Stress protein induction in skeletal muscle: comparison of laboratory models to naturally occurring hypertrophy

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Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan 66506–5602; Department of Biology, Emporia State University, Emporia, Kansas 66801; Department of Biomedical Sciences, Southwest Missouri State University, Springfield, Missouri 65804; and Department of Health and Physical Education, Southeastern Louisiana University, Hammond, Louisiana 70402

Kilgore, J. L., B. F. Timson, D. K. Saunders, R. R. Kraemer, R. D. Klemm, and C. R. Ross. Stress protein induction in skeletal muscle: comparison of laboratory models to naturally occurring hypertrophy. *J. Appl. Physiol.* 76(2): 598–601, 1994.—The purpose of the study was to compare stress protein [heat shock protein (HSP) 72] response in laboratory models of hypertrophy to naturally occurring work-induced hypertrophy. Two laboratory models of hypertrophy inducement, namely, compensatory hypertrophy and stretch hypertrophy, were compared with hypertrophy resulting from migratory flight in the blue-winged teal. We hypothesized that HSP 72 would be expressed more strongly in hypertrophied muscle than in control muscle. Furthermore, we hypothesized that changes occurring in laboratory models would also occur in work-induced enlargement. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were used to assess HSP 72 levels in control and hypertrophied muscle. Laboratory models elicited similar responses, with increased HSP 72 content in hypertrophied muscle. Work-induced hypertrophy or disuse atrophy did not change the degree of HSP 72 expression in the blue-winged teal. The presence of HSP 72 in these conditions may indicate that stressors eliciting changes in muscle protein expression, including the loss of muscle mass, may elicit HSP 72 synthesis.

heat shock protein; heat shock protein 72; compensatory hypertrophy; stretch hypertrophy

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STRESS PROTEINS, first identified as heat shock proteins (HSP), are a family of varied molecular mass proteins (28–174 kDa) that have several putative functions. Historically, the best characterized of these functions is protection of the cell from heat shock. Other functions attributed to these proteins include translocation of nascent proteins, dissociation of certain protein complexes, protection from phenotype alterations, and participation in protein folding and stabilization. Stress proteins are expressed constitutively in cells from all organisms and increase in type and concentration in response to physiological stressors (for review see Ref. 22). The most strongly induced forms of stress proteins have molecular masses of ~70 kDa. This subset of proteins (HSP 70) is highly conserved among species, is constitutively expressed in muscle tissue (9, 13), and is often associated with the myofibrillar constituents of skeletal muscle (14). Certain HSP 70 isoforms, namely HSP 72, are induced by hyperthermia, hypoxia, and glucose deprivation. Of particular interest to exercise scientists is the suggestion that tissue trauma (6), endurance training, or intermittent exercise may induce the expression of HSP 72 proteins (18, 20).

Stress proteins have been identified in several tissues after exhaustive, intense, or prolonged exercise. Spleen cells, lymphocytes, and leukocytes all have displayed enhanced expression of several stress proteins, including HSP 72 (18). Similarly, HSP 72 appears in the rat soleus, plantaris, and gastrocnemius postexercise (20).

In the absence of exercise or stress, a lower degree or lack of HSP 72 has been observed, a finding that is fairly consistent between species. It has been found that HSP 72 is not expressed in nonstressed bovine muscle (9) or found in nonstressed type II rat skeletal muscle (12, 13). It has been further determined that constitutive expression of HSP 72 is dependent on the prevalence of type I fibers within the muscle (20). Muscles with higher numbers of type I fibers have proportionally higher levels of the protein.

The cause for the increased synthesis of HSP 72 after exercise has yet to be described, although there are several proposed mechanisms. Damage to the muscle is one possibility. Exhaustive, intense, or prolonged exercise can induce myofibrillar damage. Damage to the muscle cell is attributed to a number of potential causes: load-initiated ruptures of the myofibrillar protein architecture (8), contraction-induced hypoxia (3), or increased lysozyme activity after prolonged and exhaustive exercise (27). The myofibrillar damage caused by these sources may be sufficient to initiate the synthesis of new stress proteins, although we know of no studies demonstrating such a correlation.

A limitation of previous work with stress proteins and exercise is that the models and methods used often manipulate muscle tissue in an artificial manner to achieve results. These models may or may not truly reflect the physiological changes seen as a result of work (23). The purpose of this investigation was to compare common laboratory animal models of hypertrophy (compensatory hypertrophy and stretch hypertrophy) with a natural work-induced hypertrophy of migratory fowl. We hypothesized that HSP 72 would be expressed more strongly in hypertrophied muscle than in nonhypertrophied muscle and, furthermore, that changes in HSP 72 seen in the laboratory models would also be seen in the field study.

METHODS

Experiments were conducted on three different species under three different conditions: *1)* Wistar rats for the compen-

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satory hypertrophy model, 2) Cornish Cross chickens for the stretch hypertrophy protocol, and 3) blue-winged teal for the field study.

Compensatory hypertrophy. Six male 120-day-old Wistar rats [mean body wt 500.6 ± 16.57 (SD) g; Harlan Sprague Dawley, Indianapolis, IN] were used. The medial head of the gastrocnemius of one hindlimb of each animal was ablated. Removal of the gastrocnemius causes the synergistic plantaris to hypertrophy. The gastrocnemius of the opposing limb was left intact. This yielded both a hypertrophied experimental group resulting from ablation and a nonhypertrophied control group. Rats were anesthetized with 1 ml/0.4 kg body wt of pentobarbital sodium via intraperitoneal injection. Ablation of the gastrocnemius was performed in a manner previously described (1) with the exception that the medial head of the gastrocnemius was removed and the lateral head was left intact.

Animals were allowed to move freely in their individual cages for 28 days after recovery. They were maintained in a climate-controlled room with a 12:12-h photoperiod. After the 28-day postsurgery period, animals were killed with ether. The plantaris muscles were then carefully isolated from the surrounding musculature, removed in one piece via cuts at the origin and insertion, trimmed of visible fat and connective tissue, rinsed in saline, blotted, and weighed. Intact muscles were placed in iced glycerol, immediately frozen, and stored at −70°C until analysis.

Stretch hypertrophy. Five 6-wk-old Cornish Cross roosters (mean body wt 2.40 ± 0.13 kg) were used for this portion of the study. Weights (10% of body wt) were attached to one wing of each animal. The application of weight to the wing placed a chronic stretch on the anterior latissimus dorsi and resulted in an increase in muscle mass (21). The opposing wing was not weighted, yielding a nonhypertrophied control muscle. The roosters were allowed to move freely in their cages for 30 days after weighting. Cage banks were maintained in a climate-controlled room with a 12:12-h photoperiod. Food and water were available ad libitum.

After the 30-day period, animals were killed by cervical dislocation. Anterior latissimus dorsi muscles were carefully removed, rinsed, blotted, and weighed. The muscles were placed in chilled O.C.T. tissue medium, frozen rapidly, and stored at −70°C until analysis.

Field study. Fourteen blue-winged teal (*Anas discors*) were captured and killed at three points during their annual migratory cycle: 1) immediately after migration (April), when hypertrophy of the pectoralis was at its peak; 2) at full molt (July), when the pectoralis had returned to a nonhypertrophied baseline level; and 3) just before flight (August) when the pectoralis has undergone slight hypertrophy (not work induced). Two immature birds were taken during this period to provide sources of tissue that had not undergone the stress of migration. After the birds were killed, the pectoral muscles of each animal were carefully excised and frozen in isopentane precooled in liquid nitrogen and then stored at −70°C until analysis.

Laboratory animals were given appropriate care consistent with the guiding principles of the American Physiological Society and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [DHHS Publ. No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Blue-winged teal were obtained with the authorization and assistance of the US Department of Fish and Wildlife.

Total protein assay. Entire muscles from each animal were first weighed. Then muscle samples were analyzed for total protein content by using the bicinchoninic acid assay (17) or

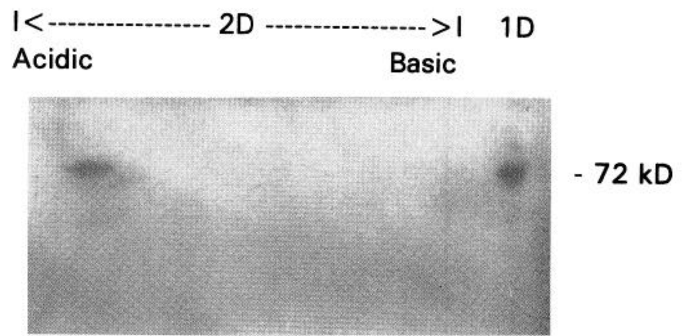

FIG. 1. Specificity of SPA-810 (C92) antibody for heat shock protein (HSP) 72. Homogenates of blue-winged teal pectoralis muscle were separated by isoelectric focusing and then separated in 2nd dimension by SDS-polyacrylamide gel electrophoresis (PAGE) (2D). Identical sample was separated by SDS-PAGE only (1D).

the Bradford assay (4) with bovine serum albumin as a standard.

Tissue preparation. Samples of thawed muscle tissue were homogenized in 5–10 vol of homogenization buffer [10 mM tris(hydroxymethyl)aminomethane-acetate, pH 7.6, 10 mM NaCl, 0.1 mM EDTA, and 15 mM mercaptoethanol]. Homogenates were centrifuged at 12,000 *g* for 20 min. Supernatants were prepared for electrophoresis by adjusting sample protein concentrations with homogenization buffer to 10 μg/μl, followed by the addition of 10 μl of reducing sample buffer (11) to the 15-μl sample.

Electrophoresis. Proteins from tissue homogenates were separated by molecular mass with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel slabs were prepared with a 4% acrylamide stacking gel (pH 8.8) and a 12% separation gel (pH 6.8). Sample wells were loaded with 10 μg of total protein and separated under denaturing conditions on a Mini-Protean II dual-slab apparatus (Bio-Rad, Richmond, CA). Samples were loaded into sample wells and electrophoresed in running buffer (pH 8.3) at 250 V for 40–45 min.

Isoelectric focusing. Muscle homogenates were diluted with an equal amount of sample buffer (9.5 M urea, 2.0% Triton X-100, 2% 3/10 ampholytes, 5% β-mercaptoethanol, diluted to 10 ml). The upper chamber of the Mini-Protean II tube module (Bio-Rad) was filled with degassed 20 mM NaOH and the lower chamber with 10 mM H_3PO_4. Samples were then focused on tube gels (pH 3/10 ampholytes, 9.2 M urea, 4% acrylamide) for 10 min at 500 V and then for 4 h at 700 V. Tube gels were then extruded into SDS-PAGE running buffer and allowed to equilibrate for 15 min. Equilibrated gels were then separated in a second dimension by SDS-PAGE electrophoresis, as described in the previous section.

Western blot. After electrophoresis or isoelectric focusing and electrophoresis, proteins from the gels were transferred overnight (Mini Trans-Blot, Bio-Rad) to polyvinylidine difluoride membranes (Millipore, Bedford, MA) by using a modification of the method described by Towbin et al. (24). After transfer, blots were blocked for 1 h in Tropix I-Block and then incubated overnight with a monoclonal antibody specific for HSP 72 (SPA-810, diluted 1:5,000, StressGen, Victoria, British Columbia, Canada; Ref. 28; Fig. 1). A 2-h incubation with a biotinylated goat anti-mouse immunoglobulin G (Sigma Chemical, St. Louis, MO; diluted 1:10,000) and a 45-min incubation with an avidin-alkaline phosphatase conjugate (diluted 1:10,000; Sigma Chemical) followed. All incubations were carried out at room temperature. Membranes were then prepared for autofluorography with AMPPD substrate (TROPIX, Bedford, MA) and visualized on Kodak XAR film. Films were scanned on a Hoefer GS300 densitometer to obtain integrated peak areas to quantify relative amounts of membrane-bound HSP 72.

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TABLE 1. *Body and muscle weights of laboratory models of compensatory hypertrophy (rat) and stretch hypertrophy (chicken)*

Animal/Muscle	n	Body Weight, g		Muscle Weight, mg	
		Initial	Final	Control	Hypertrophy
Rat plantaris	6	494.5±12.5	525.5±17.4	459.2±28.7	524.2±31.5*
Chicken anterior latissimus dorsi	5	2,404.0±134.5	3,864.0±373.1*	2,340.0±205.0	4,180.0±788.0*

Values are means ± SD. Paired t tests were conducted on muscle and body weight changes. * Significantly different from initial or control values ($P < 0.05$).

RESULTS

Muscle weights. Muscles from limbs that were experimentally treated in the laboratory were significantly ($P < 0.05$) larger than nonhypertrophied muscles from the same group in all cases (Table 1). The largest increase in mass was found in the stretch hypertrophy model, with a 78.6% increase in mass. The smallest increase came in the modified compensatory hypertrophy model, with a gain of 14.2%. The muscles obtained from the teal immediately after completing migration were 54.7% larger in mass (75.8 g ± 2.08 g; $P < 0.05$) than those of the molting, inactive teal (49.0 ± 4.59 g). The pectoralis muscles also showed a significant enlargement (58.7 ± 5.25 g; $P < 0.05$) just before commencing the next migration.

HSP 72 expression. HSP 72 was present in various degrees in muscle homogenates from all groups studied. The two laboratory models showed similar results when compared densitometrically (Table 2). Western blots of nonhypertrophied muscle showed small but detectable amounts of HSP 72 in both the predominantly type II rat plantaris and the slow tonic (type I) chicken anterior latissimus dorsi (Fig. 2). Hypertrophied muscle samples showed a significantly larger presence of HSP 72 ($P < 0.05$, compensatory hypertrophy; $P < 0.05$, stretch hypertrophy). To test the reliability of the experimental protocol, a companion trial was conducted simultaneously on rat soleus muscle (type I fibers, HSP 72 positive), and, as in previous studies, the anti-HSP 72 antibody bound to extracts of nonhypertrophied soleus muscle (Fig. 2).

The field study of blue-winged teal pectoralis muscle showed a different response than either of the two laboratory models (Fig. 2). HSP 72 was expressed strongly in each condition observed (Table 2). There was a large interanimal variation yielding large standard deviations, and, subsequently, a trend for increase or reduction in HSP 72 content with hypertrophy was not apparent. Muscles from immature birds, obtained from first-season birds immediately before migration, also showed detectable amounts of HSP 72 (Fig. 2).

TABLE 2. *Densitometric analysis of anti-HSP 72 binding*

Animal	n	Nonhypertrophied	Hypertrophied
Rat	3	1,793±1,214	6,348±2,173*
Chicken	5	3,306±1,503	5,709±2,301*
Teal	8	2,925±1,142	2,773±1,283

Values are means ± SD. Units represent peak areas as determined by gaussian integration. Statistical analysis of rat and chicken data done by paired t test. Blue-winged teal data analyzed by unpaired t test. * Significantly different from control ($P < 0.05$).

DISCUSSION

The purpose of this study was to quantitatively compare the stress protein content in skeletal muscle undergoing naturally occurring hypertrophy to muscular enlargement resulting from two commonly used laboratory protocols. Whereas the laboratory methods both elicited similar results, with increases in HSP 72 and in muscle mass occurring concomitantly, the results of the field study did not reflect this phenomenon. The pectoralis muscles of the teal underwent large changes in mass during the experimental period. These changes included both increases and decreases in mass, and in each condition similar amounts of HSP 72 were found.

Blue-winged teal offer an excellent opportunity to observe a series of transitions of muscle between hypertrophied and baseline states without researcher intervention. On arrival at their nesting grounds, the birds have completed migration of ~2,000 miles. This extended period of work elicits a large increase in the mass of working muscles, such as the pectoralis, and a lesser increase in total protein concentration of the same muscles (19). After the migration, the birds nest and molt. Their flight muscles are no longer active, and a rapid loss of muscle mass occurs. This sequence of events allows the collection of data at times corresponding to peak hypertrophy and peak atrophy.

In this portion of the study, the muscle examined (pectoralis) was composed almost entirely of type II fibers. Initially, we hypothesized that, after migration, HSP 72 would be strongly expressed in muscle hypertrophied from flight and would not be expressed in the nonhyper-

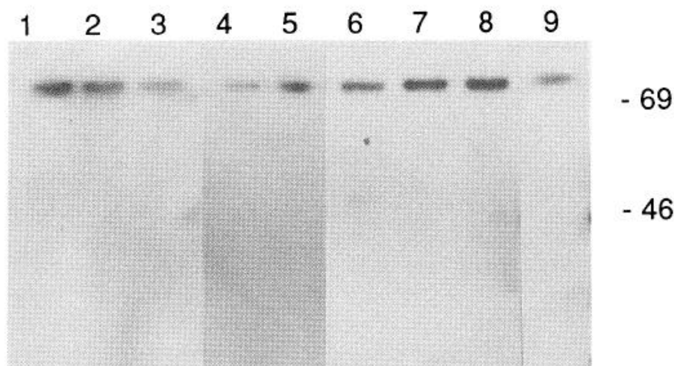

FIG. 2. Detection of HSP 72 in skeletal muscle by Western blot analysis. *Lane 1*, hypertrophied rat plantaris; *lane 2*, control rat soleus; *lane 3*, nonhypertrophied rat plantaris; *lane 4*, nonhypertrophied chicken anterior latissimus dorsi; *lane 5*, hypertrophied chicken anterior latissimus dorsi; *lane 6*, hypertrophied teal pectoralis (hormonally induced); *lane 7*, hypertrophied teal pectoralis; *lane 8*, nonhypertrophied teal pectoralis; *lane 9*, immature teal pectoralis. Numbers on *right* are molecular mass markers.

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trophied muscle, but several investigations have supported the claim that stress proteins may act as chaperones for nascent polypeptides as they emerge from the ribosome (2, 7). During the process of atrophy, there are substitutions of myofibrillar isomers (24, 25), meaning that synthesis of certain proteins is enhanced and others are depressed. This change in muscle protein profile is similar to hypertrophy in that, during hypertrophy, myofibrillar substitutions are characteristic. These alterations during hypertrophy have been documented in several animal species, including rodents and birds (10, 15, 16). As both of these conditions yield an alteration of muscle fiber architecture through synthesis and degradation of proteins, we might then predict expression of HSP 72 in both hypertrophic and atrophic conditions. The data collected during this experiment support this claim. The presence of HSP 72 in both hypertrophied and atrophied muscle may indicate that any set of conditions that favors changes in muscle protein profile is sufficient "stress" to elicit HSP 72 synthesis. Evaluation of laboratory models of atrophy, such as limb casting and tail suspension, would be appropriate to test this hypothesis.

The present investigation shows that in blue-winged teal HSP 72 is expressed in response to both exercise and disuse, both periods of muscular remodeling. The laboratory models also corroborated previous research by demonstrating that HSP 72 was only faintly or not constitutively expressed in nonstressed rat plantaris, a predominantly type II muscle, but was expressed constitutively in the rat soleus, a type I muscle. Additionally, evidence was provided suggesting that hypertrophy of rat plantaris and chicken anterior latissimus dorsi muscles induces HSP 72 synthesis. These data provide additional evidence that stress proteins may participate in much more than acquired thermotolerance and demonstrate a need to further investigate the role of stress proteins during changes in muscular structure and function.

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