Metabolic and Structural Effects of Insulin-like Growth Factor–I and High-Protein Diet on Dystrophic Hamster Skeletal Muscle (44124)

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Abstract. **In muscular dystrophy (MD) there is an imbalance between muscle protein synthesis and protein degradation, which results in a net muscle catabolism, along with muscle wasting and weakness. Using a dystrophic hamster model (BIO 53.58), we examined the chronic (8 weeks) effects of two factors that may enhance muscle protein synthesis and inhibit protein degradation, namely, insulin-like growth factor–I (rhIGF-I) and high-protein diet (HPD). Protein synthesis was determined by measuring the incorporation of ^{14}C phenylalanine into perfused leg muscle, while protein degradation was calculated from the release of tyrosine from the same perfused muscle. Urinary 3-methylhistidine excretion was used as an indicator of myofibrillar degradation. Treatment of dystrophic hamsters with rhIGF-I, HPD, or a combination of the two for 8 weeks resulted in significant decreases in total and myofibrillar degradation when compared with untreated dystrophic animals ($P < 0.05$) but had minimal effects on protein synthesis. Significant morphologic improvements ($P < 0.05$), including a normalization and greater uniformity of muscle fibers, were also seen in rhIGF-I– and rhIGF-I + HPD–treated animals. rhIGF-I and HPD were effective in reducing the excessive proteolysis seen in dystrophic muscle, and this reduced proteolysis resulted in improvement of muscle morphology.** [P.S.E.B.M. 1997, Vol 215]

In dystrophic skeletal muscle, the normal balance between protein synthesis and protein degradation is altered, by increased protein degradation and/or decreased protein synthesis (1). The result of this imbalance is a net catabolism of muscle proteins, which may be a key factor in the progressive muscle weakness and wasting seen in muscular dystrophy (MD). While therapeutic strategies in MD have thus far been of limited benefit, there are two factors that are known to increase protein synthesis and decrease protein degradation in skeletal muscle: adequate circulating levels of amino acids and adequate levels of serum insulin (2).

In normal skeletal muscle, an increased availability of amino acids has been shown to enhance the rate of protein synthesis while decreasing protein degradation (1–3). Whereas the effects of amino acid supplementation on skeletal muscle protein metabolism has been well defined in isolated muscle and tissue culture, their effects on skeletal muscle metabolism *in situ* remain controversial. In a recent study with the 129 ReJ dystrophic mouse, we demonstrated that a high-protein diet (HPD) significantly reduced rates of muscle proteolysis but did not alter the already elevated rates of protein synthesis in dystrophic skeletal muscle (4). Insulin and insulin-like growth factor–I (IGF-I) are also potent anabolic agents in skeletal muscle. IGF-I has anabolic actions similar to or even greater than insulin with less hypoglycemic potential. IGF-I has been shown to enhance both glucose and amino acid uptake by skeletal muscle while increasing rates of protein and nucleic acid synthesis

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(5–8). IGF-I is also a potent inhibitor of muscle protein degradation both *in vitro* and *in vivo* (7, 9–11). IGF-I also significantly reduced rates of muscle proteolysis in the 129 ReJ dystrophic mouse (12). Insulin resistance is a feature seen both in human myotonic dystrophy and in some animal models of MD (13, 14). Since IGF-I binds to receptors distinct from those of insulin, it may continue to exert its anabolic effect even in insulin-resistant dystrophic muscle.

In the present study, we chose to evaluate the effect of rhIGF-I and/or high-protein diet (HPD) on muscle protein metabolism and on muscle structure in the BIO 53.58 dystrophic hamster since this model exhibits abnormalities of muscle protein metabolism as well as progressive deterioration of muscle structure and some degree of insulin resistance (14). In addition, it is a large enough animal to allow for direct *in vivo* measures of muscle protein synthesis and degradation, which are not possible in murine models.

Materials and Methods

Animals. Male dystrophic (BIO 53.58) hamsters were obtained from Bio Breeders Inc. (Watertown, MA) at 8 weeks of age. Dystrophic animals were divided into the following four groups of 12 animals each: (i) animals receiving a normal diet and placebo injections; (ii) animals receiving a high-protein diet and placebo injections; (iii) animals receiving subcutaneous rhIGF-I (0.25 mg/kg bid) and a normal-protein diet; (iv) animals receiving subcutaneous rhIGF-I injection and a high-protein diet. A group of age- and sex-matched normal, nondystrophic (BIO F1B) hamsters was included to serve as a reference for normal muscle parameters. The dose of 0.25 mg/kg was chosen from a preliminary study examining the effect of sc rhIGF-I doses of 0.1, 0.25, 0.5, and 1.0 mg/kg on plasma glucose and insulin in dystrophic and normal hamsters, a study which showed that higher doses caused significant hypoglycemia (data not shown).

Diets. The normal-protein diet consisted of 240 g of casein-based protein and 431 g of dextrose per kilogram of diet, while the high-protein diet contained 500 g of casein-based protein and 172 g of dextrose per kilogram of diet. Both normal- and high-protein diets contained 200 g/kg corn starch, 50 g/kg corn oil, 5 g/kg hamster vitamin mix, 40 g/kg hamster salt and mineral mix, and 3 g/kg I-cystine. Diets were isocaloric, and the remainder of the diet was composed of Alphacel non-nutritive bulk. Animals had free access to both food and water. Since food consumption between the five groups of hamsters was similar, pair feeding was not instituted. rhIGF-I (kindly provided by Genentech Inc., San Francisco, CA) was administered as 0.25 mg/kg subcutaneous injections twice daily. Placebo injections consisted of an equal volume of phosphate-buffered saline. Animals remained on their respective protocols for 8 weeks. All studies were carried out in accordance with NH guidelines for the care and use of laboratory animals and approved by the North Shore University Hospital Animal Care Committee.

Plasma Glucose and Insulin Response. Prior to beginning the study, blood for glucose and insulin was drawn from the suborbital sinus (under light CO2 anesthesia) at 0, 1, 2, 3, and 4 hr following subcutaneous rhIGF-I injection of 0.25 mg/kg. Plasma glucose was determined using the glucose oxidase method (Sigma Chemicals, St. Louis, MO). Plasma insulin was quantitated with a double-antibody radioimmunoassay kit (Binax Inc., Portland, ME).

Perfusions. Hindquarter perfusions were carried out following the procedure of Ruderman *et al.* (15) as modified by Bylund-Fellenius *et al.* (16). Briefly, hamsters were anesthetized with ip urethane (1.3 g/kg) and the abdomen opened with a longitudinal incision. The aorta was cannulated just below the kidneys with a 22-ga. teflon cannula and an outflow incision made in the inferior vena cava at the same level. Once the cannula was in place and flushed with heparin, muscles were equilibrated with a flow-through perfusion for 30 min (0.15 ml/min × g muscle) followed by 1 hr of recirculating perfusion (0.3 ml/min × g muscle) with medium containing radiolabeled phenylalanine. Perfusions were carried out in a custom made thermostated 37°C Plexiglas box. The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4.5% bovine serum albumin, 35% fresh, washed bovine erythrocytes, 10 m*M* glucose, and amino acids at normal hamster plasma concentrations except for phenylalanine which was at 2 m*M*, 40 ng/ml bovine insulin, and 0.2 μCi/ml ^{14}C phenylalanine (DuPont NEN, Wilmington, DE). Medium was warmed to 37°C and gassed with 5% CO_2/95% O_2.

Muscle protein synthesis was calculated from the disintegration's per minute incorporated into muscle protein divided by the specific radioactivity of phenylalanine and expressed as the nanomoles of phenylalanine incorporated per gram of muscle (15, 16). Radioactivity of the acid-soluble and acid-insoluble (radioactivity incorporated into protein) extracts of the muscle homogenates were measured by liquid scintillation spectrophotometry (Beckman model 5800 β-counter, Palo Alto, CA; Ecoscint scintillation fluid, National Diagnostics, Manville, NJ). Phenylalanine concentrations of the homogenate extracts and perfusion medium were determined by HPLC technique (Automated Series 4 Amino Acid Analyzer, Perkin-Elmer, Norwalk, CT) using lithium cation exchange and postcolumn derivitization with sodium hypochlorite-*o*-phthaldehyde to form a fluorescent product (12, 17).

Protein degradation was determined by measuring the release of tyrosine from the perfused hindquarter over the course of 1 hr as well as by urinary 3-methylhistidine/creatinine. Urine for 3-methylhistidine was collected from hamsters placed in metabolic cages for 24 hr prior to perfusion. Urinary output averaged 6.3 ± 0.31 ml/24 hr * 100 g body wt and was not significantly different among the various groups. Urinary creatinine was determined using a Sigma diagnostic kit (Sigma Chemicals, St. Louis, MO).

Urinary 3-methylhistidine provides an estimate of myofibrillar degradation. However, since 2%–10% of urinary 3-methylhistidine in rodents may originate from nonmuscle tissues, primarily, the gastrointestinal tract and skin, some overestimation of 3-methylhistidine levels may occur.

Morphological Studies. For morphometric studies, gastrocnemius muscles were fixed in 2% glutaraldehyde buffered with 0.1 *M* sodium cacodylate. The cross-sectional slices of muscle were carefully dissected perpendicular to their long axis. Muscle sections were post-fixed in cold buffered 1% osmium tetroxide, rinsed in 7.5% sucrose, and dehydrated in a graded series of ethanol's. The muscle slices were embedded flat in effapoxy resin in order to provide well-oriented cross-sectional profiles of the fibers. Specimens from three blocks of well-oriented cross-sectional profiles from each of four animals were examined from each treatment group after being photographed and enlarged to a final magnification of ×145. The sections were assessed by morphometric techniques using a Zeiss Videoplan 2 Image Analyzer (Carl Zeiss Inc., Thronwood, NY) (18, 19).

Statistical Analyses. Statistical analysis were made using one-way analysis of variance (ANOVA) followed by a Tukey test for specific critical differences (20). For statistical analysis of the morphometric parameters among the various groups, the multiple cross sections from each animal were pooled and treated as a single data point. The shapes of the muscle fiber area frequency distributions of the four groups were compared using Kolmogorov-Smimov test (21). All means were expressed ±SEM. Statistical significance was set at $P < 0.05$.

Results

The response of plasma glucose and insulin levels in dystrophic and normal hamsters to a 0.25 mg/kg sc injection of rhIGF-I is shown in Figure 1. Baseline plasma glucose levels were significantly higher in dystrophic animals than in normal hamsters ($P < 0.01$). Plasma glucose levels for both dystrophic and normal animals dropped 1 hr following rhIGF-I injection. An increase in plasma glucose levels was observed 2–4 hr postinjection in both groups of hamsters; however, glucose levels in dystrophic hamsters were significantly higher than in normal animals over this time. Plasma insulin response to rhIGF-I was similar at all time points for dystrophic and normal hamsters. A drop in plasma insulin was observed at 1 hr followed by a gradual increase to baseline levels by 4 hr.

Treatment of dystrophic hamsters with HPD, rhIGF-I, or the combination of rhIGF + HPD for 8 weeks significantly reduced rates of muscle proteolysis ($P < 0.01$, 0.05, and 0.01, respectively) from perfused lower limbs when compared with untreated dystrophic animals (Fig. 2). Urinary 3-methylhistidine/creatinine, an indicator of myofibrillar degradation, was also significantly reduced in dystrophic hamsters receiving HPD, rhIGF-I, or rhIGF + HPD ($P < 0.001$, 0.05, and 0.05, respectively, Fig. 3). Rates of muscle protein synthesis were not significantly altered in dystrophic

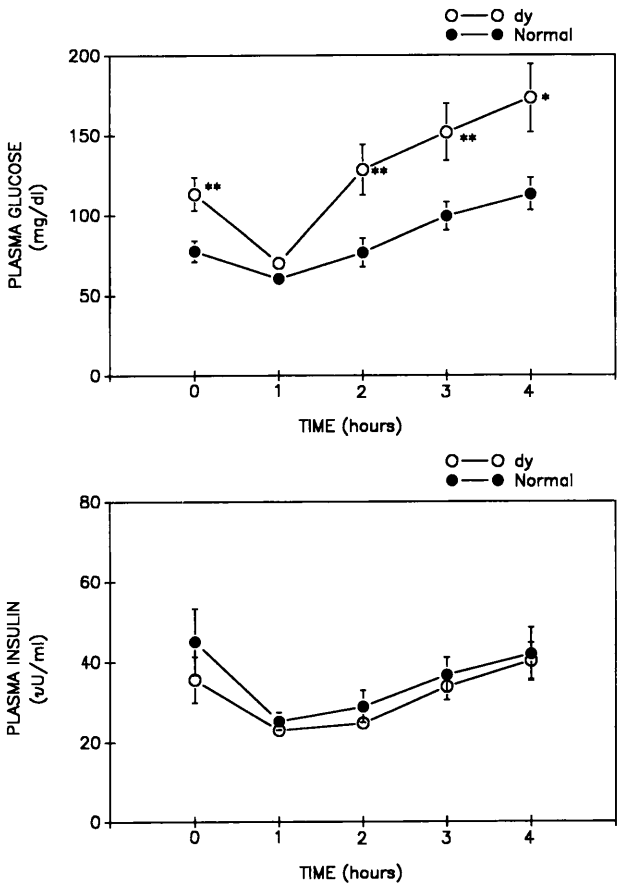

Figure 1. Plasma glucose and insulin response of dystrophic (dy) and normal hamsters following subcutaneous injection of 0.25 mg/kg rhIGF-I. Means are presented ± SEM; $n = 6–8$ animals. *, ** $P < 0.05$ and 0.01 versus normal, respectively.

hamsters treated with HPD, rhIGF-I, or the combination of the two when compared with untreated dystrophic animals (Fig. 4).

Untreated dystrophic muscle exhibited smaller muscle fibers than nondystrophic muscle, and the distribution of these muscle fiber areas was not normal when compared

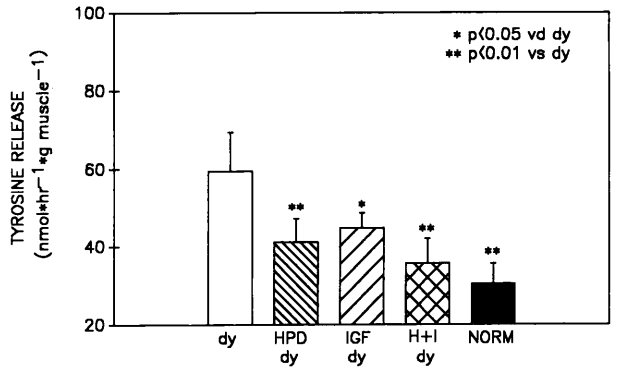

Figure 2. Tyrosine release from perfused dystrophic hamster hind limbs from animals receiving 8 weeks of no treatment (dy), high-protein diet (HPD), rhIGF-I (0.25 mg/kg), or the combination of HPD + rhIGF-I (H+I). A group of normal, nondystrophic (NORM) hamsters are included for comparison. Means are presented ±SEM; $n = 9–10$ animals/group. *, ** $P < 0.05$ and 0.01 versus untreated dystrophic hamsters, respectively.

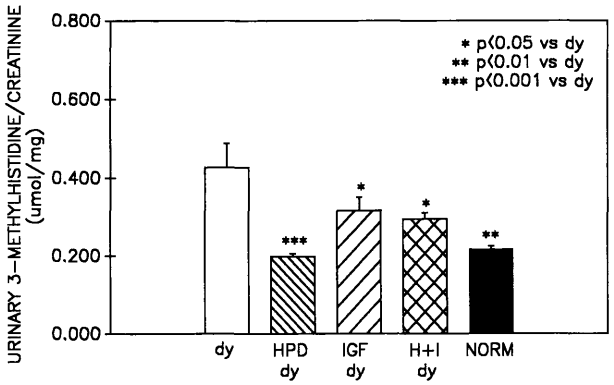

Figure 3. Urinary 3-methylhistidine/creatinine in dystrophic hamsters receiving 8 weeks of no treatment (dy), high-protein diet (HPD), rhIGF-I (0.25 mg/kg), or the combination of HPD + rhIGF-I (H+I). A group of normal, nondystrophic (NORM) hamsters are included for comparison. Means are presented ±SEM; $n = 9$ animals/group. *, **, *** $P < 0.05$, 0.01, and 0.001, versus dystrophic group, respectively.

with normal nondystrophic muscle (Fig. 5). While HPD did not alter muscle fiber size or distribution, rhIGF-I alone or combined with HPD significantly increased muscle fiber size and normalized the distribution of the muscle fiber areas when compared with untreated dystrophic muscle (Fig. 5).

Discussion

The aim of the present study was to treat dystrophic hamsters with two anabolic and anticatabolic agents, namely IGF-I and HPD with the goal of reversing the imbalance between muscle protein synthesis and degradation, which is a key finding in MD (1, 4, 13, 14). The hamster model employed in this study (BIO 53.58) has been shown to have an increased rate of muscle protein degradation when compared with normal, nondystrophic hamsters which exceeds that of protein synthesis, resulting in net proteolysis (14). Rampant muscle proteolysis results in discernible histologic lesions, which, in one study, became evident in 94% of these dystrophic hamsters by 70 days of

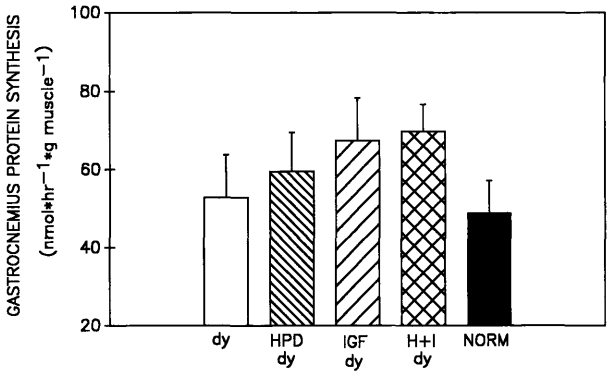

Figure 4. Protein synthesis in gastrocnemius muscle from dystrophic hamsters receiving 8 weeks of no treatment (dy), high-protein diet (HPD), rhIGF-I (0.25 mg/kg), or the combination of HPD + rhIGF-I (H+I). A group of normal, nondystrophic (NORM) hamsters are included for comparison. Means are presented ±SEM; $n = 9$–10 animals/group.

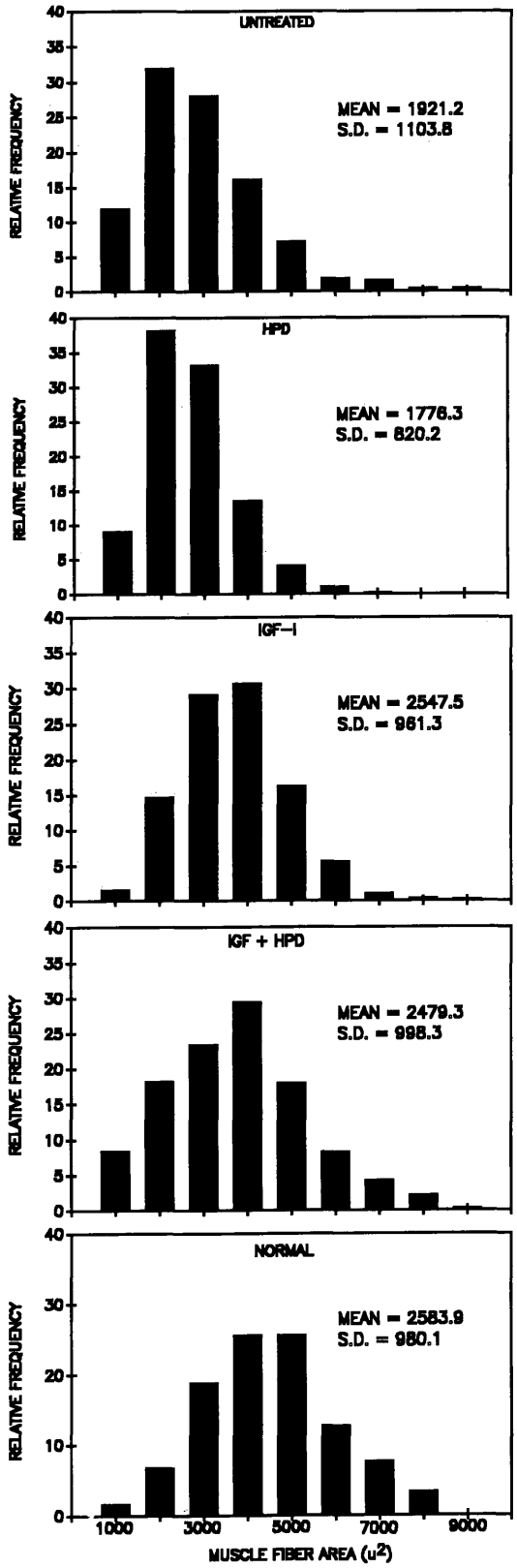

Figure 5. Frequency distributions of muscle fiber areas in gastrocnemius muscle from dystrophic hamsters receiving 8 weeks of no treatment (dy), high-protein diet (HPD), rhIGF-I (0.25 mg/kg), or the combination of HPD + rhIGF-I (H+I). A group of normal, nondystrophic (NORM) hamsters are included for comparison. $n = 4$ sections from each of four animals.

age (22). The major aberrations reported were variation in fiber size, atrophic and hypertrophic fibers, necrosis, centronucleation, and hypercontracted myofibers (23). The hamster model of MD employed in this study differs significantly from the 129 ReJ dystrophic murine model we employed in an earlier study (4, 12). Morphological abnormalities in the dystrophic mouse are markedly more severe and include excessive extrafiber spaces, decreased volume density of muscle fibers, lipid deposition, necrotic fibers, and centronucleation. Functionally, the dystrophic mouse exhibited marked atrophy and wasting of the hindlimbs along with seriously impaired muscle function and strength. While these dystrophic hamsters do not exhibit the gross hind-limb functional abnormalities, their muscle endurance is significantly reduced when compared with normal hamsters, and exercise intolerance is well documented (22, 24). Biochemically, both models exhibit characteristic increases in muscle proteolysis; however, unlike the dystrophic mouse, muscle protein synthesis was not elevated in dystrophic hamsters when compared with normal animals.

A major effect of chronic rhIGF-I administration in this study was a significant reduction in overall and myofibrillar protein degradation with minimal effects on protein synthesis. Reduction of proteolysis by rhIGF-I may help restore the balance of muscle protein metabolism such that protein synthesis may now keep up with the ongoing muscle proteolysis and thus spare muscle structure and function. The improvement in muscle protein balance may be reflected morphologically by a reduced variability and greater uniformity of muscle fibers in dystrophic hamsters receiving rhIGF-I.

Specific receptors are present in skeletal muscle for IGF-I (6, 7); however, at high enough doses IGF-I can exert an effect *via* insulin receptors. At the dosage used in this study, some lowering of plasma glucose was observed 1 hr after rhIGF-I administration, possibly indicating some cross-reaction with insulin receptors at this dosage. However, by 4 hr postinjection, plasma glucose was significantly elevated when compared with normal (nondystrophic) animals. While plasma insulin levels following rhIGF-I injection were identical for dystrophic and normal hamsters, plasma glucose was significantly higher at 0, 2, and 4 hr, possibly reflecting some degree of insulin resistance in these dystrophic animals. The fact that IGF-I acts at receptors separate from insulin means that IGF-I may still exert its effects in muscle that may be resistant to the anabolic actions of insulin.

Adequate circulating levels of amino acids are another factor that has been shown to enhance muscle protein synthesis and reduce muscle protein degradation. While a high-protein diet has been shown to increase rates of muscle protein synthesis in normal hamsters (25), we did not see any increase in muscle protein synthesis with dystrophic hamsters or, in an earlier study, with dystrophic mice (4). However, in the present study as well as in a previous murine study (4), high-protein diet significantly reduced rates of dystrophic muscle protein degradation, although the mechanism of this effect remains uncertain. A high-protein diet has been reported to increase circulating levels of insulin, IGF-I, and amino acids (1, 26, 28) as well as to enhance circulating levels of IGF binding protein–3 (IGFBP3), which may prolong the half-life of IGF-I in circulation and enhance the bioavailability of IGF-I to muscle (27, 28). While HPD did significantly reduce muscle proteolysis, it did not significantly alter the size or distribution of muscle fibers.

In a previous study with dystrophic mice, 1 month of combined IGF-I + HPD therapy caused significant increases in muscle protein synthesis as well as significant reduction in muscle proteolysis greater than that of IGF-I or high protein alone (12). In addition, the combination of rhIGF-I and high-protein diet lead to a greater improvement of muscle morphology than either treatment alone. In this study, the combination of rhIGF-I + HPD significantly reduced muscle protein degradation but had only minimal effects on protein synthesis. The combination therapy was also associated with a normalization of muscle fiber areas and distribution.

While a number of recent studies examined the effects of IGF-I on muscle metabolism, this study focused on the *in vivo* effects of rhIGF-I in an animal model of MD. A common finding in both human and animal studies of IGF-I in skeletal muscle is a reduction of proteolysis (12, 29–31). In these studies, IGF-I generally did not increase rates of protein synthesis; however, there was considerable variation in the dose and route of administration of IGF-I. The findings of this study highlight the potential usefulness of IGF-I in catabolic states such as MD, while confirming the therapeutic effectiveness of HPD as shown in a number of human muscle disorders (32–34). They also support the recently demonstrated therapeutic benefit achieved by the combined long-term administration of rhIGF-I and HPD in human myotonic dystrophy (35). Future animal and human studies of the mechanism and possible beneficial effects of these types of treatment in muscular dystrophy are warranted.

In summary, administration of rhIGF-I, high-protein diet, or the combination of the two significantly reduced skeletal muscle proteolysis in dystrophic hamsters, while the combination of the two had some stimulatory effect on muscle protein synthesis. The size of the muscle fibers was increased, and the distribution of muscle fibers areas normalized by the administration of rhIGF-I. Though the exact mechanism for the beneficial effects observed here is unclear, it may be related to the marked reduction in muscle proteolysis observed with the various treatments.

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