernators, and (2) the low demands of body function (e.g., low metabolism and T_b) that would diminish the atrophic process. While the former depicts both increased degradation and decreased synthesis of proteins, the latter emphasizes slowing of both degradation and synthesis processes. Because transcription and translation are energetically expensive events [Carey et al., 2003], the second mechanism seems to be advantageous to the hibernators for energy conservation and survival; yet, this process may not sufficiently prevent muscle atrophy unless another mechanism actively retains the myofibrillar milieu during the disuse period.

Here, we consider that arousal activities between torpor bouts could play a crucial role in preventing the atrophic process in bats. Even though each arousal is relatively brief, it occurs repeatedly over an interval of several days. For instance, greater horseshoe bats (*Rhinolophus ferrumequinum*) are observed to exhibit interbout arousals every 2–8 days with each bout lasting 2–9 h [Park et al., 2000]. Moreover, every arousal proceeds with strenuous muscle shivering and tremors that rapidly raise metabolic rate and T_b (by up to $\sim 25^\circ\text{C}$) for the first 30 min or so [Lee et al., 2002]. If the thermogenic trial elevates most of the biochemical contents in a repeated manner, the hibernator muscle may be able to maintain the myofibrillar milieu while the following low tissue temperature slows down the atrophic process [Breukelen and Martin, 2002]. A similar demonstration is

given by Harlow et al. [2004] in that periodic elevation of neck surface temperature of black bears (*Ursus americanus*) may be a causative factor for retaining muscle strength during hibernation. To clarify this mingled problem, it is necessary to delineate how and to what extent the levels of muscle proteins are retained during the prolonged disuse and what impact the periodic arousals would make, if any, on muscle properties.

The extent of myofibrillar wasting can be examined by large-scale proteomic analyses [Isfort et al., 2000]. For instance, proteomic profiles of the rat soleus muscle exposed to 3-week unloading detected significant downregulation of many sarcomeric proteins including α -actin, tropomyosin, and slow troponin isoforms. Such downregulation took place in parallel with that of molecular chaperones like heat shock proteins (HSPs), as expression of α crystallin B-chain, p20, and HSP90 decreased 0.3- to 0.4-fold compared to those of controls [Seo et al., 2006]. Importantly, these chaperones are also known to play essential roles in organization, protection and maintenance of myofilament structure and integrity against severe mechanical stresses [Koh, 2002; Srikakulam and Winkelmann, 2004]. Thus, expression levels of HSPs in the muscle cell are an important factor that may affect restraint of myofibrillar loss by unloading.

In the current study we explored proteomic and molecular assessment of the bat pectoral muscle to test the hypothesis that expression levels of major myofibrillar proteins are retained through hibernation, with periodic arousals utilized as a functional mechanism to overcome muscle atrophy. For this purpose, we examined changes in myofilament arrangements (for an index of myofibrillar integrity), tension production capacity, and levels of sarcomeric, metabolic, and heat shock proteins from muscles of bats in three different states, summer active (SA), winter hibernation (HB), and the early phase of arousal (AR) from hibernation. To the best of our knowledge, the current study is the first for 2-dimensional electrophoresis (2-DE) with skeletal muscles of hibernators.

MATERIALS AND METHODS

Subjects and Tissue Sampling

We collected male bats of the species *Murina leucogaster ognevi* in August to September for the SA group and in February for both the HB

and AR groups from a natural cave located in Gangwon Province (37°20'N, 128°10'E), Republic of Korea. All collections were made at 22:00–24:00. Rectal temperature (T_r) of the individuals was measured immediately after capture by inserting a sensing tip of a 30 American wire gauge (AWG) copper-constantan thermocouple connected to an Omega 91100-20 thermometer (Cole-Parmer Instrument, Vernon Hills, IL). The SA and HB animals were decapitated in place and frozen in liquid nitrogen. Handling time between capture and freezing was within 20 s. For the AR group, a wire-mesh cage containing each bat was lightly shaken in every 30 s for 40 min in which the animal usually displayed active wing flapping or crawling on the cage mesh for escape. The animal was then subject to T_r measurement, decapitated, and frozen in liquid nitrogen. Additional animals were carried to our laboratory where they were kept quietly in a dark humid room at 5–7°C for two full days and used in muscle contractile experiments (see below). Experimental procedures involving animals were approved by the University Animal Care and Use Committee. The animals were treated in accordance with the NIH guidelines for the care and use of experimental animals.

Electron Microscopy

General procedures for electron microscopy (EM) are described by Choi et al. [2005]. Small pieces of the pectoral muscles were obtained from three bats per group. The tissues were prefixed for 4 h in 2.5% glutaraldehyde, cut into 1 × 1 × 2 mm blocks, rinsed for 15 min in 0.1 M phosphate buffer (pH 7.2) and post-fixed for 2 h in 1% OsO₄. The fixation and rinsing processes were conducted at 4°C to minimize autolysis and extraction. The fixed samples were dehydrated twice in a series of ethanol solutions (50%, 60%, 70%, 90%, and 100%). The samples were then infiltrated with propylene oxide, affixed sequentially on dodecenyl succinic anhydride, epon 812, nadic methyl anhydride, and tri(dimethylaminomethyl) phenol (DMP-30) for 1 h each at room temperature and then polymerized at 60°C overnight. After trimming and semi-thin sectioning, muscle samples were dried on a hot plate (80°C) and were prestained with 0.5% toluidine blue. The semi-thin section was trimmed, ultra-thin sectioned with an ultra-microtome and double-post-stained with 1% uranyl acetate for 15 min and lead citrate for

5 min. Finally, the ultra-thin section (0.05 μm) was supported on a grid. Cross sections were then adjusted to 120,000× magnification for determining numbers of actin and myosin filaments, or 15,000× magnification for measuring myofiber cross-sectional area (CSA) under an electron microscope (TEM, JEM-1200EX, Jeol, Tokyo, Japan). To determine myofibril thickness, longitudinal sections were adjusted to 10,000× magnification under the same electron microscope. All images were photographed with a digital TEM camera (Megaview III), and analyzed using an Image Analysis System (SIS, Munster, Germany).

From the photographed micrographs, we chose 4–7 images per tissue showing the best resolution of myofibrillar structures (sarcomeres, actin and myosin filaments, myofibrils). We divided each image into 1–2 quadrates using Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA), totaling 36–39 quadrates for each group. Actin and myosin filaments were then counted within each quadrate (100 nm × 100 nm), with those appearing on the edge of the quadrate being excluded. The CSA and myofibril thickness were measured in each quadrate of relevant images using SigmaScan 5.0 software (Systat Software, Inc., San Jose, CA).

Proteomic Analysis

Protein extraction. The overall procedure, including protein identification, was conducted with the assistance of the Yonsei Proteome Research Center in Seoul, Korea. We carried out three sets of 2-DE experiments, with each set composed of six samples (two samples/group × three groups, thus $n = 6$ per group). The 50–100 mg frozen tissues were suspended in 4–5 volumes of sample buffer containing 7 M urea, 2 M thiourea, 100 mM DTT, 4% (w/v) CHAPS, 40 mM Tris, 0.002% Bromophenol blue and protease inhibitors (Roche, Penzberg, Germany) and sonicated for protein extraction. The extracted protein concentration was determined with a Bradford assay kit (Bio-Rad, Hercules, CA). For the first dimensional analysis, the 1.0 mg protein samples were diluted in 400 μl sample buffer with 2% (v/v) ampholyte and loaded onto 18 cm long IPG strips with a 3–10 non-linear pH range (GE Healthcare, Fairfield, CT). Focusing was carried out for about 80,000 Vh using a Multiphor II Isoelectric Focusing System (GE Healthcare). For

the second dimensional separation, electrophoresis was performed on 9–17% gradient polyacrylamide gels until the dye front reached the lower end of the gel.

2-DE image analysis. For quantifying the relative densities of proteins, gels were stained with Coomassie Brilliant Blue G-250. Stained gels were scanned using a GS-710 imaging densitometer (Bio-Rad) and analyzed with the Image Master Platinum 5.0 program (GE Healthcare). Reproducibility of the 2-DE gels and homogeneity of samples were ensured by preparing and running all samples under identical conditions and were further checked with preliminary tests on two replicates per group. Spot densities for each gel were normalized by the total density of ~550 detectable spots on the gels. To remove any irrelevant variations among the three sets of 2-DE runs, the normalized spot densities were converted to % values of the average of two control (SA) values in each experimental set. Finally, the inter-group differences were further evaluated for key proteins (e.g., HSPs) by immunoblotting analysis (see below).

Protein identification. Among more than 550 spots on each gel, 109 clear spots were excised and digested using 5 ng trypsin in 50 mM NH_4HCO_3 (Promega, Madison, WI). For MS analysis, the tryptic peptides were desalted and purified using Poros R2, Oligo R3 resin (Applied Biosystems, Foster City, CA) as described previously [Choi et al., 2003]. The mixture of eluted peptides and matrix (α -cyano-4-hydroxycinnamic acid) was spotted directly onto the MALDI plate (Opti-TOF™ 384-well Insert, Applied Biosystems). Spectra were obtained using a 4800 TOF/TOF spectrophotometer (Applied Biosystems) and calibrated with the trypsin auto-digested peaks (m/z 842.5090 and 2211.1046). The MS spectra were searched against the NCBI database using the MASCOT algorithm (Matrix Science, Boston, MA) for protein identification. Because the database for bat proteins has not been constructed yet, we attempted to examine databases of various taxonomic classes, with final searches restricted to the *Rattus* species that provided the highest scores.

Immunoblotting Analysis

The frozen muscle tissues were homogenized in ten volumes of tissue lysis buffer [20 mM HEPES (pH 7.4), 75 mM NaCl, 2.5 mM MgCl_2 ,

0.1 mM EDTA, 0.05% (v/v) Triton X-100, 20 mM β -glycerophosphate, 1 mM Na_3VO_4 , 10 mM NaF, 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and the protein inhibitor Complete Mini (Roche)]. The samples were incubated 15 min on ice and centrifuged for 30 min at 14,000g at 4°C. The supernatant was collected as the whole-tissue soluble lysate, and its protein concentration was determined using a Bradford assay kit (Bio-Rad). Whole-tissue lysates (50 μg) were subject to SDS-PAGE either on 8–10% gels to detect HSP90, HSP70, and HSP60, or on 10–12% gels for HSP27 and α B-crystallin, followed by electrophoretic transfer of protein bands to a nitrocellulose membrane and subsequent incubation with antibodies against HSPs. Immune complexes were detected with the ECL system (Amersham Pharmacia Biotech) and quantitated using a densitometer (Gel Document, Bio-Rad). The loading and transfer of protein was normalized to β -tubulin or GAPDH. The primary antibodies used for the assays were: mouse anti-HSP90, anti-HSP70, and anti-HSP60 monoclonal antibodies (SPA-830, 810, and 806, respectively) from Stressgen (Victoria, BC, Canada), anti-HSP27 (sc-13132) and α B-crystallin (sc-22744) from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti- β -tubulin (T4026) from Sigma (St. Louis, MO), and mouse monoclonal [6C5] to GAPDH (ab8245) from Abcam (Cambridge, UK). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG from Zymed (San Francisco, CA).

Muscle Contraction

We used the biceps brachii muscle (the part of the glenoid head) rather than the pectoral muscle because it was almost impossible to dissect a small strip from the massive pectoral muscle without myofiber damage. Both muscles work in a synchronous manner during flight, as the pectoral muscle controls the downstroke of the wings while the biceps brachii muscle extends the humerus to stretch out the wings [Vaughan, 1970]. We thus presumed that temporal workload activities are similar for both muscles year round.

Each animal was anesthetized with pentobarbital sodium (i.p., 60 mg kg^{-1}). The muscle tissue of the right arm was quickly removed and soaked in 70 ml of oxygenated Ringer's solution at 25°C (115 mM NaCl, 5 mM KCl, 4 mM CaCl_2 ,

1 mM MgCl₂, 1 mM NaH₂PO₄, 24 mM NaHCO₃, 11 mM glucose, 0.02 g L⁻¹ tubocurarine chloride, pH 7.35). One tendon was tied to a lever arm of a 300B-LR servomotor system (Aurora Scientific, Ontario, Canada), and the other tendon to a micrometer to adjust to the optimal muscle length. A custom DMC program (Aurora Scientific) was used to control both the servo system and a Grass S48 stimulator (Grass Technologies, West Warwick, RI). The stimulator was connected to a pair of bright platinum electrodes, and supplied a 1.0-ms square wave pulse train at supramaximal voltage and a stimulus frequency of 200 Hz that generated fully fused tetanic force. The force data were analyzed by custom software (DMA, Aurora Scientific) and normalized by muscle cross-sectional area as described previously [Seo et al., 2006].

Data Presentation

All data are presented as mean ± 1SEM, unless otherwise noted. Significance of intergroup differences was examined by one-way analysis of variance (ANOVA) and Scheffe's *post hoc* multiple comparison tests. The statistics were performed with SPSS/PC+ at the significance level $P = 0.05$.

RESULTS

Subjects and Muscle Properties

Table I summarizes body and muscle properties of the SA, HB, and AR groups. Figure 1 illustrates representative electron micrographs for the cross-sections (A–C) and a ratio of actin/myosin filaments of the pectoral muscle (D). Typical tetanic tension curves from the three groups are shown in Figure 2A–C. Bats showed a broad variation in average T_r depending on active or torpid conditions, whereas body mass

was comparable irrespective of their activity condition. For the muscle mass relative to body mass (M_m/M_b), as much of the AR pectoral muscles was used for other tests, we compared only SA and HB data which were found to be statistically indifferent between the two groups (independent *t*-test; $P > 0.05$). The protein concentration, myofiber CSA, and myofibril thickness of the pectoral tissue were not significantly different among the groups (one-way ANOVA and Scheffe's multiple comparison tests, $P > 0.05$). Both maximum tetanic tension and the rate of tetanic tension development (examined with the biceps brachii muscle) were also statistically indistinguishable among groups.

Proteomic Analysis

MS analyses identified 38 proteins among 109 spots that rendered accurate measurements of quantities in the gel images. Identifications, theoretical pIs and molecular weights of these proteins are summarized in Table II. In some proteins (e.g., spots No. 4, 5, 6, 9 and 10), theoretical pIs and actual pIs on the gel disagreed, which may be due to post-translational modification. Table III provides peptide sequences of five proteins analyzed by MS/MS. Due to the lack of a database for bat skeletal muscle, outcomes of peptide sequences matched to those of the *Rattus* species were very limited. Figure 3A–C presents representative gel images from the three groups with corresponding spot numbers listed in Table II. Figure 4 exemplifies five proteins that showed significant differences in spot densities among the three groups. From these analyses, we selected 28 proteins relevant to the aim of this study, sorted them into the sarcomeric, metabolic and stress categories and compared the levels of each protein across the three groups in Tables IV–VI.

TABLE I. Comparison of Body and Muscle Properties of the Summer-Active, Winter Hibernation and Arousal Groups of Bats *Murina leucogaster ognevi*

	SA	HB	AR
Body temperature (°C)	34.3 ± 2.0 (15)	6.0 ± 0.4 (16)	30.6 ± 1.5
Body mass (g)	7.75 ± 0.44 (15)	7.44 ± 0.11 (16)	7.52 ± 0.17 (15)
Muscle mass/body mass ($\times 10^3$) ^a	28.00 ± 1.09 (10)	29.33 ± 0.86 (10)	
Protein concentration ($\mu\text{g mg}^{-1}$ wet mass)	129.64 ± 3.51 (6)	128.54 ± 6.46 (6)	140.89 ± 5.18 (6)
Myofiber cross-sectional area (μm^2) ^b	0.172 ± 0.002 (3, 150)	0.176 ± 0.005 (3, 150)	0.169 ± 0.003 (3, 150)
Myofibril thickness (μm) ^b	0.235 ± 0.005 (3, 210)	0.248 ± 0.005 (3, 210)	0.243 ± 0.004 (3, 210)
Tetanic tension (kN m^{-2})	138.89 ± 18.12 (5)	133.56 ± 12.62 (5)	141.14 ± 7.71 (5)
Rate of tetanic tension development ($\text{kN m}^{-2} \text{ms}^{-1}$)	1.01 ± 0.09 (5)	0.88 ± 0.07 (5)	1.12 ± 0.11 (5)

Data: mean ± SE (n).

^aMuscle mass/body mass for the AR group was not measured in this study.

^bIn each parenthesis are numbers of animals and numbers of total counts from all animals in the group.

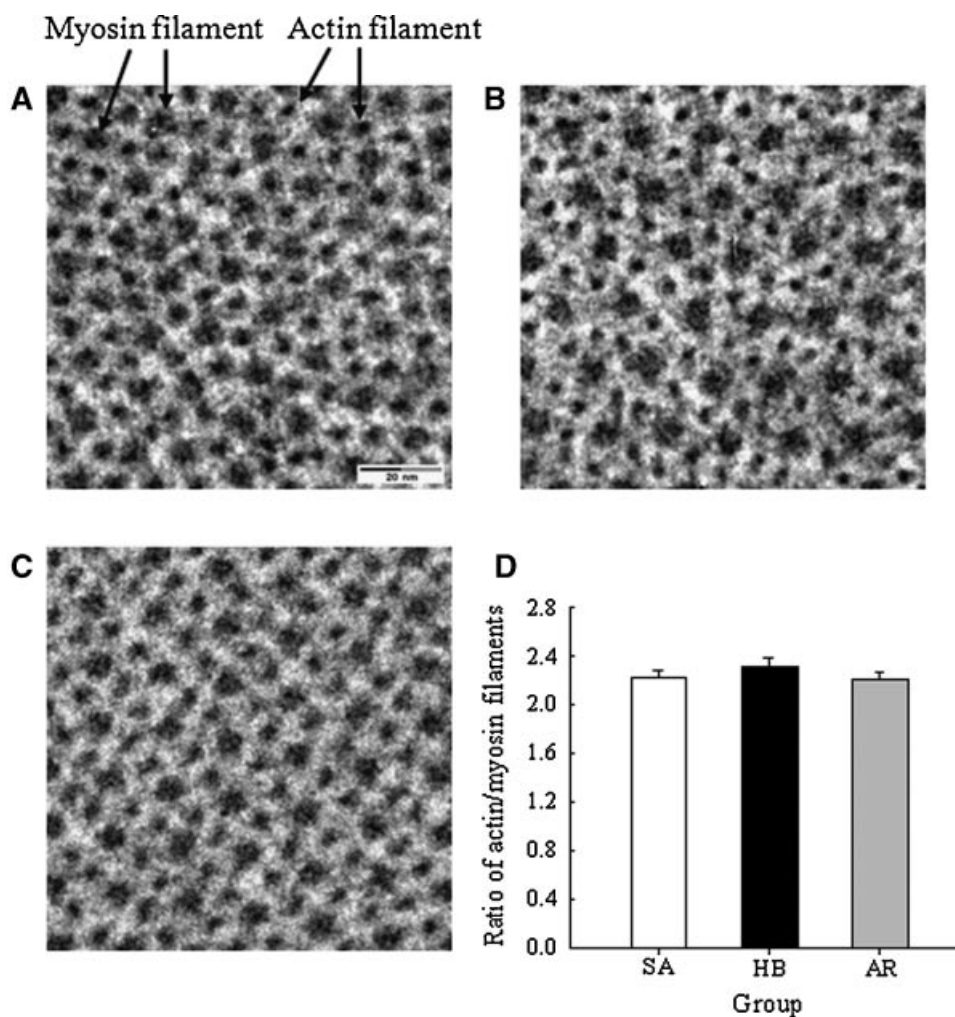


Fig. 1. Representative electron micrographs of cross sections from the bat pectoral muscle for SA (A), HB (B) and AR (C) groups. Quadrate size = 100 nm × 100 nm. The ratios of actin/myosin filaments are illustrated in (D), with histograms presented in mean ± 1SE (n = 3 per group; 39 quadrates for SA, 36 for HB, and 38 for AR).

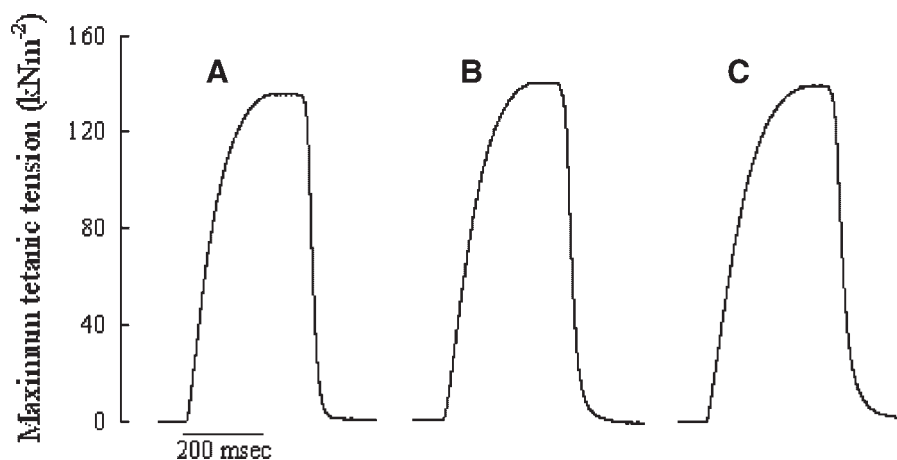


Fig. 2. Typical tension curves of the biceps brachii muscles for SA (A), HB (B), and AR (C) groups. The muscle in vitro was stimulated by a 1.0-ms square wave pulse train at supramaximal voltage and a stimulus frequency of 200 Hz in a buffer solution maintained at 25°C. Cross-sectional area (mm²) of the muscle used was 1.14 (A), 1.10 (B), and 1.47 (C).

TABLE II. A List of 38 Proteins Identified by MS for the Pectoral Muscle of the Bat *Murina leucogaster*

Spot no.	Accession number	Protein identification	MOWSE score	Coverage (%)	Peptides matched	Theoretical pI/M _w	
						pI	M _w
1	gi 51858651	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (75 kDa)	89	29	15	5.65	80.331
2	gi 51980672	NADH dehydrogenase (ubiquinone) Fe-S protein 2	60	23	11	6.52	52.927
3	gi 2119726	GRP75 precursor	83	23	18	5.87	73.984
4,5,6	gi 53734284	MGC:94869 [oxoglutarate dehydrogenase(lipoamide)]	108	23	20	6.30	117.419
7	gi 6678465	α -tubulin 3	126	45	16	4.97	50.612
8	gi 55250073	Heat shock protein 70	166	41	21	5.37	71.055
9,10	gi 16758446	Isocitrate dehydrogenase 3 (NAD ⁺) (α subunit)	100	30	18	6.47	40.044
11	gi 8250661	Myosin heavy chain	104	27	22	5.27	103.804
13,14,15,16	gi 1374715	ATP synthase (beta subunit)	192	55	25	4.92	51.171
18	gi 13786200	Voltage-dependent anion channel 1	128	52	13	8.62	30.851
19	gi 203480	Creatine kinase	73	39	10	7.88	33.423
20	gi 34861269	troponin T, fast skeletal muscle isoform (alpha/beta)	64	26	12	9.45	41.685
21	gi 57580	α B-crystallin	99	39	11	6.84	19.945
22,23,24,25,26	gi 55577	Actin	132	39	19	5.23	42.366
27	gi 54673763	Heat shock protein 90 β	90	26	16	4.93	85.161
28	gi 16741093	Heat shock protein 60	97	31	15	5.91	61.088
29	KCRS_RAT	Creatine kinase, sarcomeric mitochondrial precursor	53	12	7	8.76	47.355
30	gi 38197676	Desmin	92	33	16	5.21	53.447
31	gi 6981240	Myosin, light polypeptide 3	106	54	9	5.03	22.140
32	gi 127167	Myosin regulatory light chain 2 (MLC2)	71	46	9	4.86	11.868
33	gi 346655	Tropomyosin $\alpha\beta$ -chains	53	15	7	4.64	32.78
34	gi 74267962	ALB protein	74	15	10	5.88	71.186
35	gi 20664363	Chain B, crystallographic dimeric polylysine-actin-latrunculin A complex	80	30	7	5.46	41.558
36	gi 89243640	EPB41 protein	81	24	11	5.43	76.393
37	gi 73746712	Prostate-specific transglutaminase 4	75	26	11	7.01	77.956
38	gi 253578	Myosin light chain isoform LC17b	70	56	5	4.46	16.991
39	gi 57162093	ELMO2	74	38	9	6.15	33.487
40	gi 30962111	Albumin	71	23	10	5.23	67.862

Data search was conducted against the NCBI database and restricted to the *Rattus* species.

TABLE III. A List of Five Proteins and Their Peptide Sequences Identified by MS/MS for the Pectoral Muscle of the Bat *Murina leucogaster*

Spot no.	Accession number	Protein identification	Peptide sequence	MOWSE score	Coverage (%)	Numbers of peptides matched
13	gi 1374715	ATP synthase beta subunit	R.ESRLVEVAQHLGESTVR.T R.LVLEVAQHLEGSTVR.T R.TIAMDGTEGLVR.G R.IMNVIGEPIDERGIK.T K.AHGYSVFAGVGER.T R.TREGNDLYHEMIESGVINLK.D K.VALVYGQMNPEPPGAR.A K.VALVYGQMNPEPPGAR.A—Oxidation (M) R.VALTLGLTVAEYFR.D R.DQEGQDVLLEFDNIFR.F R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLATDMGTMQER.I R.IPSAVGYQPTLATDMGTMQER.I—Oxidation (M) R.IPSAVGYQPTLATDMGTMQER.I—2 Oxidation (M) R.AIAELGIYPVAVDPLDSTR.I K.SLQDIAALGMDELSEEDKLTWSR.A K.YRWTEYGLTFTTEK.W R.WTEYGLTFTTEK.W R.WTEYGLTFTTEK.WTDLTGLTEIIVEDQLAR.G K.WNTDNTLGTETIIVEDQLAR.G K.LTFDSSFSPNTGK.K K.LTFDSSFSPNTGKK.N K.REHINLGCDDVDFDIAGPSIR.G R.GALVLYGEGWLAGYQMNFFTSK.S K.SRVTQSNFAVGYYK.T R.VTQSNFAVGYYK.T R.VTQSNFAVGYYKTDFFQLHTNVNDGTGTFGGSYQK.V K.TDEFQLHTNVNDGTGTFGGSYQK.V K.TDLNHNENLKGDDDLDPNYVLSRR.V K.LSVEALNSLTGEFK.G K.LSVEALNSLTGEFKGK.Y K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D—Oxidation (M) K.SELVWVNEEDHLR.V R.LGSSEVEQVQLVVDGVK.L R.AVFPVIGRPR.H K.IWHHIFYNELR.V R.VAPEEHPTLLTEAPLNPK.A R.ITGVLDSGDGVTHNVPIYEGYALPHAIMR.L R.LDLAGRDLTDYLMK.I R.LDLAGRDLTDYLMK.I—Oxidation (M) R.GYSFVTTAER.E R.GYSFVTTAEREIVR.D K.SYELPDGQVITIGNER.F R.KDLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M—Oxidation (M) K.IKLIAPPER.K K.ISSVQSIIVPALEFIANHR.K R.KPLVIAEDVDGEALSTLVLR.L	137	42	16
18	gi 13786200	Voltage-dependent anion channel 1	R.ESRLVEVAQHLGESTVR.T R.LVLEVAQHLEGSTVR.T R.TIAMDGTEGLVR.G R.IMNVIGEPIDERGIK.T K.AHGYSVFAGVGER.T R.TREGNDLYHEMIESGVINLK.D K.VALVYGQMNPEPPGAR.A K.VALVYGQMNPEPPGAR.A—Oxidation (M) R.VALTLGLTVAEYFR.D R.DQEGQDVLLEFDNIFR.F R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLATDMGTMQER.I R.IPSAVGYQPTLATDMGTMQER.I—Oxidation (M) R.IPSAVGYQPTLATDMGTMQER.I—2 Oxidation (M) R.AIAELGIYPVAVDPLDSTR.I K.SLQDIAALGMDELSEEDKLTWSR.A K.YRWTEYGLTFTTEK.W R.WTEYGLTFTTEK.W R.WTEYGLTFTTEK.WTDLTGLTEIIVEDQLAR.G K.WNTDNTLGTETIIVEDQLAR.G K.LTFDSSFSPNTGK.K K.LTFDSSFSPNTGKK.N K.REHINLGCDDVDFDIAGPSIR.G R.GALVLYGEGWLAGYQMNFFTSK.S K.SRVTQSNFAVGYYK.T R.VTQSNFAVGYYK.T R.VTQSNFAVGYYKTDFFQLHTNVNDGTGTFGGSYQK.V K.TDEFQLHTNVNDGTGTFGGSYQK.V K.TDLNHNENLKGDDDLDPNYVLSRR.V K.LSVEALNSLTGEFK.G K.LSVEALNSLTGEFKGK.Y K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D—Oxidation (M) K.SELVWVNEEDHLR.V R.LGSSEVEQVQLVVDGVK.L R.AVFPVIGRPR.H K.IWHHIFYNELR.V R.VAPEEHPTLLTEAPLNPK.A R.ITGVLDSGDGVTHNVPIYEGYALPHAIMR.L R.LDLAGRDLTDYLMK.I R.LDLAGRDLTDYLMK.I—Oxidation (M) R.GYSFVTTAER.E R.GYSFVTTAEREIVR.D K.SYELPDGQVITIGNER.F R.KDLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M—Oxidation (M) K.IKLIAPPER.K K.ISSVQSIIVPALEFIANHR.K R.KPLVIAEDVDGEALSTLVLR.L	147	43	12
19	gi 203480	Creatine kinase	R.ESRLVEVAQHLGESTVR.T R.LVLEVAQHLEGSTVR.T R.TIAMDGTEGLVR.G R.IMNVIGEPIDERGIK.T K.AHGYSVFAGVGER.T R.TREGNDLYHEMIESGVINLK.D K.VALVYGQMNPEPPGAR.A K.VALVYGQMNPEPPGAR.A—Oxidation (M) R.VALTLGLTVAEYFR.D R.DQEGQDVLLEFDNIFR.F R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLATDMGTMQER.I R.IPSAVGYQPTLATDMGTMQER.I—Oxidation (M) R.IPSAVGYQPTLATDMGTMQER.I—2 Oxidation (M) R.AIAELGIYPVAVDPLDSTR.I K.SLQDIAALGMDELSEEDKLTWSR.A K.YRWTEYGLTFTTEK.W R.WTEYGLTFTTEK.W R.WTEYGLTFTTEK.WTDLTGLTEIIVEDQLAR.G K.WNTDNTLGTETIIVEDQLAR.G K.LTFDSSFSPNTGK.K K.LTFDSSFSPNTGKK.N K.REHINLGCDDVDFDIAGPSIR.G R.GALVLYGEGWLAGYQMNFFTSK.S K.SRVTQSNFAVGYYK.T R.VTQSNFAVGYYK.T R.VTQSNFAVGYYKTDFFQLHTNVNDGTGTFGGSYQK.V K.TDEFQLHTNVNDGTGTFGGSYQK.V K.TDLNHNENLKGDDDLDPNYVLSRR.V K.LSVEALNSLTGEFK.G K.LSVEALNSLTGEFKGK.Y K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D—Oxidation (M) K.SELVWVNEEDHLR.V R.LGSSEVEQVQLVVDGVK.L R.AVFPVIGRPR.H K.IWHHIFYNELR.V R.VAPEEHPTLLTEAPLNPK.A R.ITGVLDSGDGVTHNVPIYEGYALPHAIMR.L R.LDLAGRDLTDYLMK.I R.LDLAGRDLTDYLMK.I—Oxidation (M) R.GYSFVTTAER.E R.GYSFVTTAEREIVR.D K.SYELPDGQVITIGNER.F R.KDLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M—Oxidation (M) K.IKLIAPPER.K K.ISSVQSIIVPALEFIANHR.K R.KPLVIAEDVDGEALSTLVLR.L	54	34	7
23	gi 55577	Actin	R.ESRLVEVAQHLGESTVR.T R.LVLEVAQHLEGSTVR.T R.TIAMDGTEGLVR.G R.IMNVIGEPIDERGIK.T K.AHGYSVFAGVGER.T R.TREGNDLYHEMIESGVINLK.D K.VALVYGQMNPEPPGAR.A K.VALVYGQMNPEPPGAR.A—Oxidation (M) R.VALTLGLTVAEYFR.D R.DQEGQDVLLEFDNIFR.F R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLATDMGTMQER.I R.IPSAVGYQPTLATDMGTMQER.I—Oxidation (M) R.IPSAVGYQPTLATDMGTMQER.I—2 Oxidation (M) R.AIAELGIYPVAVDPLDSTR.I K.SLQDIAALGMDELSEEDKLTWSR.A K.YRWTEYGLTFTTEK.W R.WTEYGLTFTTEK.W R.WTEYGLTFTTEK.WTDLTGLTEIIVEDQLAR.G K.WNTDNTLGTETIIVEDQLAR.G K.LTFDSSFSPNTGK.K K.LTFDSSFSPNTGKK.N K.REHINLGCDDVDFDIAGPSIR.G R.GALVLYGEGWLAGYQMNFFTSK.S K.SRVTQSNFAVGYYK.T R.VTQSNFAVGYYK.T R.VTQSNFAVGYYKTDFFQLHTNVNDGTGTFGGSYQK.V K.TDEFQLHTNVNDGTGTFGGSYQK.V K.TDLNHNENLKGDDDLDPNYVLSRR.V K.LSVEALNSLTGEFK.G K.LSVEALNSLTGEFKGK.Y K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D—Oxidation (M) K.SELVWVNEEDHLR.V R.LGSSEVEQVQLVVDGVK.L R.AVFPVIGRPR.H K.IWHHIFYNELR.V R.VAPEEHPTLLTEAPLNPK.A R.ITGVLDSGDGVTHNVPIYEGYALPHAIMR.L R.LDLAGRDLTDYLMK.I R.LDLAGRDLTDYLMK.I—Oxidation (M) R.GYSFVTTAER.E R.GYSFVTTAEREIVR.D K.SYELPDGQVITIGNER.F R.KDLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M—Oxidation (M) K.IKLIAPPER.K K.ISSVQSIIVPALEFIANHR.K R.KPLVIAEDVDGEALSTLVLR.L	112	33	13
28	gi 16741093	HSP 60 precursor	R.ESRLVEVAQHLGESTVR.T R.LVLEVAQHLEGSTVR.T R.TIAMDGTEGLVR.G R.IMNVIGEPIDERGIK.T K.AHGYSVFAGVGER.T R.TREGNDLYHEMIESGVINLK.D K.VALVYGQMNPEPPGAR.A K.VALVYGQMNPEPPGAR.A—Oxidation (M) R.VALTLGLTVAEYFR.D R.DQEGQDVLLEFDNIFR.F R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLATDMGTMQER.I R.IPSAVGYQPTLATDMGTMQER.I—Oxidation (M) R.IPSAVGYQPTLATDMGTMQER.I—2 Oxidation (M) R.AIAELGIYPVAVDPLDSTR.I K.SLQDIAALGMDELSEEDKLTWSR.A K.YRWTEYGLTFTTEK.W R.WTEYGLTFTTEK.W R.WTEYGLTFTTEK.WTDLTGLTEIIVEDQLAR.G K.WNTDNTLGTETIIVEDQLAR.G K.LTFDSSFSPNTGK.K K.LTFDSSFSPNTGKK.N K.REHINLGCDDVDFDIAGPSIR.G R.GALVLYGEGWLAGYQMNFFTSK.S K.SRVTQSNFAVGYYK.T R.VTQSNFAVGYYK.T R.VTQSNFAVGYYKTDFFQLHTNVNDGTGTFGGSYQK.V K.TDEFQLHTNVNDGTGTFGGSYQK.V K.TDLNHNENLKGDDDLDPNYVLSRR.V K.LSVEALNSLTGEFK.G K.LSVEALNSLTGEFKGK.Y K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D K.SMTEQEQQLDIDHFLFDKPVSPLLLASGMAR.D—Oxidation (M) K.SELVWVNEEDHLR.V R.LGSSEVEQVQLVVDGVK.L R.AVFPVIGRPR.H K.IWHHIFYNELR.V R.VAPEEHPTLLTEAPLNPK.A R.ITGVLDSGDGVTHNVPIYEGYALPHAIMR.L R.LDLAGRDLTDYLMK.I R.LDLAGRDLTDYLMK.I—Oxidation (M) R.GYSFVTTAER.E R.GYSFVTTAEREIVR.D K.SYELPDGQVITIGNER.F R.KDLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M K.DLYANNVMSGGTTMYPGIADR.M—Oxidation (M) K.IKLIAPPER.K K.ISSVQSIIVPALEFIANHR.K R.KPLVIAEDVDGEALSTLVLR.L	37	6	2

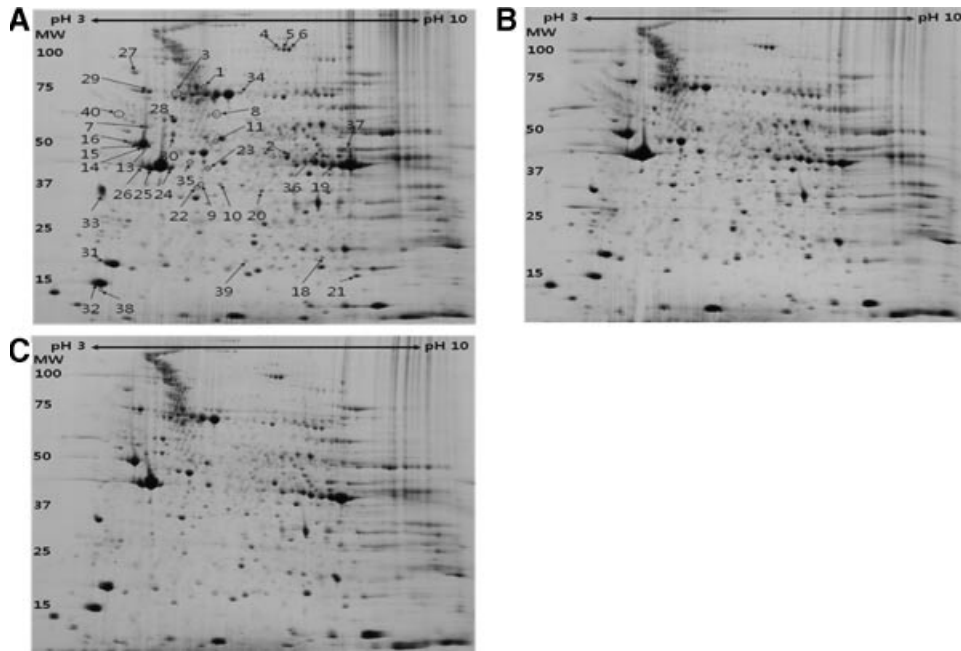


Fig. 3. Representative 2-DE gel images of the pectoral muscle for SA (A), HB (B), and AR (C). Protein samples (1.0 mg) extracted from the pectoral muscle were separated on wide-range (pH 3-10 NL) IPG strips for the first dimension followed by a 9–17% SDS–PAGE gel for the second dimension. Coomassie blue G250 was used for the gel staining. Thirty eight proteins identified using MALDI–TOF analysis were indicated by numbers in panel (A). Names of these proteins were listed in Table II.

Sarcomeric proteins. Seven proteins associated with myofibrillar structure and contractile function were identified: five isoforms of actin, desmin, myosin heavy chain, myosin light

polypeptide 3, myosin regulatory light chain 2, troponin T fast isoform and tropomyosin $\alpha\beta$ chains. We found that most of the sarcomeric proteins showed statistically similar levels

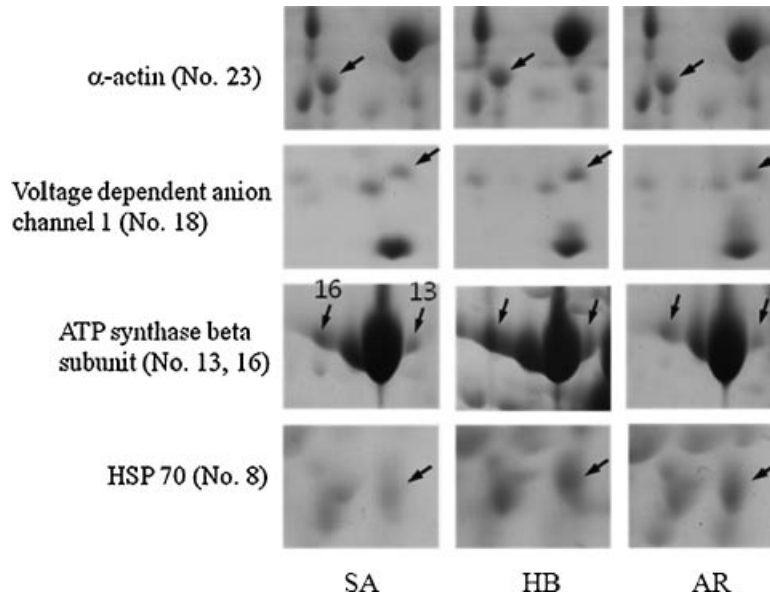


Fig. 4. Comparisons of spot densities for five proteins across the three groups. The protein names and spot numbers are given with arrows indicating the corresponding spots. Final analysis revealed levels of these proteins were significantly greater in HB and/or AR than in SA.

TABLE IV. Comparison of Expression Levels of Sarcomeric Proteins in the Pectoral Muscle Among SA, HB, and AR Groups

Protein name	Spot no.	SA	HB	AR
Actin	22	1.00 ± 0.15	1.30 ± 0.19	1.52 ± 0.27
	23	1.00 ± 0.15 ^a	1.86 ± 0.21 ^b	1.74 ± 0.27 ^b
	24	1.00 ± 0.02	1.06 ± 0.18	1.27 ± 0.39
	25	1.00 ± 0.04	1.18 ± 0.13	1.15 ± 0.10
	26	1.00 ± 0.06	1.80 ± 0.40	1.23 ± 0.25
Desmin	30	1.00 ± 0.06	1.50 ± 0.43	1.31 ± 0.26
Myosin heavy chain	11	1.00 ± 0.07	1.15 ± 0.27	1.27 ± 0.35
Myosin, light polypeptide 3	31	1.00 ± 0.03	1.12 ± 0.16	1.26 ± 0.23
Myosin regulatory light chain 2 (MLC-2)	32	1.00 ± 0.04	0.96 ± 0.12	1.09 ± 0.12
Troponin T fast isoform (alpha/beta)	20	1.00 ± 0.07	1.42 ± 0.24	1.18 ± 0.25
Tropomyosin αβ-chains	33	1.00 ± 0.03	1.01 ± 0.24	1.44 ± 0.21
α-tubulin 3	7	1.00 ± 0.13	1.12 ± 0.23	1.70 ± 0.43
Voltage dependent anion channel 1	18	1.00 ± 0.08 ^a	1.63 ± 0.26 ^b	1.54 ± 0.20 ^b

Data: mean ± 1SE (n = 6).

^{a,b}*P* < 0.05 (one-way ANOVA and Scheffe's multiple comparison tests).

among the groups. The only exception was seen in one isoform of actin (spot No. 23) for which the level was significantly higher in the HB and AR groups than in SA (one-way ANOVA and Scheffe's tests, *P* < 0.05) (Table IV).

One structural protein (α-tubulin 3, which makes up a part of microtubules) and one regulatory protein (voltage dependent anion channel 1, a pore-forming transmembrane protein in neurons and muscle cells) were also identified, and, for the latter, expression was significantly upregulated in HB and AR as compared to SA (*P* < 0.05).

Metabolic proteins. Six proteins were identified in this category, including four isoforms of ATP synthase beta subunit, creatine kinase, creatine kinase sarcomeric mitochondrial precursor, two isoforms of isocitrate dehydrogenase 3 alpha subunit and NADH dehydrogenase Fe-S proteins 1 and 2. For the ATP synthase beta subunit, four isoforms were also reported in human skeletal muscle, with comparable shifts in pI probably due to post-

translational modification [Hojlund et al., 2003]. All of these proteins are aerobic and thus associated with the mitochondrial inner membrane or matrix (Table V).

We found that most of these proteins showed similar expression levels among groups. However, two isoforms of ATP synthase (spots No. 13 and 16) showed significant upregulation in HB and creatine kinase sarcomeric mitochondrial precursor (No. 29) in AR as compared to SA (*P* < 0.05).

Stress proteins. Five proteins associated with the stress response were identified, namely GRP75 precursor, HSP90β, HSP70, HSP60 and αB-crystallin. Although most of these proteins were expressed at similar magnitudes among groups, the level of HSP70 in HB and AR was about 1.7-fold greater than in SA (*P* < 0.05) (Table VI).

Expression of HSPs

Immunoblotting analysis allowed us to compare inter-group differences in the levels of six

TABLE V. Comparison of Expression Levels of Metabolic Proteins in the Pectoral Muscle Among SA, HB, and AR Groups

Protein name	Spot no.	SA	HB	AR
ATP synthase (beta subunit)	13	1.00 ± 0.04 ^a	1.73 ± 0.32 ^b	0.96 ± 0.19
	14	1.00 ± 0.18	0.79 ± 0.18	1.38 ± 0.61
	15	1.00 ± 0.01	1.21 ± 0.20	1.09 ± 0.21
	16	1.00 ± 0.05 ^a	1.58 ± 0.17 ^b	1.18 ± 0.10
	19	1.00 ± 0.06	0.90 ± 0.19	0.94 ± 0.04
Creatine kinase	29	1.00 ± 0.10 ^a	1.37 ± 0.30	1.73 ± 0.28 ^b
Creatine kinase, sarcomeric mitochondrial precursor	9	1.00 ± 0.15	2.42 ± 0.98	2.10 ± 0.79
Isocitrate dehydrogenase 3 (NAD ⁺) (α subunit)	10	1.00 ± 0.07	2.11 ± 0.74	1.78 ± 1.13
NADH dehydrogenase (ubiquinone) Fe-S protein 1 (75 kDa)	1	1.00 ± 0.07	1.45 ± 0.27	1.02 ± 0.19
NADH dehydrogenase (ubiquinone) Fe-S protein 2	2	1.00 ± 0.14	1.05 ± 0.22	0.93 ± 0.33

Data: mean ± 1SE (n = 6).

^{a,b}*P* < 0.05 (one-way ANOVA and Scheffe's multiple comparison tests).

TABLE VI. Comparison of Expression Levels of Stress Proteins in the Pectoral Muscle Among SA, HB, and AR Groups

Protein name	Spot no.	SA	HB	AR
GRP75 precursor	3	1.00 ± 0.17	1.02 ± 0.23	0.84 ± 0.19
HSP90β	27	1.00 ± 0.22	0.81 ± 0.20	1.31 ± 0.17
HSP70	8	1.00 ± 0.13 ^a	1.77 ± 0.21 ^b	1.69 ± 0.26 ^b
HSP60	28	1.00 ± 0.08	1.14 ± 0.25	1.21 ± 0.18
αB-crystallin	21	1.00 ± 0.14	0.88 ± 0.22	1.19 ± 0.18

Data: mean ± 1SE (n = 6).

^{a,b} $P < 0.05$ (one-way ANOVA and Scheffe's multiple comparison tests).

HSPs (HSP90, HSP70, HSP60, HSP27, and αB crystallin) (Fig. 5A,B). Among these proteins, the level of HSP70 in the HB and AR groups was significantly increased in comparison to SA

($F_{2,24} = 7.39$, $P = 0.003$), while the levels of the rest of the HSPs were similar among groups. The patterns of intergroup variations for HSP90, HSP70, HSP60, and αB-crystallin

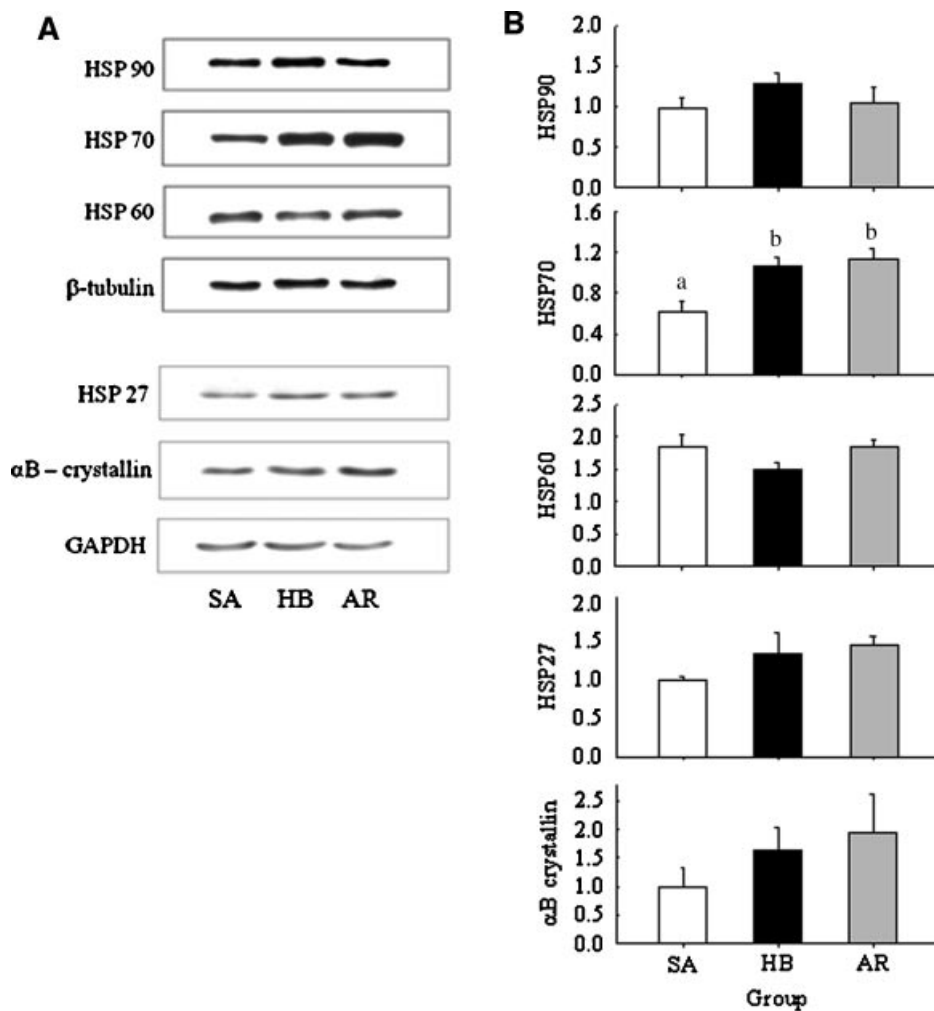


Fig. 5. Intergroup comparisons for levels of six stress-responsive proteins, HSP90, HSP70, HSP60, HSP27, and αB crystallin. Whole-tissue soluble lysates were used to determine levels of the proteins by immunoblotting analysis with respective antibodies (A), and quantitative analysis was summarized in (B). Data are reported as mean ± 1SE (n = 6). a–b, $P < 0.05$ (one-way ANOVA and Scheffe's multiple comparison tests).

were generally comparable to those respective proteins observed in the proteomic analysis (Fig. 5 and Table V).

DISCUSSION

No Sign of Atrophy in Hibernating Bat Muscle

The muscle samples we collected for HB and AR groups were those from the bats that had already spent more than 3 months of winter in hibernation. This time span is long enough to cause significant atrophy of skeletal muscle in nonhibernators. 'Three weeks' of hindlimb unloading, for instance, rendered a 35–50% reduction in muscle mass and tension of the soleus muscle in rodents [Fitts et al., 2000; Seo et al., 2006]. During this time period, levels of contractile proteins and stress proteins (HSPs) decreased by 40–70%, and the ratio of actin/myosin filaments decreased by 31% [Choi et al., 2005; Seo et al., 2006]. The loss of proteins is known to result from a reduced rate of synthesis for the first days of unloading coupled with an increased rate of degradation [Vandenburgh et al., 1999].

In bat muscle, however, we failed to observe any sign of muscle atrophy for the 3 months of dormancy. All morphological data (i.e., M_m/M_b , protein concentration, myofiber CSA, myofibril thickness, actin/myosin filament ratio) and tension production capacity remained nearly constant over the active and hibernation periods examined (Table I, and Figs. 1 and 2). Moreover, our proteomic and immunoblotting analyses revealed most sarcomeric, metabolic, and stress proteins being maintained through hibernation. In some proteins (e.g., two isoforms of actin, voltage-dependent anion channel 1, two isoforms of ATP synthase subunits, and HSP70), expressions were even significantly increased in HB and/or AR groups (Tables IV–VI and Fig. 5). Such retention of myofibrillar content and contractile function would be adaptive because the animals can perform powerful flight or an escape response as a reaction to emergent conditions (e.g., predator attack) during hibernation or immediately after arousal.

Retention of muscle properties is commonly seen in various mammalian hibernators. In our previous report [Kim et al., 2000], total soluble protein and total RNA concentrations in the pectoral muscle of bats (*M. leucogaster*) were maintained at a constant level for summer, mid-

hibernation (February), and end-hibernation (April). Myofiber cross-sectional area did not change until mid-hibernation, but decreased by 32% towards the end of hibernation, suggesting a significant depletion of lipids and glycogen in muscle cells after the 5-month dormancy. In the golden-mantled ground squirrel (*Spermophilus lateralis*), the gastrocnemius and soleus muscles exhibited a 14–20% reduction in their relative mass over 6 months of hibernation [Wickler et al., 1991], but this reduction is substantially less than that of nonhibernators observed after such a length of muscle disuse [Hudson and Franklin, 2002]. More impressive is the case of black bears (*Ursus americanus*) that were found to display little change in muscle mass, contractile properties (e.g., contraction time, peak rate of tension development) or fatigue resistance for about four months of winter dormancy [Tinker et al., 1998; Lohuis et al., 2007b]. Vyskocil and Gutmann [1977] reported a similar outcome of muscle contractility in golden hamsters (*Mesocricetus auratus*) after 3 months of disuse. Taken together, these results suggest that a potential regulatory mechanism exists in hibernators that would allow them to overcome muscle atrophy for the prolonged dormancy.

A Potential Mechanism Preventing Muscle Atrophy in Bat

We initially hypothesized interbout arousals as a potential mechanism that prevents muscle atrophy of the hibernating bat. We did not measure the duration and frequency of arousal of the bats in this study. However, it has been well documented that all hibernators studied so far adopt periodic arousals during hibernation, and that bats in the temperate region tend to arouse at a relatively frequent rate (ca. in 2- to 8-day intervals; Park et al. [2000]). Like repeated strenuous exercise, the arousal activities would boost most cellular and biochemical processes between torpor bouts, through which myofibrillar contents are maintained or even augmented above the normothermic level.

In support of this hypothesis, our proteomic and immunoblotting analyses show significant elevation in HSP70 expression for HB and AR (Table VI and Fig. 5). The observation that levels of the remaining HSPs and GRP75 precursor, which otherwise should have been decreased during dormancy, remained fairly constant across the three groups further

supports our hypothesis (Table VI and Fig. 5). HSPs are known to act as molecular chaperones during protein folding, repair, and membrane translocation under heat stress, oxidative stress, toxin exposure, infectious disease, etc. Previous reports demonstrate that most of these proteins including HSP70 and small HSPs (HSP27, α B-crystallin) are upregulated under mechanical stress, where they stabilize or protect the organization of actin and myosin filaments against the physical force [Koh, 2002; Srikakulam and Winkelmann, 2004; Paulsen et al., 2007]. Moreover, as a part of HSP70 members, GRP75 is known to be associated with survival and protection of cells in response to various stresses like oxygen deficiency or glucose deprivation [Lee et al., 2002]. Therefore, upregulation or at least balanced regulation of these proteins might provide a potential mechanism for protecting and retaining myofibrillar proteins during rewarming.

In association with these results, it is worth noting several studies on biochemical mechanisms that underlie muscle mass preservation in hibernators. Because the animals are at low T_b and metabolism during dormancy, they must have a defense mechanism against oxidative stress during rewarming. Hudson and Franklin [2002] considered this oxidative problem and drew a relationship between muscle atrophy and mass-specific metabolic rates in various mammals. Based on this relationship, they postulated that a declined production of reactive oxygen species (ROS) during dormancy may limit the extent of oxidative damage on myofibers and thereby lower the rate of muscle wasting. Relevant to this, oxidative capacity (e.g., gauged by citrate synthase activity) is found to be increased in hibernating animal muscles, which raises thermogenic capacity for arousal activities [Wickler et al., 1991; Kim et al., 2000]. The incremental nature of oxidative capacity was also seen in our hibernating bat muscle (e.g., two isoforms of ATP synthase beta subunit in Table V). Because this elevated capacity may inevitably cause oxidative damage to muscle cells, antioxidants are expected to be upregulated to counteract the damage during repeated arousals. Indeed, several studies have reported elevation of antioxidant levels in various tissues including skeletal and cardiac muscle, and brown adipose tissue in hibernators [Hudson and Franklin, 2002; Ohta et al., 2006; Yan et al., 2006; Morin and Storey, 2007]. Our

results extend these findings by offering the mechanistic basis of such elevation in antioxidants and aerobic capacity that the previous studies did not present.

With regard to the mechanistic basis, we affirm the proposition of Breukelen and Martin [2002], who considered interbout arousals as a route to recover cellular conditions from the 'biochemical freeze,' and low T_b of torpor to reduce rates of biochemical reactions (as expected from the temperature effect). This view is partly supported by our data, as seen in most myofibrillar contents including chaperones retained or elevated during arousal. Several studies demonstrate that protein degradation and synthesis occur in a slow, yet balanced manner in the pectoral muscle of bats [Yacoe, 1983] and the vastus lateralis muscle of black bears [Lohuis et al., 2007a,b]. Interestingly, bears appear to use bouts of muscle activity for retaining muscle strength, albeit without whole-body arousal from torpor [Harlow et al., 2001]. Spikes of neck surface temperature generated four times a day in a range of 2–30°C during denning suggest the functional restoration role of mechanical and heat stimulation, and may thus be interpreted as that of interbout arousals seen in small hibernators like bats.

Lastly, there are of course many genes of which expression is seasonally regulated in hibernators (see a review of Carey et al. [2003]). For instance, genes encoding α_2 -macroglobulin, apolipoprotein A1, uncoupling proteins, NADH-ubiquinone oxidoreductase, and antioxidant proteins like superoxide dismutase are found to be overexpressed at the mRNA level in the liver, heart, or skeletal muscle of hibernating ground squirrels [Boyer et al., 1998; Fahlman et al., 2000; Epperson and Martin, 2002; Yan et al., 2006]. However, these gene products (and others not mentioned here) are mostly responsible for improving microcirculation (by reducing blood clotting), transporting lipids, augmenting metabolic/thermogenic potential, or removing reactive oxygen species. Thus, despite their crucial roles for cytoprotection and survival, upregulation of these gene products might marginally affect the extent of muscle retention during the long disuse period.

In conclusion, arousal would allow bats an opportunity to drink, feed, mate, urinate, find favorable roost sites, or restore metabolic

imbalance (summarized in Park et al. [2000]). Among these, our study emphasizes the role of arousal for restoring metabolic imbalance, with dramatic increments of mechanical and thermal stimulation, and thereby minimizing muscle loss and contractile depression. Since hibernation has been considered to be a useful model for an extended spaceflight [Blackstone et al., 2005], periodic arousals between torpor bouts may provide an insight into countermeasures against muscle atrophy that would have to be overcome for the long space trip.

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