Insulin-Like Growth Factor-I and High Protein Diet Decrease Calpain-Mediated Proteolysis in Murine Muscular Dystrophy

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Abstract. **In muscular dystrophy (MD) the imbalance between muscle protein synthesis and degradation may be an important factor leading to muscle wasting. The three major pathways of muscle proteolysis identified in skeletal muscle are: the lysosomal cathepsin pathway, the calcium-dependent calpain pathway, and the ATP-dependent ubiquitin pathway. Insulin-like growth factor I (IGF-I) and a high-protein diet (HPD) have been shown to reduce proteolysis in skeletal muscle. We examined the effect of 6 weeks of recombinant human IGF-I (rhIGF-I) alone or in combination with HPD treatment on the proteolytic pathways in skeletal muscle of 129 ReJ dystrophic (*dy*) mice. (A group of normal (Norm) nondystrophic (129 J) mice were included as controls). Untreated *dy* mice exhibited increased net proteolysis ($P < 0.05$), elevated net calpain activity ($P < 0.01$), and increased ubiquitin levels when compared to control mice ($P < 0.05$). Our evidence suggests that HPD and rhIGF-I decrease proteolysis in the 129 ReJ *dy* mouse. This effect appears attributable, at least in part, to reduced calpain-mediated myofibrillar breakdown ($P < 0.05$) due to decreased calpain autolysis or increased calpastatin levels. In contrast to calpain, cathepsin B activity was increased in HPD and rhIGF-I + HPD-treated *dy* muscle ($P < 0.05$) and unaltered in the rhIGF-I treated animals. Levels of free and protein-conjugated ubiquitin were also increased in rhIGF-I, and rhIGF-I + HPD treated *dy* animals ($P < 0.05$). The amelioration of muscle wasting in the 129 ReJ *dy* model by HPD and/or rhIGF-I may have potential implications in the treatment of human MD.** [P.S.E.B.M. 1998, Vol 218]

In muscular dystrophy (MD), net muscle protein loss and wasting are enhanced. While this may be due to increased protein degradation, reduced protein synthesis or a combination of the two (1), a number of studies have suggested that excess muscle proteolysis is the key factor in the muscle wasting and weakness associated with MD (1–7). In general, degradation of cellular proteins occurs *via* three pathways: lysosomal; calcium-dependent; and ATP-dependent pathways (3). Studies indicate increased activity of all three mechanisms in atrophied, injured, or dystrophic muscle (2–8). However, the significance of these proteolytic pathways in the degenerative process of MD remains poorly understood.

Previously, we showed that a 4–8-week administration of recombinant human insulin-like growth factor-I (rhIGF-I) and/or a high-protein diet (HPD) increased protein synthesis, reduced total proteolysis, and preserved muscle structure in the 129 ReJ *dy* mouse and Bio 53.58 hamster models of MD (9–11). The present study was intended to investigate the effects of treatment on the cathepsin, ubiquitin, and calpain pathways in muscle protein breakdown in the dystrophic 129 ReJ *dy* mouse during these therapeutic interventions.

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The results suggest a reduction in net calpain-mediated proteolysis, and that may contribute to the overall improvement in net muscle protein levels.

Materials and Methods

Animals and Experimental Protocol. Male dystrophic (129 ReJ *dy*) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 30 days of age and divided into the following four experimental groups for a period of 6 weeks: [A] Normal protein diet (NPD) and placebo injections of phosphate-buffered saline (*dy* NPD) (n = 14); [B] NPD, rhIGF-I treated (10 μg b.i.d. s.c.) (*dy* NPD + rhIGF-I) (n = 13); [C] HPD and placebo injections (HPD) (*dy* HPD) (n = 11); [D] rhIGF-I + HPD (*dy* rhIGF-I + HPD) (n = 12). In addition to the four experimental *dy* groups, age- and sex-matched, untreated, nondystrophic 129 J mice (NORM) receiving NPD were included as a reference for normal muscle parameters (n = 14). During this experimental treatment period, animals had free access to water and the solid diets described below. At the conclusion of the study, animals were anesthetized with i.p. urethane (1.3 g/kg), and gastrocnemius and quadriceps muscles were removed bilaterally for biochemical and molecular analysis. Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH), and the protocol was approved by the North Shore University Hospital Animal Care and Utilization Committee.

Diet Formulation. The normal protein diet (NPD) contained 22.9% casein and 47.1% sucrose, whereas the HPD contained 57.4% casein and 17.1% sucrose. Both diets contained 15.0% corn starch, 4.7% corn oil, 4.0% American Institute of Nutrition (AIN76) mineral mix, and 1.0% AIN vitamin mix (ICN, Costa Mesa, CA). The remainder of the diets were made up of Alphacel non-nutritive bulk, and both diets were isocaloric. Additionally, nondystrophic mice were fed the same normal protein diet that untreated dystrophic mice received. Since food consumption between the various groups of mice did not differ, pair feeding was not necessary.

Total Proteolyis. Total proteolysis was determined by measuring the release of tyrosine from incubated muscles (12). Briefly, gastrocnemius muscles were rapidly removed bilaterally and placed in Krebs-Henselit bicarbonate buffer (pH 7.4, 35°C) containing 0.13% albumin, 10 m*M* glucose, 0.1 m*M* leucine, 0.1 m*M* isoleucine, 0.2 m*M* valine, and 0.5 m*M* cycloheximide, gassed with 5% CO_2/95% O_2. Muscles were preincubated for 30 min and then transferred to tubes containing fresh medium for an additional 2 hr incubation. At the end of the incubation, muscles were removed, rinsed, and blotted. Tyrosine concentration of the medium and muscles was determined fluorometrically (13) and expressed as a function of tyrosine release in nmol/hr × g dry weight of tissue. Cyclohexamide (0.5 m*M*) was included in the incubation medium to inhibit protein synthesis and prevent recycling of amino acids.

Cathepsin and Calpain Activity. Freshly isolated quadriceps muscles were homogenized in 20 volumes of tris-HCl buffer (pH 7.4) containing 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol, 15 m*M* cysteine, and 0.25 *M* sucrose. Muscle homogenates were centrifuged at 10,000*g* for 15 min. Activity of cathepsin B in muscle homogenates was measured fluorometrically according to the method of Barrett and Kirschke (14) using the specific substrate Z-Phe-Arg-N-methyl coumarin. The fluorescence of free aminomethylcoumarin product was measured at 370 nm excitation and 460 nm emission and quantitated against an aminomethylcoumarin standard (0.5 μ*M* of standard was equal to 0.1 μU of activity in the reaction tube). Cathepsin activity was measured in mUnits of activity per gram of dry weight of tissue. Calcium-dependent calpain activity was measured spectrophotometrically in muscle homogenates using the method of Moss *et al.* (15) with azocasein as the substrate. Calpain activity was measured both in the presence and absence of calcium in the incubation mixture and the difference used to calculate specific calpain activity. One unit of calpain activity was defined as the amount of enzyme that produced an absorbance change of 1.0 in a 1 cm cuvette at 366 nm under standard assay conditions. Calpain activity was expressed in mUnits of activity per gram dry weight of tissue.

Ubiquitin Determination. Free ubiquitin (FUB) and protein-conjugated ubiquitin (CUB) were quantitated using Western blot. Gastrocnemius muscles were rapidly removed under anesthesia, frozen in liquid nitrogen, pulverized in a liquid nitrogen–cooled mortar, and homogenized in 1.0 ml of ice-cold 50 m*M* tris-HCl buffer (pH 7.4) containing 0.25 *M* sucrose, 5 m*M* EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 0.1 unit/ml aprotinin, 1 μ*M* leupeptin, 1 μ*M* pepstatin, 5 m*M* N-ethylmaleimide and 1 m*M* phenymethylsulfonyl fluoride (16). Aliquots of the homogenate were diluted to 1 μg/ml of protein with 30 m*M* phosphate buffer (pH 7.0) containing 7.5% (w/v) SDS, 0.15% (w/v) dithiotrietol, 0.05% (w/v) bromophenol blue, and 30% glycerol. Diluted samples were boiled for 5 min, and 5 μg of protein was separated using SDS-polyacrylamide gel (15%) electrophoresis.

Following separation, proteins were transferred onto Polyscreen membranes (Dupont Inc., Wilmington, DE) overnight at low voltage in 25 m*M* Trizma buffer (pH 8.3) with 144 m*M* glycine and 0.01% SDS. After transfer, the membrane was blocked for 2 hr with high salt TBS (pH 7.4) containing 25 mg/ml BSA and 5% nonfat dry milk. The membrane was then incubated overnight (4°C) with a polyclonal antibody that recognized both FUB and CUB (antibody was the generous gift of Dr. Arthur L. Haas, Department of Biochemistry, Medical College of Wisconsin). The polyclonal antibody employed had identical sequence homology to mouse ubiquitin (17). After repeated washes, the membrane was incubated for 2 hr with a horseradish peroxidase–conjugated goat-anti-rabbit second antibody. Detection was carried out using the NEN Renaissance chemiluminescence system (Dupont Inc, Wilmington, DE). Rela-

tive quantitation of proteins was performed using a scanning microdensitometer (Biorad model GS-700, Biorad Inc, Hercules, CA). Ubiquitin was expressed in units that were defined as optical density (o.d.) × $mm.^2$

Statistical Analysis. Statistical comparisons between the four groups of animals were performed using a one-way analysis of variance (ANOVA) followed by a Tukey's test for specific critical differences (18). Statistical significance was set at the 95% level ($P < 0.05$). All means were expressed ± the standard error of the mean (SEM).

Results

General Characteristics and Weight Data. Food intake between the *dy* groups did not differ (Table I). *dy* groups had a significantly lower % weight gain as compared to NORM ($P < 0.05$); however, the *dy* HPD and *dy* HPD + rhIGF-I-treated groups had increased weight gain compared to the *dy* NPD group ($P < 0.05$). The weights of the *dy* NPD + rhIGF-I treated animals did not differ from the *dy* NPD group.

The quadriceps of NORM weighted roughly two times more than the *dy* NPD group. Additionally, the *dy* HPD and *dy* HPD + rhIGF-I treated groups both had an increase in quadriceps weight compared to the *dy* NPD group. By contrast, the NPD + rhIGF-I-treated group failed to show any increase in quadriceps weight compared to *dy* NPD. The weight of the gastrocnemius in NORM weighed approximately four times that of *dy* NPD. Furthermore, gastrocnemius muscles of all *dy* animals weighed less than that of NORM. *dy* HPD-treated animals showed an increase in gastrocnemius weight compared to *dy* NPD. Although having an increase in gastrocnemius weight, the *dy* HPD + rhIGF-I group did not significantly differ from *dy* NPD.

Net Proteolysis. *dy* NPD animals had significantly increased tyrosine release when compared to NORM muscles ($P < 0.05$) indicative of increased muscle catabolism (Fig. 1). Both HPD and HPD + rhIGF-I-treated *dy* animals exhibited a reduction in tyrosine release when compared to their untreated controls (*dy* NPD) ($P < 0.05$; $P < 0.01$). Tyrosine release in the NPD + rhIGF-*dy* treated animals was still greater than NORM muscle ($P < 0.05$). No differences were seen between the *dy* HPD and *dy* HPD + rhIGF-I-treated groups and the NORM.

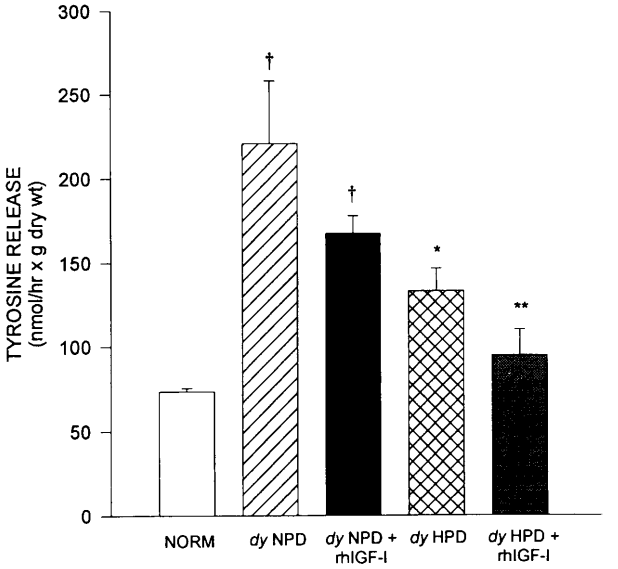

Figure 1. Tyrosine release from gastrocnemius muscle. Results of tyrosine release, and indicator of net proteolysis, measured from gastrocnemius muscles in NORM ($n = 14$), *dy* NPD + rhIGF-I ($n = 13$), *dy* HPD ($n = 11$), and *dy* HPD + rhIGF-I ($n = 12$). Means are shown ± SEM. Tyrosine release was measured in nmol of tyrosine per hour × gram of dry weight. ** $P < 0.01$ vs *dy* NT; * $P < 0.05$ vs *dy* NT; † < 0.05 vs NORM.

Calpain Activity. *dy* NPD animals exhibited increased calpain activity compared to NORM animals ($P < 0.01$) (Fig. 2). All treated groups (*dy* HPD, *dy* NPD + rhIGF-I, and *dy* HPD + rhIGF-I) showed significantly reduced calpain activity compared to *dy* NT ($P < 0.05$). The *dy* HPD, *dy* NPD + rhIGF-I and *dy* HPD + rhIGF-I groups' calpain activity did not differ from NORM.

Cathepsin B Activity. Cathepsin activity in the *dy* NPD group was indistinguishable from NORM mice (Fig. 3). The *dy* HPD and *dy* HPD + rhIGF-I groups all showed elevated cathepsin activity when compared to *dy* NPD ($P < 0.05$). By contrast, *dy* NPD + rhIGF-I-treated animals showed no enhanced cathepsin activity.

Table I. General Characteristics

	Food intake (g)	% Weight change	Quadriceps weight (g)	Gastrocnemius weight (g)
NORM $n = 14$	4.6 ± 0.70[a]	30.5 ± 1.56[a]	0.057 ± 0.002[a]	0.043 ± 0.005[a]
dy NPD $n = 11$	2.13 ± 0.2[b]	13.8 ± 0.33[b]	0.023 ± 0.003[b]	0.011 ± 0.001[b]
dy NPD + rhIGF-I $n = 13$	2.19 ± 0.30[b]	14.0 ± 0.81[b]	0.029 ± 0.004[b,c]	0.013 ± 0.002[b]
dy HPD $n = 14$	2.61 ± 0.29[b]	22.2 ± 1.03[c]	0.035 ± 0.002[c]	0.024 ± 0.003[c]
dy HPD + rhIGF-I $n = 12$	2.48 ± 0.18[b]	23.2 ± 0.59[c]	0.033 ± 0.004[c]	0.021 ± 0.002[b,c]

Note. Data of food intake (g), body weight change (%), and muscle weights (g) from NORM, *dy* NPD, *dy* NPD + rhIGF-I, *dy* HPD, and *dy* HPD + rhIGF-I treated animals. Number of animals in each group are displayed in the table. Means ± SEM. Values in the same column that do not share a superscript are significantly different ($P < 0.05$).

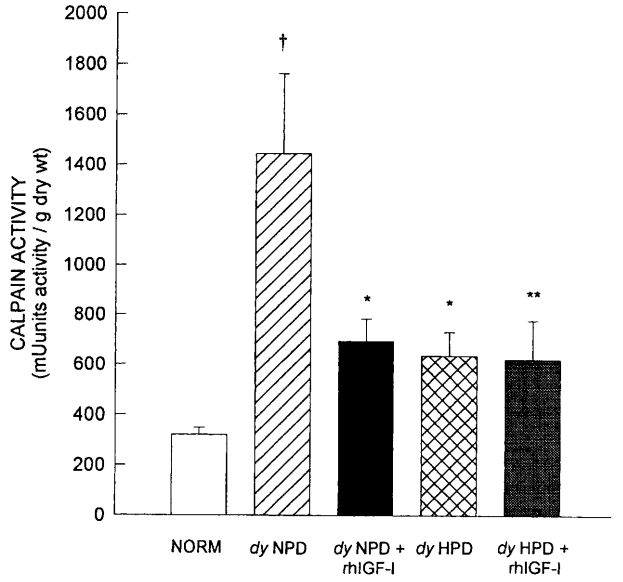

Figure 2. Calcium-mediated calpain activity. Results of calcium-mediated calpain activity measured in quadriceps muscles in NORM (n = 14), *dy* NPD (n = 14), *dy* NPD + rhIGF-I (n = 13), *dy* HPD (n = 11) and *dy* HPD + rhIGF-I-treated (n = 12) animals. Calpain activity was measured in mUnits of enzymatic activity per gram of tissue dry weight. One unit of calpain activity was defined as the amount of enzyme that produced an absorbance change of 1.0 in a 1 cm cuvette at 366 nm under standard assay conditions. Results are expressed as Means ± SEM. $^{**}P < 0.01$ vs *dy* NT; $^{*}P < 0.05$ vs *dy* NT; † < 0.05 vs NORM.

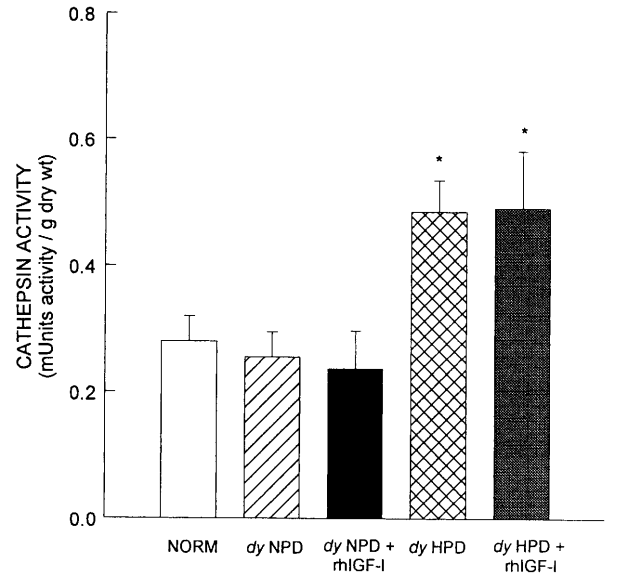

Figure 3. Lysosomal cathepsin activity. Results of lysosomal cathepsin activity measured in quadriceps in NORM (n = 14), *dy* NPD (n = 14, *dy* NPD + rhIGF-I (n = 13), *dy* HPD (n = 11), and *dy* HPD + rhIGF-I-treated (n = 12) animals. Cathepsin activity was measured in mUnits of activity of enzymatic activity per gram of tissue dry weight. 0.5 μ*M* of aminomethylcoumarin standard was equal to 0.1 μU of activity in the reaction tube. Results are expressed as Means ± SEM. $^{*}P < 0.05$ vs *dy* NT.

Ubiquitin Quantification. The *dy* NPD + rhIGF-I and *dy* HPD + rhIGF-I muscle had elevated total ubiquitin compared to *dy* NPD ($P < 0.01$), attributable to an increase in both free and conjugated ubiquitin ($P < 0.05$) (Table II). Total ubiquitin in all *dy* animals was elevated when compared to NORM ($P < 0.05$).

Western Blot of Free and Conjugated Ubiquitin. Western blot was performed on gastrocnemius muscle. The gel shows both free and conjugated ubiquitin (Fig. 4). Lane A, NORM; Lane B, *dy* NPD; Lane C, *dy* HPD; Lane D, *dy* NPD + rhIGF-I; Lane E, *dy* HPD + rhIGF-I. The blot shows significant increases in free ubiquitin in the NPD + rhIGF-I and HPD + rhIGF-I-treated *dy* groups. Similarly, conjugated ubiquitin was only increased in the NPD + rhIGF-I and HPD + rhIGF-I-treated groups. The approximate molecular weight of the major bands are included in the figure.

Ubiquitinization of Specific Muscle Proteins. The ubiquitinization of specific muscle proteins is shown in Figure 5. In general, larger molecular weight proteins (120–200 kDa) appeared to be preferentially ubiquinated over smaller muscle proteins (Figure 5A) shown by the increase in band density of these proteins. The *dy* NPD muscles appeared to show greater ubiquitinization of lower molecular weight proteins (10–50 kDa) than NORM muscles (Figure 5B). Treatment with rhIGF-I and/or HPD further increased ubiquitinization of the 32-kDA protein whereas the lower molecular weight (27-kDa) protein was decreased.

Discussion

Summary of Findings. The key finding of this study is that the reduction in proteolysis with all three treatments is likely in part to be mediated by a decrease in calpain activity. All three treatments reduced calpain activity with cathepsin results varied and conjugated ubiquitin only slightly elevated in the rhIGF-I-treated groups. Taken together, the data suggest an important role for calpain in therapeutic interventions for MD.

Pathophysiology of the 129 ReJ *dy* Mouse. The key findings of this study with regard to the pathology of the 129 ReJ *dy* mouse are: (a) increased total proteolysis; (b) increased calcium-dependent calpain protease activity; (c) unaltered muscle lysosomal cathepsin activity; and (d) increased ubiquitin proteolysis. The increase in total proteolysis appears to be due mainly to the increased net calcium-dependent calpain protease activity.

The increased calcium-dependent proteolysis observed in the 129 ReJ *dy* mouse may be related to altered sarcolemmal integrity and enhanced calcium flux in dystrophic muscle that results from the genetic lack of the basement membrane protein laminin (22, 23). Additionally, calpain enzymes have much broader range of activity in dystrophic muscle and are more widely distributed throughout dystrophic myocytes, whereas in normal muscle, they tend to be localized to the z-band region of the sarcomere (19). Another factor that may enhance calpain proteolysis in *dy* muscle is a deficiency of calpastatin, an endogenous inhibitor of calpain, that has been reported to be present in certain models of MD (20, 21, 24). Increased calpain activity has

Table II. Ubiquitin Quantification (Units = O.D. × mm^2)

	Total ubiquitin	Free ubiquitin	Conjugated ubiquitin
NORM			
$n = 5$	46.38 ± 1.14[a]	15.1 ± 1.30[a]	30.2 ± 2.30[a]
dy NPD			
$n = 5$	56.39 ± 2.63[b]	20.26 ± 2.63[a,b]	35.94 ± 2.63[a,b]
dy NPD + rhIGF-I			
$n = 5$	72.07 ± 2.02[c]	25.49 ± 0.82[b]	44.55 ± 4.59[a,b]
dy HPD			
$n = 5$	58.79 ± 2.27[b]	22.56 ± 2.48[a,b]	34.43 ± 3.18[a,b]
dy HPD + rhIGF-I			
$n = 5$	72.00 ± 1.58[c]	25.64 ± 1.54[b]	45.69 ± 3.24[b]

Note. Results of free and bound ubiquitin quantification from NORM ($n = 5$), *dy* NPD ($n = 5$), *dy* NPD + rhIGF-I ($n = 5$), *dy* HPD ($n = 5$), and *dy* HPD + rhIGF-I ($n = 5$) treated animals. Values are expressed in units defined as the optical density × mm^2. All means are expressed ± SEM. Values in the same column that do not share a superscript are significantly different.

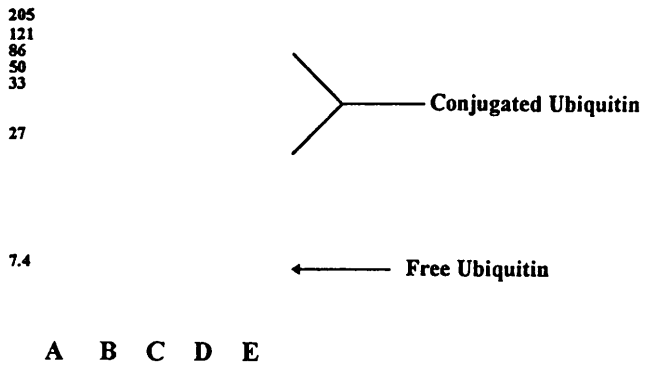

Figure 4. Western Blot of free and conjugated ubiquitin. Western blot was performed on gastrocnemius muscle as described under "Materials and Methods." The gel shows both free and conjugated ubiquitin. Lane A, NORM; Lane B, *dy* NPD; Lane C, *dy* HPD; Lane D, *dy* NPD + rhIGF-I; Lane E, *dy* HPD + rhIGF-I. Approximate molecular weights are expressed in kDa.

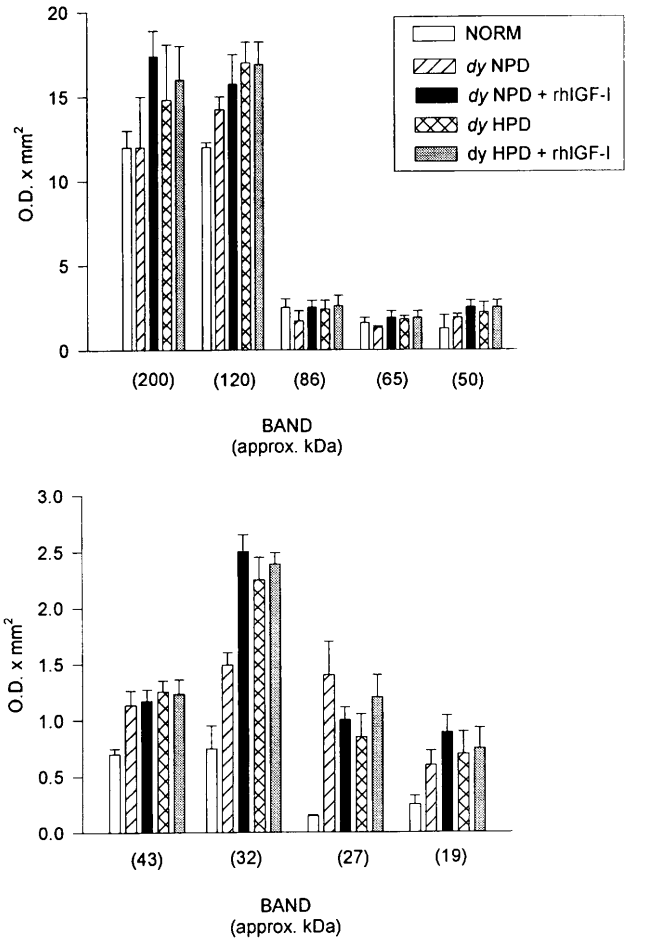

Figure 5. Ubiquitinization of specific muscle proteins. Histogram distribution of specific ubiquitinated muscle proteins measured in gastrocnemius muscles of NORM; *dy* NPD; *dy* NPD + rhIGF-I; *dy* HPD; and *dy* HPD + rhIGF-I ($n = 5$ per group). Data are measured in units defined as optical density × mm^2. Means are expressed ± SEM.

also been reported in the *mdx* mouse, dystrophic hamster, and human Duchenne MD (19–21). In our system, calpain activity was measured in crude muscle homogenates to estimate net calpain activity *in vivo*. Thus, what was measured was the activity of calpain in relation to its specific inhibitor calpastatin, which was also present in the crude homogenate.

Lysosomal cathepsins were not elevated in the 129 ReJ *dy* mouse even though they have long been known to be responsible for the degradation of a number of soluble cytoplasmic proteins in normal muscle. Several animal and human studies have reported an increase in activity of cathepsins in dystrophic, atrophied, or insulin-deficient skeletal muscle (5, 7, 22, 23). Takeda (7) suggested that cathepsin B might participate in the regeneration process by degrading damaged muscle fibers. Our finding of normal cathepsin activity in untreated dystrophic muscle is consistent with recent findings showing that cathepsins did not play a major role in the degradation of septic or catabolic muscles (25, 3). Part of the discrepancy in the findings between these studies may lie in the different animal models employed and the varying methodologies used in the measurement of cathepsins.

Ubiquitin has been reported to be involved in the breakdown of damaged or abnormal proteins in skeletal muscle (2). Labeling of muscle proteins with the 86-kDa ubiquitin peptide appears to act as a "tag" for targeting proteins for destruction by a multienzyme degrading complex (17). Increased ubiquitinization of high molecular weight proteins

has been observed in exercise-injured, atrophied, and denervated skeletal muscle, and in human occulopharyngeal MD (4, 15, 26). This also appears to be the case in dystrophic muscle since the proteins showing the highest ubiquitinization were in the 120–200-kDa range (27, 28). The increased ubiquitinization of certain proteins and increased levels of total ubiquitin observed in untreated 129 ReJ *dy* mouse muscle may imply a role for this pathway in the degrading of specific proteins within dystrophic muscle rather than a nonspecific degradative process such as the calpain pathway. However, since specific activity of ubiquitin proteolysis was not measured, the relative effects of this proteolytic pathway compared to the lysosomal, or calcium-mediated pathways remains unknown.

Effect of Dietary and Pharmacological Treatment on Proteolysis in the 129 ReJ *dy* Mouse. Two factors shown to reduce rates of proteolysis in dystrophic muscle are rhIGF-I administration and a high-protein diet (HPD) (8–10). In both dystrophic mice and hamsters, long-term treatment with rhIGF-I and/or HPD was effective in reducing overall and myofibrillar proteolysis in these animals. When rhIGF-I and HPD were combined, a concomitant increase in muscle protein synthesis was also observed (9, 10).

Our present data suggest that the HPD-induced reduction in proteolysis may be due largely to a decrease in net calcium-dependent calpain activity. In previous studies, we reported significant preservation of skeletal muscle structure and morphology in dystrophic mice fed a HPD (10). Although the mechanism by which HPD affects muscle proteolysis is uncertain, HPD has been shown to increase circulating levels of both IGF-I and insulin (1, 29), two factors that directly reduce muscle proteolysis. In addition, increased intake of dietary protein has been reported to induce synthesis of IGF-binding proteins that in turn may increase the circulating half-life and bioavailability of IGF-I when administered concomitantly (30, 31).

In previous studies we reported that HPD and rhIGF-I preserved muscle structure in MD. This study presents a theory by which improved muscle structure can be related to a decrease in muscle proteolysis through a decrease in net calcium-dependent calpain activity.

With the present data, we have strong evidence to support the view that rhIGF-I preserves muscle structure in the 129 ReJ *dy* mouse (9) due to a decrease in calcium-dependent calpain activity. This preservation of muscle structure may be associated with a preservation of sarcolemmal integrity and possible normalization of calcium fluxes that may decrease activation of calcium-dependent proteases. The "leakiness" of dystrophic muscles to calcium is well established and has been correlated with the lack of essential membrane components such as dystrophin and laminin (35, 36). It is also clear that elevations in intracellular calcium are integral to the enhanced proteolysis that is observed in dystrophic muscle. Based upon our earlier morphologic findings (9, 10) and the reduction in calpain activity we observed in our present study, we are putting forth the hypothesis that preservation of muscle structure and integrity with HPD/IGF-I may be associated with reduced leakiness of dystrophic muscles to calcium. This reduced cytosolic calcium accumulation in turn may explain the reduced calpain activity we reported, the reduced calpain activity may in part explain the reduction in overall muscle proteolysis that occurred.

On the other hand, protein intake may be the relevant factor noted in the HPD and the HPD-rhIGF-I treatments on cathepsins. It is possible that HPD increases the release of thyroid stimulating hormone that may stimulate cathepsin activity, the enzyme responsible for the cleavage of thyroglobulin into active thyroid hormone (32, 33); however, induction of this pathway with HPD treatment remains to be ascertained.

While treatment of *dy* mice with rhIGF-I or rhIGF-I and HPD increased both free and conjugated forms of ubiquitin, it is difficult to conclude with certainty that there is a change in the amount of protein slated for degradation since these amounts are not directly proportional to the absolute levels of ubiquinated proteins that are present (27, 28). Furthermore, the tagging of proteins marked for hydrolysis by ubiquitin is not unequivocal as it has been demonstrated that ubiquitin can be bound to certain normal muscle proteins that are both functional and stable (34).

Implications for MD. It has been known for some time that protein supplementation can ameliorate muscle wasting in humans (37–40). The data presented here support the therapeutic potential of HPD and IGF-I by reducing muscle proteolysis in the 129 ReJ *dy* mouse, probably *via* a reduction in net calcium-dependent calpain activity. This may be the approach by which the severe and debilitating muscle wasting, characteristic of this model of MD, can be delayed.

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