Effects of Insulin-Like Growth Factor-1/Binding Protein-3 Complex on Muscle Atrophy in Rats

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Muscle atrophy and wasting is a serious problem that occurs in patients with prolonged debilitating illness, burn injury, spinal injury, as well as with space flight. Current treatment for such atrophy, which often relies on nutritional supplementation and physical therapy, is of limited value in preventing the muscle wasting that occurs. Considerable recent attention has focused on the use of anabolic growth factors such as insulin-like growth factor (IGF-1) in preventing muscle atrophy during limb disuse or with various catabolic conditions. However, potential side effects such as hypoglycemia appear to be limiting factors in the usefulness of IGF-1 for clinical treatment of muscle wasting conditions. The formulation of IGF-1 used in this study (IGF-1/BP3) is already bound to its endogenous-binding protein (BP3) and, as a result, has a greater specificity of action and significantly less hypoglycemic effect. Using a rat model of hind limb suspension (HLS) for 10 days, we induced marked muscle atrophy that was accompanied by enhanced muscle proteolysis and reduced muscle protein content. When HLS rats were treated with IGF-1/BP3 (50 mg/kg, b.i.d.), they retained greater body and muscle mass. Muscle protein degradation was significantly reduced and muscle protein content was preserved. The rate of protein synthesis, although somewhat reduced in HLS muscle, was not significantly elevated by IGF-1/BP3 treatment. Volume density of HLS-treated muscles were increased compared to untreated HLS rats and the actual number of fibers per area of muscle was likewise increased. The results of the current study suggest that IGF-1/BP3 might be useful for inhibiting muscle proteolysis in catabolic conditions and thus preserving muscle protein content and mass. Exp Biol Med 228:891–897, 2003

Key words: IGF-1/BP3; muscle atrophy; muscle morphology; hind-limb suspension

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When skeletal muscles are not used, they undergo a rapid atrophy that is characterized by decreases in muscle size, loss of muscle protein, and reduced muscle strength. Muscle atrophy is a serious problem in conditions such as burn injury, stroke, limb disuse, debilitating arthritis, as well as in catabolic conditions such as sepsis, AIDS, cancer, and space flight (1–7).

An important feature of muscle atrophy due to limb disuse is an imbalance in muscle protein metabolism with rates of muscle protein synthesis being reduced while rates of protein degradation are markedly increased (8–12). This net catabolism of muscle proteins may be largely responsible for the loss of muscle weight and muscle proteins that is observed.

Several studies report that anabolic hormones and growth factors such as insulin-like growth factor 1 (IGF-1) may be of value in preserving muscle mass in catabolic states such as caloric restriction, chronic illness, burn injury, and cancer cachexia (13–16). A limiting factor in the use of IGF-1 as a therapeutic agent is its significant glucose-lowering effect, which restricts the dose that can be used and thus the anabolic potential of this agent. In this study, we used a novel IGF-1/BP3 complex (kindly provided by Celtrix Pharmaceuticals, Santa Clara, CA) in which recombinant human IGF-1 is administered already bound to its endogenous-binding protein, IGF-1-binding protein-3 (IGF-1/BP3). Administration of IGF-1 in this form may not only enhance the bioavailability of IGF-1 (17), but because the IGF-1/BP3 complex does not readily interact with insulin receptors and may be given at significantly higher doses without marked hypoglycemia. Using a rat model of hind-limb suspension (HLS), the current study examined the potential usefulness of IGF-1/BP3 administration on reducing muscle protein degradation and increasing muscle protein synthesis during muscle atrophy while preserving normal muscle mass and structure in this condition.

Materials and Methods

Animals. Male Wistar rats (150 g) were obtained from Charles River Laboratories (Wilmington, MA). After several days of acclimatization in our animal facility, rats were randomly assigned to one of three groups (n = 8 rats

per group). Rats in Group 1 were hind-limb suspended for 10 days and received twice daily placebo injections of subcutaneous phosphate-buffered saline (PBS; HLS group). Rats in Group 2 were hind-limb suspended for 10 days and received twice daily subcutaneous injections of IGF-1/BP3 (50 mg/kg twice daily; HLS + IGF-1/BP3 group). Rats in Group 3 were not suspended but received twice daily placebo injections of subcutaneous PBS (control group). Before beginning the experimental protocol, a pilot group of six rats was injected with 50 mg/kg IGF-1/BP3 and blood was withdrawn hourly from the suborbital sinus over a 6-hr period for measurement of blood glucose. Concentrations of blood glucose were determined using the glucose oxidase method (Sigma Chemical, St. Louis, MO). The use of animals in this study was approved by the IACUC at the Massachusetts College of Pharmacy and Health Sciences.

HLS. Before HLS, rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The tail was then wrapped with soft cotton gauze and a flexible wire loop was firmly taped around the gauze at the base of the animal's tail. The animal then had its hind limbs suspended several centimeters off the floor of the cage by a thin wire attached to a swivel secured to the rods of the cage top. This swivel arrangement allowed the animal freedom to move about the cage and groom using its front limbs. HLS has been shown to be well tolerated by rats for significant periods of time (8, 18). Adequate blood flow to the taped tail was monitored by the pink color of the exposed tail region. HLS rats had free access to food pellets placed inside the cage and to water via an extra long waterspout. Food and water intakes were measured daily in all three groups of rats. Rats remained suspended for 10 days.

At the end of the suspension period, HLS, HLS + SK, and control rats were weighed and anesthetized with intraperitoneal pentobarbital (50 mg/kg). Leg muscles were rapidly removed intact bilaterally by cutting at their sites of attachment. The soleus muscle was selected for subsequent detailed analysis because previous studies of HLS in rats reported this muscle, which is composed of predominately type I fiber, to be most affected by HLS (8, 18, 19).

Muscle Protein Metabolism. Net protein degradation was measured by quantitating the release of tyrosine from incubated muscles. Under anesthesia, soleus muscles were rapidly removed intact and were immediately incubated at 35°C in Krebs-Henseleit bicarbonate buffer (pH 7.4, gassed with 5% CO_2/95% O_2) containing 10 m*M* glucose, 0.1 m*M* isoleucine, 0.1 m*M* leucine, 0.2 m*M* valine, and 0.5 m*M* cycloheximide to inhibit protein synthesis. Muscles were preincubated for 30 min to equilibrate intracellular and extracellular tyrosine pools, and were transferred to tubes containing fresh medium for an additional 2 hr. Tyrosine in the incubation medium was determined using the fluorometric method of Wong *et al.* (20). Rates of muscle protein synthesis were determined in contralateral muscles, as described previously (21), by quantitating the rate of incorporation of ^{3}H-phenylalanine (0.2 μCi/ml of medium) into muscle protein. Muscles for measurement of protein synthesis were incubated as above for protein degradation but without cycloheximide and with the addition 200 μ*M* cold phenylalanine and 0.5 U/ml of bovine insulin to the incubation medium. After incubation, muscles were rinsed with saline, blotted, and homogenized in phosphate buffer (pH 7.4). The homogenate was precipitated with 50% trichloroacetic acid (TCA) and was centrifuged to pellet proteins. The pellet was then washed sequentially with 5.9% TCA and a 1:1 mixture of ether:ethanol. The final pellet was digested with NaOH (5 mol/L) and the radioactivity incorporated into muscle protein was measured in a β-scintillation counter. Protein synthesis was calculated as the ratio of radioactivity incorporated per gram of muscle protein and the specific radioactivity of free phenylalanine.

Biochemical Analyses. Total protein content was determined in muscle homogenates using a modified Lowry method with bovine serum albumin (BSA) as the standard (22). Total nucleic acids were extracted from muscles using established methods (23). Briefly, flash frozen muscles were homogenized on ice in a buffer containing 0.15 *M* NaCl, 50 m*M* Trizma-HCl, 5 m*M* EDTA, and 1% SDS at pH 8.3. Homogenates were extracted with an equal volume of phenol:chloroform:isoamyl alcohol and were centrifuged. After additional washes with the phenol:choloform: isoamyl mixture and chloroform alone, the final isolate was neutralized with one-tenth volume of 2.2 *M* sodium acetate and nucleic acids were precipitated at –20°C with ethanol. After two more ethanol washes, the concentration and purity of nucleic acids was determined from the absorbance at 260/280 nm. DNA concentration was measured using the Hoechst dye method (23). RNA content was calculated from the difference of total nucleic acids and DNA.

Muscle Morphology. At the conclusion of the study, muscles were fixed in 0.2 mol/L glutaraldehyde buffered with 0.1 mol/L cacodylate (pH 7.3). After fixation, tissue was rinsed in 0.1 mol/l cacodylate with 0.20 mol/L sucrose and was carefully dissected out perpendicular to its long axis. The sections of muscle were then postfixed in cold-buffered osmium tetroxide, rinsed again in sucrose, and dehydrated in a graded series of ethanols. Muscle slices were embedded flat in effapoxyresin (24). The cross-sectional muscle sections were assessed by morphometric techniques using SigmaScan Image Analysis software (Jandel, San Rafael, CA). For morphometric studies, samples from four blocks of well-oriented cross-sectional profiles from each animal were photographed and enlarged. Quantitative structural variables assessed included muscle fiber volume density, VV (volume of muscle fibers/volume of tissue); muscle fiber areas and diameters; frequency distribution of muscle fiber areas, a measure of the variability of the muscle fibers; and the shape factor of the muscle fibers (F) (24, 25).

Statistical Analysis. Comparison of statistical differences between the various groups was carried out using one-way analysis of variance (ANOVA) with a Student-Newman-Keuls test for specific critical differences. For sta-

Table I. Body Weight Change and Muscle Weights from HLS Rats, HLS Rats Receiving IGF-1/BP3, or Nonsuspended Rats Receiving Placebo (Control)

	Change in body weight (g)		
HLS	+27.8 ± 4.7[a]		
HLS + IGF-1/BP_3	+37.5 ± 3.5[b]		
Control	+52.2 ± 2.4[b]		
	Muscle weight (g)		
	HLS	HLS + IGF-1/BP3	Control
Soleus	0.042 ± 0.003[a]	0.052 ± 0.002[a,b]	0.114 ± 0.006[b]
Plantaris	0.114 ± 0.01[a]	0.142 ± 0.005[a,b]	0.174 ± 0.007[b]
Gastrocnemius	0.224 ± 0.008[a]	0.253 ± 0.007[a,b]	0.332 ± 0.014[b]

Note. Means are presented ± SEM, n = 6–8 per group.
[a] Minimum $P < 0.05$ vs control.
[b] Minimum $P < 0.05$ vs HLS rats.

tistical analysis of the morphological parameters VV and F, and for muscle fiber area distributions, the multiple cross-sections from each animal were pooled and treated as a single data point. The shapes of the muscle fiber area distributions were compared with normality using the Kolmogorov-Smirinov test.

Results

The dose of IGF-1/BP3 used in this study (50 mg/kg), although significantly higher than the dose of IGF-1 used in earlier studies (0.50 mg/kg), did not cause severe or prolonged hypoglycemia in treated animals. Although there was a modest (10%-15%) decrease in plasma glucose levels 2 hr after injection, by 4 hr postinjection, plasma glucose levels had returned to baseline.

Animal Growth and Muscle Weights. Food and water intake did not vary significantly between HLS, HLS + IGF-1/BP3, and control rats. Despite similar intakes of food (14.2 ± 1.1 g/day for HLS rats vs 16.0 ± 0.9 g/day for control rats) and water, HLS rats showed significantly lower gains in body weight over the 10-day period than control rats. HLS + IGF-1/BP3-treated animals grew at rates comparable with controls (Table I). Final weights of soleus (predominately type I fibers), plantaris (predominately type II fibers), and gastrocnemius (mixed fiber type) muscles from all three groups of rats are also shown in Table I. Soleus, plantaris, and gastrocnemius muscle weights were significantly reduced in HLS rats after 10 days of suspension when compared with controls. However, IGF-1/BP3-treated HLS rats showed significantly greater mass for all three muscles.

Muscle Protein Metabolism, Protein Content, and Nucleic Acid Content. Net muscle protein degradation was significantly elevated after 10 days of HLS when compared with control rats (Fig. 1). In HLS rats receiving concomitant IGF-1/BP3 injections, net muscle protein degradation was reduced to levels significantly below HLS animals and comparable with control animals. Rates of muscle protein synthesis, although not statistically different from one another, were lowest in untreated HLS rats and were somewhat higher in HLS + IGF-1/BP3 and control animals (Fig. 2). Protein content of soleus muscles was reduced after 10 days of HLS when compared with control animals, but remained significantly higher in HLS rats receiving IGF-1/BP3 (Table II).

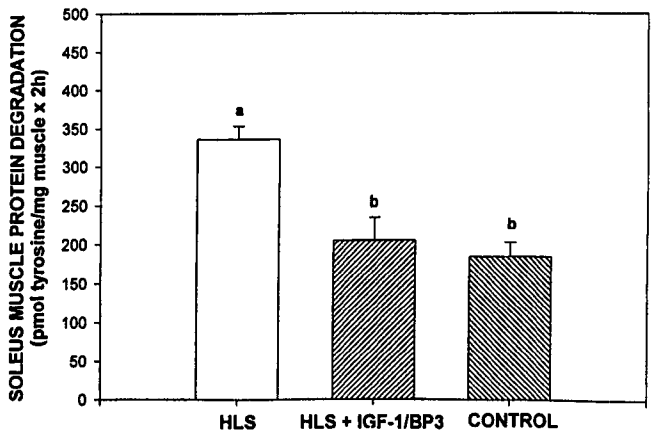

Figure 1. Soleus muscle protein degradation rates from hind-limb suspended rats (HLS) receiving no treatment, HLS rats receiving IGF-1/BP3 or non-suspended rats receiving placebo (CONTROL). N = 8 rats per group, a = minimum p<0.05 vs control, b = minimum p<0.05 vs HLS group.

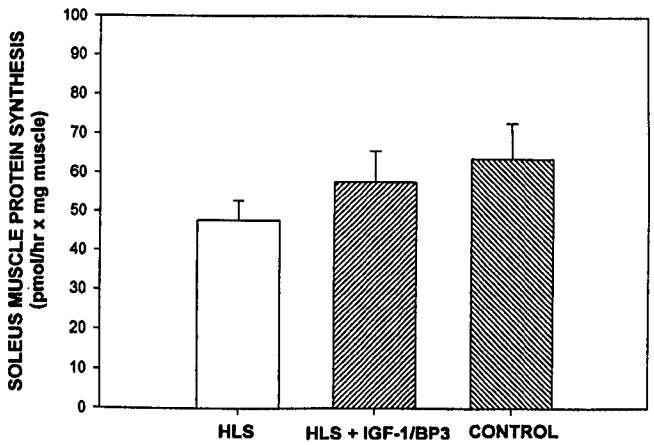

Figure 2. Soleus muscle protein synthesis from hind-limb suspended rats (HLS) receiving no treatment, HLS rats receiving IGF-1/BP3 or non-suspended rats receiving placebo (CONTROL). N = 8 rats per group, a = minimum p<0.05 vs control, b = minimum p<0.05 vs HLS group.

Table II. Soleus Muscle Protein and Nucleic Acid Content From HLS Rats, HLS Rats Receiving IGF-1/BP3, or Nonsuspended Rats Receiving Placebo (Control)

	HLS	HLS + IGF-1/BP3	Control
Protein (mg/g muscle)	100.7 ± 10.7[a]	144.6 ± 10.5[b]	132.7 ± 3.8
Total nucleic acids (μg/mg muscle)	0.843 ± 0.13[a]	0.936 ± 0.08[a]	1.68 ± 0.10[b]
DNA (μg/mg muscle)	0.26 ± 0.04	0.29 ± 0.04	0.37 ± 0.007
RNA (μg/mg muscle)	0.57 ± 0.9[a]	0.64 ± 0.5[a]	1.31 ± 0.10[b]

Note. Means are presented ± SEM, $n = 8$ rats per group.
[a] Minimum $P < 0.05$ vs Control.
[b] Minimum $P < 0.05$ vs HLS.

Total nucleic acids, DNA, and RNA were significantly reduced in HLS-treated rat muscle when compared with control (Table II). Although IGF-1/BP3 treatment increased nucleic acid content somewhat in HLS muscles, the increase was not statistically significant.

Muscle Morphology. Muscle sections from rats after 10 days of HLS exhibited a significant reduction in volume density (volume of muscle fibers per volume of tissue), greater rounding of muscle fibers (F closer to 1.0), and reduced mean fiber areas and diameters when compared with control muscles (Table III). HLS rats treated with IGF-1/BP3 showed increased muscle volume densities when compared with HLS muscles, as well as an increased surface/volume ratio of individual muscle fibers. However, the mean fiber areas of HLS + IGF-1/BP3 muscles remained significantly reduced when compared with both HLS and control muscle (Table III). The shape factor (F) of IGF-1/BP3-treated muscle was not different from that of control muscle. When the distribution of muscle fibers areas for each of the three groups was plotted on a frequency histogram, differences in fiber sized distribution between the different groups became more evident (Fig. 3). Although all three groups of muscle fibers showed a normal distribution by statistical testing, HLS muscles had significantly smaller mean fiber areas than control and HLS muscles. The fiber area distribution of IGF-1/BP3-treated HLS muscles was significantly narrower than HLS or control muscles with a correspondingly smaller standard deviation.

Discussion

The wasting of skeletal muscles is a serious problem associated with a number of catabolic states such as prolonged illness, cancer, AIDS, stroke, burns, and space flight (1–7). In patients with catabolic illness, loss of lean body mass can significantly alter response to drug therapy, impair immune function, and increase risk of sepsis, thus placing the patient in a vulnerable physiologic state that can prolong or impede the recovery process. The etiology of the muscle wasting process that accompanies catabolic states is unclear but may involve altered ratios of circulating hormones such as insulin and glucagon, as well as increased release of catabolic cytokines such as interleukins and tumor necrosis factor (26–28). A key finding in muscle wasting associated with chronic catabolic illness is a loss of equilibrium in muscle protein metabolism. In normal skeletal muscle, there is a tight regulation between rates of muscle protein synthesis and degradation such that no net change in muscle mass occurs. However, in catabolic illness, muscle protein degradation rates are markedly increased while rates of protein synthesis appear to be depressed.

In the present study, 10 days of HLS caused significant impairment of body weight and muscle weight gain. It is interesting to note that muscle weights were reduced in all three muscle groups studied, regardless of their primary fiber type composition. In light of previous reports that HLS induces minimal atrophy in on muscles that are predominately fast-twitch fibers, we might have expected to less decrease in muscle mass for gastrocnemius and plantaris muscles in response to HLS, but this was not the case. In future studies, we will expand our biochemical, metabolic, and morphological analyses to these other muscle types to quantify the effects of HLS on muscles of various fiber composition groups.

The reduced muscle mass observed in HLS rats was

Table III. Morphology Measurements in Soleus Muscle from HLS Rats, HLS Rats Receiving IGF-1/BP3, and Nonsuspended Rats Receiving Placebo (Control)

	HLS	HLS + IGF-1/BP3	Control
Volume density (μ^3 muscle/μ^3 tissue)	0.93 ± 0.01[a]	0.99 ± 0.005[b]	0.98 ± 0.009[b]
Surface/volume (μ^2/μ^3)	0.013 ± 0.001	0.017 ± 0.005[a,b]	0.013 ± 0.007
Mean fiber area (μ^2)	1087.7 ± 23.9[a]	935.2 ± 16.3[a,b]	1486.9 ± 9.3[b]
Mean fiber diameter (μ)	148.6 ± 3.4[a]	141.7 ± 2.1[a]	181.4 ± 9.3[b]
Shape factor (F)	0.63 ± 0.007[a]	0.61 ± 0.007	0.58 ± 0.007[b]

Note. Means are presented ± SEM, $n = 4$ muscle sections from each of eight rats per group.
[a] Minimum $P < 0.05$ vs Control.
[b] Minimum $P < 0.05$ vs HLS.

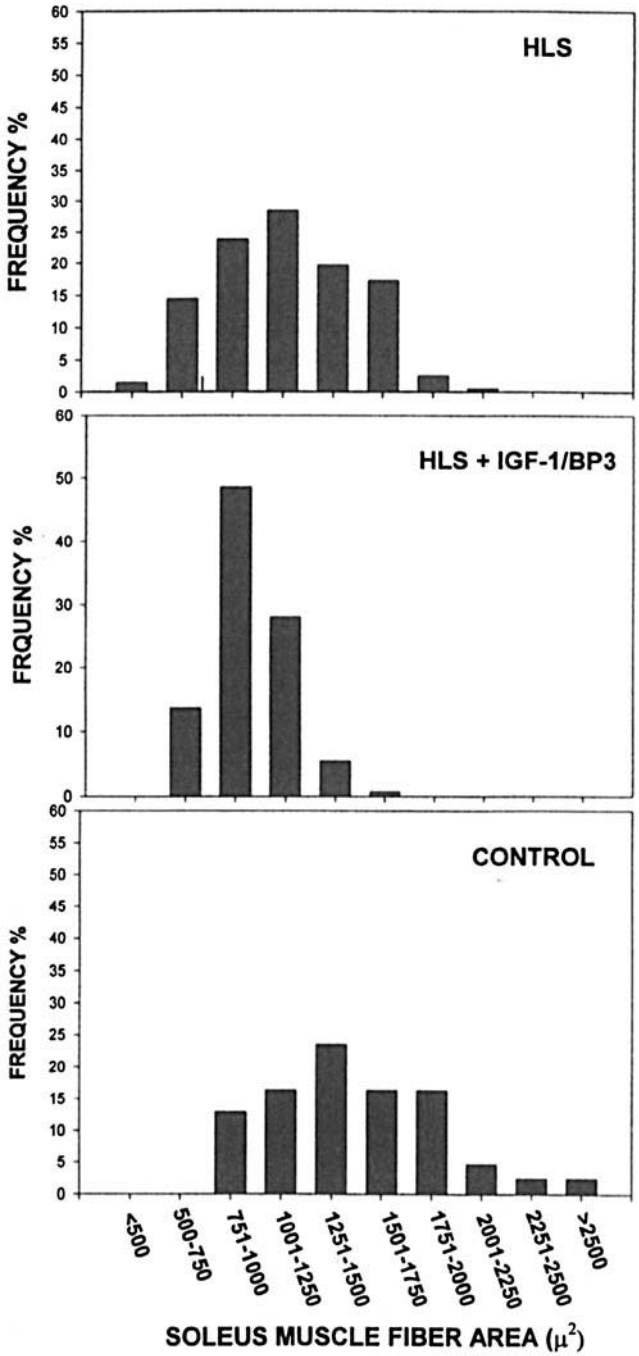

Figure 3. Distribution of soleus muscle fiber areas from hind-limb suspended rats (HLS) receiving no treatment, HLS rats receiving IGF-1/BP3 or non-suspended rats receiving placebo (CONTROL). N = 4 muscle sections from each of 8 rats per group.

accompanied by markedly increased rates of protein degradation and reduced muscle protein content. The major beneficial effect of IGF-1/BP3 in this study appeared to be reduced muscle proteolysis. IGF-1/BP3 significantly reduced net protein degradation rates in muscles from HLS rats. Preservation of muscle weight and protein content paralleled this reduced muscle proteolysis. In a previous study with highly catabolic muscle from dystrophic hamsters, we reported a 27% decrease in muscle protein degradation rates with rhIGF-1 (29); here with IGF-1/BP3, we report a near 40% decrease. A key component of muscle proteolytic pathways, namely calpain-mediated myofibrillar degradation, was also reduced in rhIGF-1-treated dystrophic mice (30). The effects of IGF-1/BP3 on activity of key components of the proteolytic pathways in HLS muscle will be investigated in future studies.

Although a number of other studies support our finding of IGF-1's effectiveness at reducing protein degradation in various catabolic models (14, 31), the effects of IGF-I on muscle protein synthesis are much more variable. The extent to which IGF-I affects protein synthesis appears to be a function of dosage, route of administration, and method used to measure protein metabolism. Infusion of low-dose rhIGF-1 did not stimulate protein synthesis in humans despite a 300% increase in plasma IGF-I level (32). Whereas other studies in normal human and fasted lambs using leucine clamps did report some increase in muscle protein synthesis with IGF-I (33, 34), a recent study by Fang *et al.* (31) likewise reported an increase in muscle protein synthesis in burn-injured rats treated with IGF-I (1). Interestingly, Fang *et al.* (31) reported that maximal rates of protein synthesis occurred at lower doses of IGF-1 (100 ng/ml), whereas protein degradation continued to be inhibited by IGF-1 doses up to 1 μg/ml. However, in their study, isolated muscles were incubated directly with IGF-1 in the medium. In our previous studies with subcutaneously administered rhIGF-1 in dystrophic mice and hamsters, we did not see significant increases in muscle protein synthesis with rhIGF-1 (0.50 mg/kg) (21, 29). However, we did see an increase in muscle protein synthesis rates in dystrophic mice when rhIGF-1 was given in conjunction with a high-protein diet, something we may try in the future with IGF-1/BP3. In the present study, a subcutaneous IGF-1/BP3 dose of 50 mg/kg did not significantly increase muscle protein synthesis rates. The lack of IGF-1/BP3 effect on muscle RNA levels in this study supports the finding that this compound did not increase protein synthesis in HLS muscle and that its major effect was on proteolysis. In future studies, administration of IGF-1/BP3 by implanted osmotic pump might lead to more consistently elevated levels of drug and possibly enhanced anabolic effects.

The effects of HLS on skeletal muscle morphology reported in this study are similar to those reported in previous studies. HLS has been shown to cause significant atrophy in slow twitch muscles such as the soleus, while having little or no effect on fast twitch muscles like the EDL (18, 19). Atrophy of the soleus manifested as a significant reduction in muscle fiber areas and decreased volume density of muscle. Although there is a reduction in fiber areas during atrophy, the distribution of atrophied fibers remains normal with the histogram basically shifted to the left. Treatment of HLS rats with IGF-1/BP3 significantly increased the volume density of HLS muscles. Although the mean fiber areas of IGF-1/BP3-treated HLS muscles remained smaller than control muscles, the number of muscle

fibers per unit area was significantly increased. Although we did not examine changes in specific muscle fiber types within the soleus, earlier studies in adult rats have reported that HLS does not have any effect on fiber type composition (18). However, in young rats similar to those used in this study, HLS has actually been shown to arrest the conversion of type II to type I fibers in soleus muscles (18). It would be interesting to examine the effects of IGF-1/BP3 on fiber conversion or the overall proportion of specific fiber type in HLS muscles because a recent study by Lynch (35) reported that IGF-1 treatment increased the proportion of type IIa fibers and reduced the proportion of type I fibers in dystrophic muscles.

The anabolic potential of IGF-1/BP3 complex has also recently been investigated in other catabolic conditions such as burn injury and osteoporosis. In severely burned adults, IGF-1/BP3 (1-4 mg/kg/day) increased net protein synthesis in leg muscles (36). Children with severe burns showed reduced levels of proinflammatory mediators such as IL-1 and TNF-α after IGF-1/BP3 administration and decreased muscle protein catabolism (37, 38). Increased cytokine levels have been shown in previous studies to stimulate catabolism of skeletal muscles (26, 27). In another recent study in semistarved rats, IGF-1/BP3 complex but not IGF-1 alone was able to stimulate muscle protein synthesis under these catabolic conditions (17). A second study by Wang *et al.* (39) in rats with cancer cachexia showed that IGF-1/BP3 was able to increase food intake, attenuate weight loss, and improve glucose metabolism without effecting tumor size.

In summary, IGF-1/BP3 was used in this study at significantly higher doses than standard IGF-1 and without significant hypoglycemia. The major effects of IGF-1/BP3 on hind-limb suspended animals was a marked reduction in muscle protein degradation rates that resulted in preservation of muscle mass, protein content, and volume density. The beneficial effects of IGF-1/BP3 on muscle catabolism and its lack of side effects may hold significant potential for treatment of muscle wasting in human catabolic conditions such as prolonged illness, cancer, AIDS, and burn injury. IGF-1/BP3 may also be of value when used as a possible prophylactic agent to limit skeletal muscle wasting during space flight.

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