Effects of Exercise on Canine Skeletal Muscle Proteolysis: An Investigation of the Ubiquitin-Proteasome Pathway and Other Metabolic Markers*

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ABSTRACT

The effects of long-term athletic training are associated with excessive skeletal muscle turnover attributable to increased rates of myofibrillar protein synthesis and proteolysis, which are mechanisms poorly understood in the athletic dog. A physiologic field study using 44 English pointers and Labrador retrievers that had been purposely bred for bird hunting and retrieving was conducted to examine changes in the ubiquitin-proteasome (UP) pathway, which has been implicated in exercise-induced proteolysis. Muscle biopsy samples were collected from all dogs in September (preseason, pretraining) and February (peak season, peak activity). Western blot analysis was used to assess changes in expression of various components of the UP pathway in the biopsy samples. Citrate synthase and glycogen levels were also measured in a subset of these samples. Results across the population indicated pronounced up-regulation of ubiquitinated conjugates and the p31 regulatory capping subunit during the peak hunting period compared with the preseason period. In contrast, the catalytic core of the proteasome (β–subunits) showed no apparent up-regulation in response to increased physical activity. Increased

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physical activity during the hunting season was associated with increased muscle glycogen levels and citrate synthase activity in these dogs. Overall, up-regulation of specific components of the UP pathway was an indication that it plays a role in the proteolytic process associated with skeletal muscle turnover during long-term athletic training, as previously believed.

INTRODUCTION

The role of long-term exercise (i.e., physical conditioning and training) on skeletal muscle metabolism and myofibrillar dynamics has been well studied in relation to turnover rates of skeletal muscle protein.[1–4] It is known that structural components of skeletal muscle can undergo degradation to provide substrates for metabolism.[5,6] The metabolic changes associated with exercise include increases in mitochondrial volume and substrate sequestration, such as glycogen levels in muscle.[5,7,8] Of particular interest is the increased rate of skeletal muscle turnover, namely, the myofibrillar components that have been extensively studied using radiolabeled phenylalanine, as well as up-regulation of myofibrillar messenger RNA and protein expression.[9–12] Although increases in the synthetic rates are important, exercise-induced changes in muscle proteolysis are equally important but have been poorly defined in the literature. Recent findings have suggested that the activity and expression of the ubiquitin-proteasome (UP) pathway are increased by acute, strenuous bouts of exercise.[13–19] However, it is not known whether specific components of this pathway (i.e., protein ubiquitination or 26S proteasome expression) are differentially regulated during prolonged periods of physical activity and/or training.

The primary purpose of this study was to examine several components of the UP pathway in a large population of athletic dogs during periods of minimal and peak physical activity. Exercise-induced changes in expression were assessed using Western blot analysis on muscle tissue samples obtained from a biopsy of the biceps femoris. Ubiquitination of intracytosolic proteins was evaluated based on the expression of soluble ubiquitinated conjugates; the 26S proteasome was examined through the catalytic core β-subunit of the 20S proteasome and the p31 subunit of the PA700 regulatory cap. A recent finding by the authors' laboratory indicated that the expression of the PA700 regulatory cap may be regulated differently than that of the catalytic core. As a result, it is necessary to evaluate components of the catalytic 20S-core portion of the 26S proteasome and the regulatory PA700 portion to determine changes in regulation of the UP pathway.[20] Although normal training regimens are known to up-regulate myofibrillar protein synthetic rates, it is hypothesized that, in canine athletes, these regimens and the concomitant increase in physical activity can also up-regulate portions of the proteolytic UP pathway responsible for myofibrillar protein degradation.

MATERIALS AND METHODS

Test Subjects and Training

A group of 44 English pointers and Labrador retrievers (23 males and 21 females) between 2 and 10 years of age was selected for the study with the informed consent of the plantation owner and complying with standards set by the Institutional Animal Care and Use Committee. The dogs were from a privately owned hunting kennel in which they were used on a regular basis for quail hunting and retrieving. They were not trained or used for hunting during the off-season, which extended from March through September. Training and physical conditioning were initiated in September and continued until the beginning of the quail-hunting season in mid-November.

Using commonly accepted practices in the sport, training and conditioning of the dogs during the upcoming hunting season involved 20 to 30 minutes of "roading" (i.e., simulated cart pulling) two to three times per week. The dogs were also trained using simulated hunting events at the same frequency. During the hunting season, the frequency and duration of hunting for all dogs averaged 1.6 hunts per week and 64 minutes per hunt. Dogs were housed singly in a 6 × 20-foot run with continuous access to water. During the course of the season, the dogs were fed one of three diets containing 26% to 31% protein, 17% to 21% fat, and 31% to 38% carbohydrate (as-fed basis) according to regular kennel practices. The dog handlers monitored each dog's body weight and condition throughout the year. Body weights and body condition scores based on a 5-point scale were also measured by a veterinary technician at baseline and peak-season sampling time points.

Sample Acquisition

A muscle biopsy sample was obtained from each dog in September (pretraining) and early March (peak activity) by a licensed veterinarian using standard veterinary procedures. Each dog was briefly anesthetized using intravenous administration of 1 ml of Telazol®/22.5 kg of weight (50-mg delivery of tiletamine and zolazetam each) to obtain a small percutaneous biopsy of the biceps femoris. The average biopsy sample was approximately 80 to 100 mg of skeletal muscle tissue. The collected tissue was immediately snap-frozen and stored at −80°C until analysis. The March sample (peak activity) was obtained 24 hours after each dog had been subjected to a 40-minute hunting event on the previous day.

Sample Preparation

Each biopsy sample was homogenized using a Brinkman tissue homogenizer in cold 4°C homogenizing buffer (buffer H: 20 mM Tris [hydroxymethyl], 20 mM sodium chloride, 1 mM EDTA, 1 mM mercaptoethanol; pH 7.6; equilibrated to 1L). An average of 75 mg of tissue was homogenized in 750 µl of buffer H. After homogenization, the samples were centrifuged at 12,000 *g* for 20 minutes at 4°C in a microcentrifuge. After protein concentrations were determined using the Bradford technique[22] on a DU-640 spectrophotometer, the samples were equilibrated to a final concentration of 2.5 µg/µl using buffer H and 5× sodium dodecyl sulfate (SDS) loading buffer (0.312 M Tris-hydrochloride [HCl]; pH 6.8; 10% SDS, 25% β-mercaptoethanol, 0.05% bromophenol blue).

Western Blot Analysis

Muscle extracts (50 µg of total protein per sample) were subjected to SDS-polyacrylamide gel electrophoresis on either 4% to 12% Laemmli-gradient gels (ubiquitin conjugates) or 12% Laemmli gels (β- and p31 subunits). Preseason and peak-season samples for an individual dog were resolved on the same gel, along with approximately 0.5 µg of purified p31 (from transformed *Escherichia coli*) or 20S proteasome (from rabbit) for comparative purposes. The use of these control proteins and antibodies for comparative purposes in expression has been validated by Ordway and colleagues.[23] Proteins were electrotransferred to nitrocellulose membranes, and the membranes were blocked in 5% nonfat fried milk in Tris-buffered saline with 0.05% Tween®-20. Blots were then incubated with rabbit polyclonal antibodies against the proteasome β-subunit (1:1000) or the p31 subunit of PA700 (1:2500) or mouse monoclonal antibody against ubiquitin conjugates (1:1000), with subsequent incubation with antirabbit or antimouse immunoglobulin G (IgG) horseradish peroxidase-labeled antibodies (1:5000). Blots were visual-

ized by the enhanced chemiluminescence-detection method and analyzed by densitometry using Total Lab software (Amersham Biosciences, Piscataway, NJ). Subsequent amido black staining was conducted on each p31 blot to ensure that equal loading of protein was completed for each pair of samples.

Glycogen and Citrate Synthase Analysis

Glycogen analysis was conducted on muscle tissue obtained from 33 dogs according to the protocol described by Lo and colleagues.[24] Glycogen analysis could not be conducted on 11 dogs because a minimum of 20 mg of tissue was required for the analysis. Citrate synthase analysis was also conducted on preseason and peak-season samples for 11 dogs to evaluate this marker of mitochondrial volume. After Western blot and glycogen analyses, 40 mg of remaining tissue was homogenized in 400 µl of homogenizing medium using a Brinkman tissue homogenizer at 4°C. Homogenizing medium consists of 150 mM sucrose, 2 mM EDTA, and 100 mM Tris-HCl at a pH of 7.45. The homogenate was then centrifuged at 2500 rpm at 4°C for 20 minutes. The supernatants were collected and assayed on a DU 640 spectrophotometer for citrate synthase activity according to methodologies adapted from Srere.[25]

Data Analysis

All statistical analyses were conducted using Microsoft Excel® software version '98. Densitometry results were evaluated using a two-tailed paired *t*-test to assess the influence of athletic training and physical activity on various components of the 26S proteasome pathway and ubiquitin conjugation blots with the α-value set at 0.05. Baseline and peak-training glycogen levels in muscle, citrate synthase activity, and body weights were also evaluated for significance ($P < .001$) using a two-tailed paired *t*-test with an α-value set at 0.05. Diet, gender, and breed effects were removed from the model after preliminary analysis showed no dietary-, gender-, or breed-related differences for any parameter (data not shown). In addition, the mass of muscle does not influence the parameters tested in general, as proven by Ordway and colleagues.[23]

RESULTS

26S Proteasome

There was no significant change in the regulation of the β-subunit of the 20S proteasome catalytic core based on densitometric evaluation of Western blots when baseline and peak-training results were compared. The average percentage change in the densitometric readings between pretraining and peak training was 10.6% ± 49.6% (Figure 1). The p31 expression, as an indication of regulatory capping expression, showed a moderate degree of exercise-induced up-regulation (48% ± 69%; $P < .001$) when comparing baseline and peak-training expression (Figure 2). Typical immunoblots of p31 and β-subunit expression showing clean blotting procedures and distinct banding are shown in Figure 3. All blots contained control proteins run in conjunction with a molecular weight marker for proper band identification.

Ubiquitin Conjugates

Densitometry on the ubiquitin conjugate films showed a marked increase (89% ± 136%; $P < .001$) in the ubiquitinated conjugates in skeletal muscle during peak training (Figure 4). A typical blot of the ubiquitin conjugates demonstrating the range of conjugated proteins in the cytosol is shown in Figure 3.

Muscle Glycogen, Citrate Synthase Activity, and Body Weight

There was a marked increase ($P < .001$) in the glycogen content of skeletal muscle of these dogs at peak training compared with that in

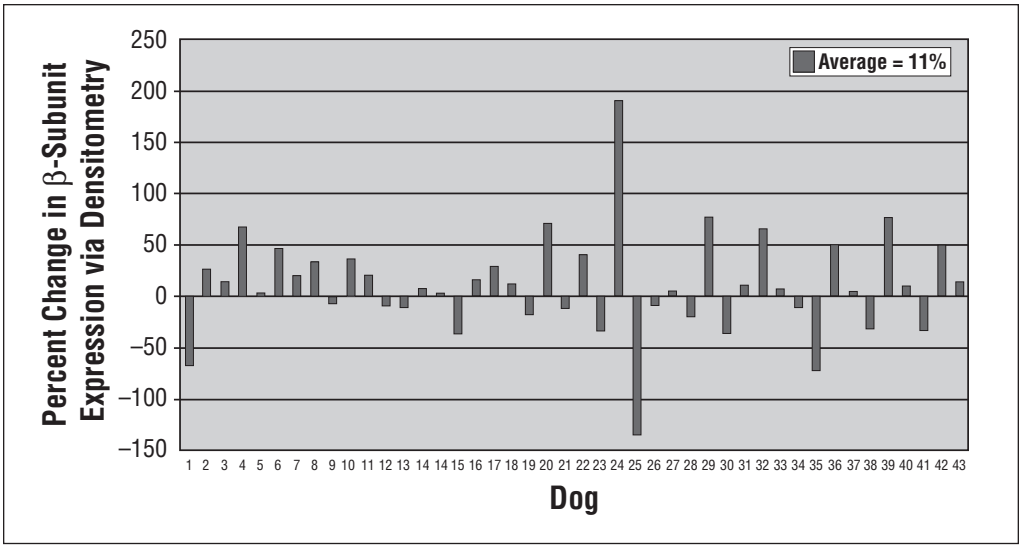

Figure 1. *The 20S proteasome β-subunit immunoblot percentage of change in expression between baseline and peak training, based on densitometry results for each dog.*

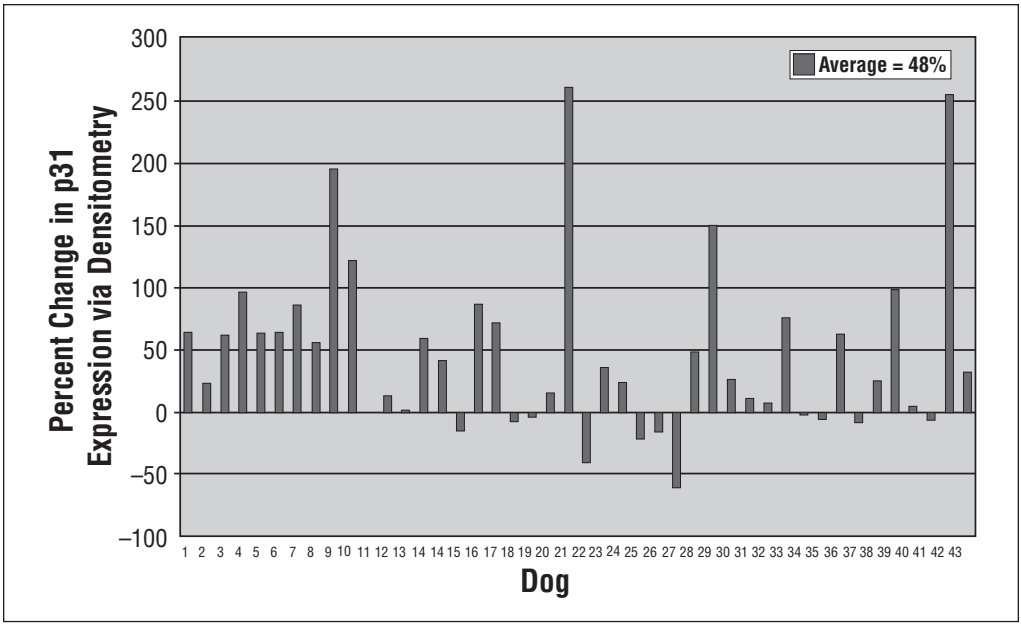

Figure 2. *The PA700 regulatory cap p31 subunit immunoblot percentage of change in expression between baseline and peak training, based on densitometry results for each dog.*

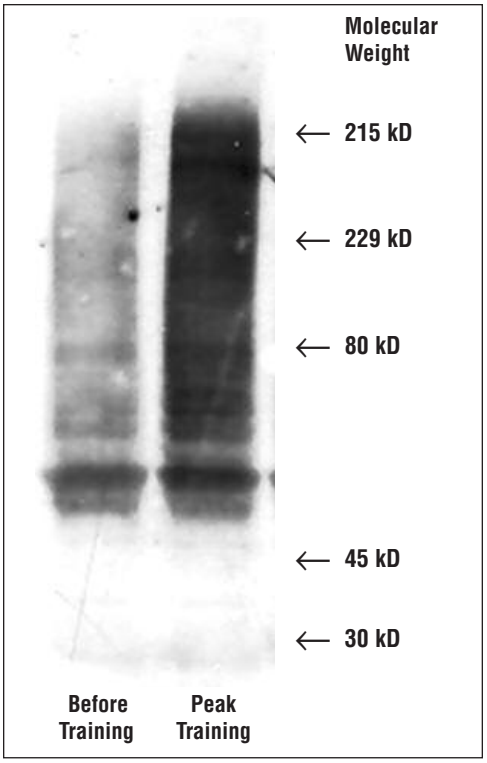

Figure 3A

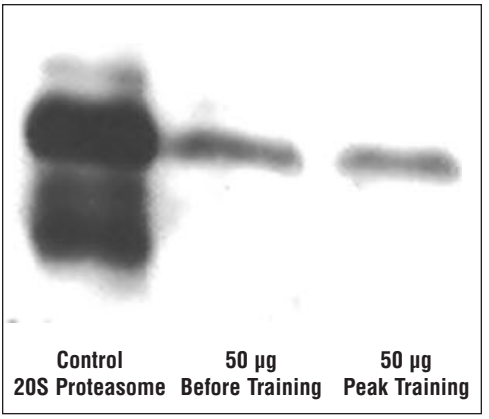

Figure 3B

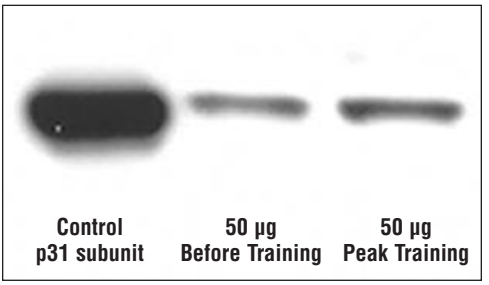

Figure 3C

Figure 3. *Typical immunoblot results for each ubiquitin-proteasome–pathway parameter assessed at baseline versus those at peak training.* (**A**) *Ubiquitin conjugates,* (**B**) *20S proteasome* β*-subunit expression, and* (**C**) *PA700 regulatory cap p31 subunit expression.*

preseason resting samples (Table 1). Citrate synthase activity also showed a significant increase ($P < .01$) at peak training in these dogs compared with pretraining values (Table 1). The body weight of the dogs also increased ($P < .001$) from a baseline average of 19.14 kg to an average of 20.05 kg at peak training (Table 1). Body condition scores across the population showed no significant differences, averaging 2.95 at pretraining and 3.05 at peak training on a 5-point scale.

DISCUSSION

The metabolic pathways involved in skeletal muscle proteolysis include the lysosomal pathway, the calpain pathway, and the UP pathway. Lysosomal proteolysis is involved in nonmyofibrillar proteolysis associated with endocytic pathways and is responsible for the turnover of selected proteins from the extracellular matrix or plasma membrane. The calpain proteolytic system has been implicated in the structural disassembly of the myofibrillar unit, including actin and myosin. The adenosine triphosphate-dependent UP pathway is involved in the catabolism of long- and short-lived intracytosolic and some membrane-bound proteins. This pathway is responsible for

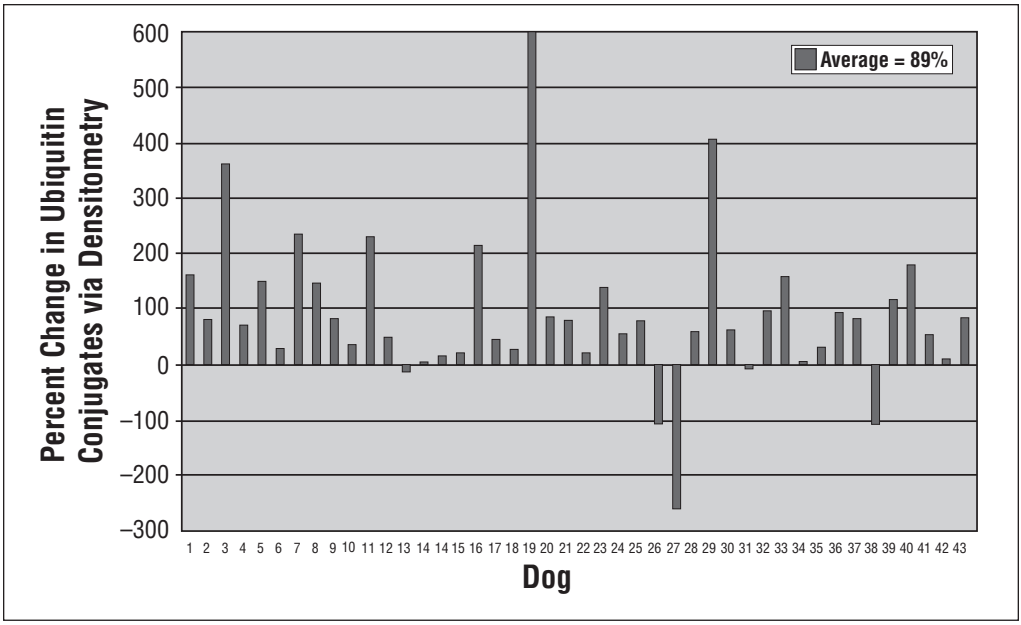

Figure 4. *Ubiquitin conjugates immunoblot percentage of change in expression between baseline and peak training, based on densitometry results for each dog.*

TABLE 1. Average Skeletal Muscle Glycogen Content, Citrate Synthase Activity, and Body Weight of Study Dogs in September (Pretraining) and March (Peak Training)

Parameter	*September (Pretraining)*	*March (Peak Training)*	P *Value*
Glycogen (g /100 mg of tissue)	4.85 ± 2.39	7.26 ± 2.69	*P* < .001
Citrate synthase (μm substrate oxidized/min)	26.8 ± 10.0	32.7 ± 11.1	*P* < .01
Body weight (kg)	19.14 ± 2.09	20.05 ± 1.95	*P* < .001

the actual liberation of amino acids via cleavage of long-lived proteins, such as myosin.[19]

The UP pathway has multiple components, including the 20S subunit of the proteasome, which is a multisubunit complex consisting of four ring-like structures with seven subunits in each ring. This cylindrical peptidase contains nearly homologous α- and β-subunits and is controlled by a lid and cap. The lid sits on either end or both ends of the 20S cylindrical unit and consists of a ring-like structure with six subunits that are essential for proteolysis. Positioned on the lid structure is a capping complex, which may be involved in substrate recognition for subsequent proteolysis. The exact number of constituents in the capping structure has yet to be determined, but there are at least seven subunits involved. The cap and lid portion is collectively known as the *PA700* or *19S regulatory complex*. The association of a tar-

geted protein to this entire 26S proteasome complex (20S and PA700 complex) involves ubiquitin. During the ubiquitination process, ubiquitin covalently binds to lysine residues on the target proteins through a series of chaperone and ligation enzymes (E1, E2, E3). Covalent binding of ubiquitin induces the formation of a polyubiquitin tail on the protein of interest. After they have been ubiquitinated, the ubiquitin conjugates are subjected to proteolysis via the 26S proteasome.

During the past 10 years, it has become increasingly clear that the UP pathway is a major pathway involved in skeletal muscle proteolysis during various disease processes that lead to muscle atrophy.[26,27] It is also becoming apparent that subtle changes in this proteolytic pathway occur in healthy animals as well. These subtle influences occur through diet, aging, and exercise and can have substantial influences on lean body constitution over time.[13–18,28,29] The role of exercise on ubiquitin-dependent proteolysis in skeletal muscle is apparently profound, based on these results. It is possible that increased proteolysis is needed to repair exercise-induced damaged or oxidized protein and/or to counterbalance increased myofibrillar synthesis. Recent literature has reported increased ubiquitin expression and conjugation immediately after strenuous eccentric exercise in nontrained individuals.[14,17] These results showed that repeated contractions of untrained muscle during a single bout of exercise up-regulated the ubiquitination of skeletal muscle proteins. In contrast, the observed up-regulation of ubiquitination in this study's group of canine athletes may have resulted from the prolonged athletic training program. The 40-minute simulated hunt conducted 24 hours before the February sampling would not be considered strenuous exercise for these physically conditioned dogs, suggesting that the observed ubiquitination was likely an overall training effect and not the result of a single acute bout of exercise. Previous publications and data from Ordway and colleagues[23] suggest that an extremely rigorous bout of contractile activity is needed to markedly up-regulate the UP pathway and that in those cases, the 20S portion may also be up-regulated, which was not observed in this population of study dogs.

To confirm that these dogs were exercised on a regular basis, two additional metabolic parameters of exercise in the muscle of these dogs were evaluated: citrate synthase activity and glycogen content. Physical training is associated with increased mitochondrial volume in skeletal muscle and increased ability to store glycogen.[5,7,8] Although citrate synthase activity was measured in only 11 dogs and glycogen levels in 33 dogs, the results showed significant increases in the peak-training samples compared with preseason samples. These results confirm that the level of physical activity of these dogs during the hunting season was sufficient to increase the parameters often associated with long-term training and aerobic events.

The 26S proteasome has been implicated in the degradation of myofibrillar components, and its up-regulation in various situations can lead to lean body wasting, such as protein loss, starvation, and cytokine and hormonal stimulation.[25,30–33] Therefore, the goal was to observe small but significant changes in the various components of the catalytic core (β-subunit) or the capping complex (p31 subunit). Unfortunately, the β-subunit expression showed no significant changes in these dogs from pretraining to peak training. Interestingly, the p31 subunit expression increased significantly ($P < .001$), with an average increase of nearly 50%. Although p31 is only one of many subunits in the regulatory PA700 protein complex, it is critical to proteasome function. Research[34] has

shown that elimination of the yeast homologue of p31, known as *NIN1*, from the regulatory complex increases ubiquitin conjugates and causes the cells to become dormant. Therefore, p31 is believed to be critical in the regulatory capping function. These findings suggested that the regulatory cap, which may be involved in recognition of ubiquitinated substrates, may be differentially expressed in situations of physical stress, resulting in increased proteolysis. Although expression does not equate to activity, a study by Radak and colleagues[15] confirmed that exercise can induce a higher proteolytic rate, which may be the result of up-regulation of the PA700 subunits and/or increased substrate ubiquitination. Although speculative, it is likely that the proteasome 20S core remains undifferentially expressed, considering it is essential for proteolysis of many cytosolic components regardless of ubiquitination, whereas polyubiquitination of a substrate results in preferential degradation by the capping complex.[35]

When considering that the group of dogs used in this study displayed a slight increase in body weight between pretraining and peak training, it was assumed that the changes in p31 and/or the ubiquitinated conjugates were not attributed to loss of lean body mass or weight loss. In fact, a collaborative study from the same group of authors has recently shown that loss of lean body mass is associated with reduction in p31-subunit expression, due presumably to a survival mechanism to conserve lean body mass during long-term protein deprivation.[20] Other environmental and physiologic factors that have been shown to affect the UP pathway include cytokines (tumor necrosis factor, interleukin-6), sepsis, neoplasia, renal failure, and glucocorticoids. However, it is highly unlikely that any of these conditions existed within this group of dogs based on standard veterinary examinations conducted at the initiation and conclusion of the hunting season. All dogs were shown to be healthy throughout the season. Therefore, the large number of the biopsy samples that showed up-regulation of the UP pathway in the peak-training period compared with the resting, pre-training period suggests that increased physical activity was the primary factor responsible for increased expression of p31 and ubiquitinated conjugates. The variability of ubiquitin conjugation and p31 up-regulation cannot be explained by breed or dietary differences; however, because of the field study design, other factors may have played a role in this interdog variability, such as stress of the hunt, genetic influences, and individual physical exertion or lack thereof. All of these variables could not be accounted for or measured in multivariable modeling for influences on the UP pathway.

Overall, an examination of the UP pathway in canine skeletal muscle provides strong evidence that ubiquitin conjugation and the p31 proteasome subunit are up-regulated during athletic training and physical activity. Although there could be other confounding factors that were not or could not be measured in this study, the large sample size and profound changes observed in distinct portions of the pathway are consistent with findings in the literature, suggesting that the UP pathway is a major pathway involved in exercise-induced proteolysis. It is likely that up-regulation of the UP pathway during a long-term exercise regimen provides a homeostatic balance between increased rates of myofibrillar protein synthesis and degradation and as a mechanism to repair exercised-induced muscle damage or oxidation.

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