Mature IGF-I excels in promoting functional muscle recovery from disuse atrophy compared with pro-IGF-IA

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Park S, Brisson BK, Liu M, Spinazzola JM, Barton ER. Mature IGF-I excels in promoting functional muscle recovery from disuse atrophy compared with pro-IGF-IA. J Appl Physiol 116: 797–806, 2014. First published December 26, 2013; doi:10.1152/japplphysiol.00955.2013.—Prolonged disuse of skeletal muscle results in atrophy, and once physical activity is resumed, there is increased susceptibility to injury. Insulin-like growth factor-I (IGF-I) is considered a potential therapeutic target to attenuate atrophy during unloading and to enhance rehabilitation upon reloading of skeletal muscles, due to its multipronged actions on satellite cell proliferation, differentiation, and survival, as well as its actions on muscle fibers to boost protein synthesis and inhibit protein degradation. However, the form of IGF-I delivered may alter the success of treatment. Using the hindlimb suspension model of disuse atrophy, we compared the efficacy of two IGF-I forms in protection against atrophy and enhancement of recovery: mature IGF-I (IGF-IS) lacking the COOH-terminal extension, called the E-peptide, and IGF-IA, which is the predominant form retaining the E-peptide. Self-complementary adeno-associated virus harboring the murine Igf1 cDNA constructs were delivered to hindlimbs of adult female C57BL6 mice 3 days prior to hindlimb suspension. Hindlimb muscles were unloaded for 7 days and then reloaded for 3, 7, and 14 days. Loss of muscle mass following suspension was not prevented by either IGF-I construct. However, IGF-IS expression maintained soleus muscle force production. Further, IGF-IS treatment caused rapid recovery of muscle fiber morphology during reloading and maintained muscle strength. Analysis of gene expression revealed that IGF-IS expression accelerated the downregulation of atrophy-related genes compared with untreated or IGF-IA-treated samples. We conclude that mature-IGF-I may be a better option than pro-IGF-IA to promote skeletal muscle recovery following disuse atrophy.

THE LACK OF MUSCLE ACTIVITY inevitably leads to a loss of muscle mass (26). Muscle atrophy is a common clinical phenomenon observed in multiple rehabilitation settings. It is characterized by reductions in several morphological and physiological parameters, including decreases in muscle fiber size and number, protein content, and protein/DNA ratios, as well as losses in muscle strength and shifts in contractile properties toward those of fast muscle fiber types (2, 13). Disuse atrophy is often a secondary consequence of disease or injury, such as in systemic diseases that require bed rest or orthopedic injuries that demand cast immobilization or a change in weight-bearing status (9, 32). Although the loss in muscle mass and strength can be significant, disuse atrophy can be reversed with effective rehabilitation interventions, such as load-bearing and progressive resistance training (1, 33). However, significant time is required to fully restore functional muscle mass, where recovery time from disuse atrophy can surpass the time during which loss of muscle mass occurred. Further, loss of force-generating capacity is exacerbated at the transition from unloaded to reloaded conditions (36). Thus, therapies must counter not only the loss of mass and strength associated with inactivity, but also the extensive weakness that accompanies the early phases of reloading.

Insulin-like growth factor I (IGF-I) has long been recognized as one of the critical factors for coordinating muscle growth, enhancing muscle repair, and increasing muscle mass and strength. Initially, IGF-I was considered a prime therapeutic candidate for prevention of disuse atrophy through its prosurvival and pagrowth actions. However, IGF-I transgenic mice subjected to hindlimb suspension, an animal model for disuse atrophy, exhibited a loss of muscle mass similar to wild-type mice, showing muscle atrophy was not prevented by increased IGF-I (11). Therefore, the consensus is that IGF-I is ineffective in combating disuse atrophy, even though it modulates most of the pathways associated with maintenance of muscle mass. Previous evidence showed an attenuation of atrophy after hindlimb suspension by electroploration of IGF-I into rat skeletal muscles (3), even though IGF-I levels and the IGF-I signaling are reduced (18, 22). Regardless of the modest benefit IGF-I may have on prevention of disuse atrophy, the potential for IGF-I to accelerate the healing process following reloading has largely been ignored. Only recently was it shown that increased IGF-IA production by recombinant virus administration improved the efficiency of recovery following cast immobilization (34). Therefore, employing IGF-I in the recovery phase of disuse atrophy may prove to be beneficial.

Viral expression of IGF-IA, which is the most abundant isoform of IGF-I (5), leads to the accumulation of pro-IGF-I forms in the muscle tissue (12). These forms retain the COOH-terminal extension called the E-peptide, and a large proportion of pro-IGF-I is glycosylated. This extension may improve storage of this growth factor (20), but at the expense of efficient receptor binding (12). As a consequence, expression of IGF-IA may not be the ideal form of IGF-I to deliver to enhance recovery from disuse atrophy, where association of glycosylated pro-IGF-I with the extracellular matrix could inhibit the ability of this growth factor to activate the IGF-I receptor. In contrast, mature IGF-I, which is stabilized by its association with IGF binding proteins (15), may utilize a different mechanism of release to enable receptor activation.

The goal of this study was to compare the efficacy of IGF-IA and mature IGF-I for preventing disuse atrophy and promoting recovery following reloading. We utilized the hindlimb suspension model in mice, which exhibits the salient features of
disuse atrophy, including loss of muscle mass and function during unloading, and diminished strength during reloading.

**MATERIALS AND METHODS**

**Animals and viral injections.** All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Female C57BL/6 mice (12- to 14-wk old) were purchased from Jackson Laboratory (Bar Harbor, ME) and were randomly assigned to each experimental cohort. Recombinant self-complement adeno-associated virus serotype 2/8 (AAV2/8) harboring the Igf1 cDNA of murine class I IGFI-IA and mature IGFI-sequences was produced at the Children’s Hospital of Philadelphia Vector Core and was used to express these IGFI-forms in skeletal muscle, as described previously (12). Briefly, IGFI-1A included the sequence to encode the class I signal peptide, IGFI-I, and the EA-peptide. IGFI-1S lacked E-peptide sequence, and a stop codon was inserted at the end of the mature IGFI-I (A70X) as previously described (7). Virus particles (5 × 10^{10}) in 50 µl PBS were delivered to the anterior and posterior compartments of one hindlimb targeting tibialis anterior (TA), extensor digitorum longus, soleus, and gastrocnemius, respectively. After viral injections, mice returned to normal activities for 3 days until suspended.

**Suspension and reloading.** To induce disuse atrophy only in hindlimbs, the hindlimb suspension model was used with a slight modification from the original model for rats (27). Three days after viral delivery, mice were suspended under light anesthesia (intraperitoneal injection of 50 µl of 17 mg/ml ketamine and 2.5 mg/ml xylazine in sodium chloride). The tails of mice were attached to a short metal chain by foam tape (Skin Trac; Zimmer, Warsaw, IN). The metal chain was then connected to a crossbar lining along at the center of a specially designed cage, and the length of the metal chain was adjusted so that the hindlimbs of mice were suspended in the air while the forelimbs were able to grasp metal grids on a cage floor, which provided traction for mice to move freely. Thus mice maintained normal activities, such as eating, drinking, excreting, and grooming. After 7 days of hindlimb suspension, mice were returned to a normal cage and reloaded their hindlimb muscles for 3, 7, or 14 days (R3D, R7D, and R14D, respectively). A total of four to seven mice were utilized for each treatment and time point.

**Tissue collection and muscle function testing.** Animals were killed at five time points: before and after suspension and 3, 7, and 14 days after reloading of hindlimbs. Skeletal muscles were harvested for morphological, biochemical, and functional analyses. Gastrocnemius, TA, and soleus muscles were blotted, weighed, and flash frozen either in liquid nitrogen for biochemical analysis or in melting isopentane at a fixed position, surrounded by optical cutting temperature compound (Sakura, Torrance, CA) for morphological analysis.

Harvested soleus muscles were immediately pinned to a Sylgard base in a small dish containing Ringer solution, which was continuously equilibrated with 95% O_2-5% CO_2. Sutures were attached to each end of the tendons making loops so that the soleus could be mounted in an isolated muscle mechanics bath (model 300B; Aurora Scientific, Aurora, ON, Canada) containing gas-equilibrated Ringer solution at 22°C for functional measurements. After determining optimum length (Lo) by supramaximal twitch stimulation, three maximum isometric tetanic forces (Po) were acquired using a train of 100-Hz, 500-ms supramaximal electrical pulses with highest Po recorded. A 5-min resting period was allowed between each tetanic contraction. Muscle specific forces (N/cm²) were calculated by normalizing the maximum muscle tension to the muscle cross-sectional area (CSA). Physiological CSA was estimated using the following formula: CSA = muscle mass (g)/Lo (cm) × 0.691 × 1.06 (g/cm²), where 0.69 is the fiber length to muscle length ratio, and 1.06 is the density of muscle. Soleus muscles were then processed for morphological analysis as described earlier.

**Determination of IGFI levels and activity.** IGFI-I protein levels in untreated, IGFI-IA-treated, and IGFI-1S-treated muscles were assessed with immunoblotting and ELISA. Immunoblotting with an IGFI antibody (R&D Systems, Minneapolis, MN) was performed to evaluate forms of IGFI-I presented in viral-treated TA muscles during the reloading period. IGFI-I protein levels in gastrocnemius muscles were also quantified by a commercially available rat IGFI-I ELISA kit (R&D Systems) as previously described (7, 12). Immunoblots of TA samples for phosphorylated Akt and total Akt determined the activation of IGFI-I signaling pathways with secondary horseradish peroxidase-conjugated mouse and rabbit recognizing antibodies (Cell Signaling Technology, Beverly, MA). GAPDH or Tubulin (Sigma-Aldrich, St. Louis, MO) was blotted as loading controls for all samples tested.

**Muscle fiber morphology.** Frozen cross-sections (10 µm) from the midbelly of soleus muscles were subjected to immunohistochemistry to determine fiber types. Laminin (rabbit Ab-1; Neomarkers, Fremont, CA) marked outlines of each muscle fiber, and fast and slow muscle fiber types were distinguished by antibodies specific for myosin heavy chain (MHC) 2A (SC-71) and MHC 1/f (BAF-5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), respectively. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Images of muscle sections were obtained on a Leica epifluorescence microscope (Leica Microsystems, Bannockburn, IL), and OpenLab software (Improvis, PerkinElmer, Waltham, MA) was employed to analyze digital images and obtain fiber areas from entire muscle cross-sections.

**Satellite cell activation.** Frozen cross-sections (10 µm) from the midbelly of soleus muscles were subjected to immunohistochemistry for the assessment of satellite cell activation during recovery periods. The frozen sections were dried at room temperature for 5 min followed by 2% formaldehyde fixation. The fixed sections were sequentially dehydrated and rehydrated with ethanol and Citrosof (Fisher, Waltham, MA). Antigen retrieval was achieved in boiling 10 mM sodium citrate, and the sections were permeabilized by 0.5% Triton X-100 treatment. The specimens were further treated with mouse Ig blocking reagent following the manufacturer’s protocol (Mouse on Mouse, Vector Laboratories) prior to primary antibody incubation. A combination of three immunohistochemistry sets was employed to determine the quantity and activation state of satellite cells. Satellite cells were identified with mouse anti-Pax7 (R&D Systems) and rabbit anti-laminin, proliferating cells were marked with rabbit anti-Ki67 (Sigma-Aldrich) and mouse anti-dystrophin (Dys2, Novacasta, Vector Labs), and mouse anti-Pax7 and rabbit anti-Ki67 were used to assess the proportion of proliferating satellite cells. All nuclei were counterstained with DAPI, and stained sections were visualized under the same condition described previously. All Pax7, Ki67, and Pax7-Ki67 positive nuclei were counted individually and normalized by total fiber number of the same soleus muscle.

**Atrophy-related and IGF binding protein gene expression.** Total RNA extracted from gastrocnemius muscles was subjected to single-strand reverse transcription (Applied Biosystems, Foster City, CA). With obtained cDNA templates, quantitative polymerase chain reaction (qPCR) was performed using the Applied Biosystems 7300 and reagents (LightCycler FastStart DNA MasterPLUS SYBR Green I; Roche Applied Science, Indianapolis, IN) to quantify gene expression with oligonucleotide sequences specified in Table 1. Genes of interest were compared with 18S as a housekeeping gene, where the difference in cycle threshold (CT) between any gene and 18S was utilized to calculate the level of expression (ΔCT). All fold changes were calculated relative to ΔCT values of the nonsuspended control muscle group, with no fold change set to a value of one [fold change = 2^{ΔCT(sample)−ΔCT(control)}].

**Statistics.** Data are presented as means ± SE unless stated otherwise. Two-way ANOVA was utilized for comparisons among control, IGFI-IA, and IGFI-1S groups at each time point with Tukey’s multiple comparison tests. P < 0.05 was accepted as statistical significance.
RESULTS

IGF-I does not prevent disuse atrophy, but IGF-IS restores functional capacity following suspension. To estimate the level of IGF-I production and receptor activity following viral delivery of IGF-IA and IGF-IS, immunoblotting for AKT phosphorylation and ELISA measurements of IGF-I were performed on the TA and gastrocnemius muscles, respectively. Both IGF-I forms caused ~40% elevated P-AKT levels compared with untreated muscles, which was significantly different in the IGF-IS condition. After 7 days of hindlimb suspension, the extent of P-AKT in any sample compared with their respective nonsuspended cohorts was not altered (Fig. 1, A and B). Similarly, the IGF-I content of gastrocnemius muscles was significantly higher following viral delivery of IGF-IA and IGF-IS compared with untreated muscles from both nonsuspended and suspended mice (Fig. 1C). The ELISA measurements are more sensitive to nonglycosylated IGF-I forms (12), and so it is likely that the IGF-IA content is underestimated. IGF-I production resulted in significant hypertrophy of the muscles from nonsuspended mice (Fig. 1D), but, despite the progrowth effects of IGF-IA and IGF-IS, atrophy was not prevented after 7 days of hindlimb suspension (Fig. 1D). The untreated and IGF-IA-treated soleus muscles experienced about 22% reduction in muscle mass (normalized to body weight), and IGF-IS-treated soleus relative mass was lowered by 19% in the mice subjected to hindlimb suspension compared with nonsuspended controls. This trend was consistent for the TA and gastrocnemius muscles under the same conditions (Table 2). Analysis of soleus fiber areas was consistent with the changes in mass, where muscles subjected to either IGF-IA or IGF-IS treatment in nonsuspended animals displayed a slight increase in median fiber size (Fig. 2D). However, in all cases, the fiber size diminished following hindlimb suspension (Fig. 2, A–D). Analysis of fiber type and number showed no apparent differ-

Table 1. Oligonucleotide sequences for real-time reverse-transcription polymerase chain reaction (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotides (5′-3′)</th>
<th>Sense Antisense</th>
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<td>aacctggacatctggcaactcg</td>
</tr>
<tr>
<td>Igfbp4</td>
<td>ttgtgcacgggcaaggcgt</td>
<td>cggggtcttcggcagggtctcg</td>
</tr>
<tr>
<td>Igfbp5</td>
<td>gccggcaaanacgagtaga</td>
<td>ggtctctcggcagctctcg</td>
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<td>Myostatin</td>
<td>ccggcaacggcggccagccgga</td>
<td>gcagttcaggaacttccttcctc</td>
</tr>
<tr>
<td>Fbxo40</td>
<td>tctggaagtctccacatcaga</td>
<td>cagctgttggatagcagccccga</td>
</tr>
<tr>
<td>Atrogin1</td>
<td>aacaggaggttatgcagtaagg</td>
<td>aacagtacctgtggactcttg</td>
</tr>
<tr>
<td>Murf1</td>
<td>aggcttccccacccactg</td>
<td>tgtgcttctcagggcactc</td>
</tr>
<tr>
<td>18 s</td>
<td>ctctgttcccctactgtctg</td>
<td>aatgaccattcgacgtttc</td>
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Fig. 1. Viral expression of insulin-like growth factor-IS (IGF-IS) prevents muscle weakness associated with hindlimb suspension induced atrophy. A: representative immunoblots for P-AKT and T-AKT in tibialis anterior (TA) muscle lysates following viral injection and/or suspension. B: quantification of Western blot (A) revealed ~40% increase in P-Akt by both viral constructs in muscles from nonsuspended mice, but no difference in Akt phosphorylation following hindlimb suspension. C: estimation of IGF-I content in gastrocnemius muscles measured by ELISA showed 10- to 60-fold increase in IGF-I content in viral injected muscles. D: soleus mass normalized to body mass demonstrated that both viral constructs caused a significant increase in muscle mass in nonsuspended mice, but that in all cases, suspension caused a significant loss of mass, which was at a similar proportion regardless of viral construct. E: specific force did not differ among treatment groups in soleus muscles from nonsuspended mice, and significantly decreased in untreated suspended muscles. However, only IGF-IS treatment significantly improved specific force following suspension compared with untreated muscles. NS, nonsuspended; S, suspended muscles. Bars represent means ± SE from 4–7 mice of each condition and time point. *P < 0.05 compared with control treatment at each time point; †P < 0.05 compared with the NS time point within the same treatment.
Fig. 1. Percent fiber size in soleus muscles following reloading. A–C: cumulative fiber size distributions for control, IGF-IA, and IGF-IS treated soleus muscles from each time point determined by immunohistochemistry for laminin. A leftward shift of a line indicates a fiber area distribution with smaller fibers. Each time point is marked with different symbols; the thicker line represents NS, and the dotted line represents S. D: median fiber areas determined from the cumulative percent fiber distribution showed that IGF-IS treatment fiber size returned to nonsuspended values by 7 days reloading, whereas control and IGF-IA treatments achieved nonsuspended values by 14 days reloading. Data are means ± SE for n = 3–5 samples. *P < 0.05 compared with control treatment at each time point; †P < 0.05 compared with the nonsuspended (NS) time point within the group under same treatment; ‡P < 0.05 compared to the nonsuspended levels (comparing Fig. 1E) with the data in Fig. 4C.}

**Table 2. Normalized muscle mass (mg/g)**

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Treatment</th>
<th>NS</th>
<th>S</th>
<th>R3D</th>
<th>R7D</th>
<th>R14D</th>
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<tbody>
<tr>
<td>Tibialis Anterior</td>
<td>Control</td>
<td>1.78</td>
<td>1.59</td>
<td>1.65</td>
<td>1.62</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>1.91</td>
<td>1.73</td>
<td>1.79</td>
<td>1.88</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>2.29</td>
<td>1.95</td>
<td>1.99</td>
<td>2.18</td>
<td>2.19</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>Control</td>
<td>4.71</td>
<td>4.31</td>
<td>4.55</td>
<td>4.50</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
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<td>4.51</td>
<td>4.67</td>
<td>5.28</td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>6.26</td>
<td>5.28</td>
<td>5.37</td>
<td>5.68</td>
<td>5.96</td>
</tr>
</tbody>
</table>

Data were collected from n = 3–4 mice for each treatment at each time point. Muscle mass was normalized to body weight (mg muscle/g body wt). Two-way ANOVA followed by Tukey’s multiple comparison test was utilized to determine significant difference. *P < 0.05 compared with control group at the same time point; †P < 0.05 compared to the nonsuspended (NS) time point within the group under same treatment; ‡P < 0.05 between insulin-like growth factor-IA (IGF-IA) and IGF-IS at the same time point. S, suspended; R3D, R7D, and R14D: reloaded hindlimb muscles for 3, 7, or 14 days.
of mass was more efficient with IGF-I treatment, where IGF-
IA-treated soleus muscles were larger by 7 days of reloading,
and, in IGF-IS-treated soleus muscles, significant gains in mass
were already apparent by 3 days of reloading (Fig. 4
C).
Functional measurements of soleus muscles revealed an
~50% drop in specific force at 3 days of unloading in both untreated
and IGF-IA-treated muscles compared with muscles from non-
suspended mice, and this weakness persisted through 7 days
(Fig. 4D). In contrast, IGF-IS-treated soleus muscles main-
tained specific forces during the entire reloading period, unlike
those treated with IGF-IA or having no treatment at all. Thus
IGF-IS protected against the loss of force-generating capacity
associated with reloading.

To examine how changes in mass occurred during suspen-
sion and reloading, muscle fiber size, number, and type were
measured in the soleus. The cumulative fiber area distributions
(Fig. 2, A–C) shifted leftward during suspension, indicating
smaller muscle fibers, and progressively returned to nonsus-
pension patterns during reloading. IGF-IA-treated muscles ex-
hibited a more extreme leftward shift at 3 days of reloading,
before beginning to regain fiber size. IGF-IS-treated muscles
exhibited similar transitions during suspension and reloading,
but returned to nonsuspended fiber distribution by 14 days,
unlike untreated or IGF-IA-treated muscles. Median fiber areas
of all samples also confirmed the accelerated restoration of the
fiber area by IGF-IS treatment (Fig. 2
D). While the untreated

Fig. 3. Fiber type and fiber number analysis of soleus muscles via immunohistochemistry. A: percent fibers categorized into fast-twitch muscle (2A) and slow-twitch muscle (1/B) via immunohistochemistry. The normal fiber type ratio of 60:40 (2A:1/B) was retained in most muscles at all time points, but the control muscles showed significant increases in fast-twitch muscle composition during muscle reloading [for 3 days (R3D) and 7 days (R7D)], as did IGF-IA-treated muscles at R3D. Bars with no pattern, control muscles; stippled bars, IGF-IA treatment; striped bars, IGF-IS treatment. Overlay of no color or grey represents myosin heavy chain I/B and IIA, respectively. X-axis labels specify the time point. B: average fiber numbers of solei muscles showed that the viral injected muscles do not show a significant change in fiber number after suspension and during the muscle reloading while the control muscles showed increases in solei fiber numbers during the muscle reloading. Data are means ± SE for n = 3–5 samples. †P < 0.05 compared with the NS time point within the same treatment.

Fig. 4. Diminished function caused by reloading is rescued by IGF-IS. A: immunoblotting of TA muscles for IGF-I before suspension and 3, 7, and 14 days after reloading shows that IGF-IA produces primarily pro- and glycosylated pro-IGF-I, whereas IGF-IS produces only mature IGF-I. Protein loading was 80 µg for control, 40 µg for IGF-IA, and 20 µg for IGF-IS. Increased protein loading (80 µg) of IGF-IA samples and longer exposure (right) reveals increased presence of mature IGF-I in the IGF-IA-treated muscles only during reloading. GAPDH was utilized as loading control for each blot. B: gastrocnemius IGF-I content measured by ELISA showed significant differences in IGF-I content in viral injected muscles throughout the reloading period after induced atrophy. C: the muscle-to-body mass ratios for soleus muscles demonstrated that muscles treated with IGF-IS had constantly higher ratios than those of control muscles during reloading. IGF-IA-treated muscles regained more mass by R7D than untreated controls. D: specific forces of soleus muscles following reloading showed that the control and IGF-IA-treated muscles were significantly weaker until R14D, but IGF-IS in treated muscles maintained the muscle strength during the entire reloading period. Dotted lines are average specific forces of nonsuspended (NS) and suspended (S) control soleus muscles from Fig. 1E. R3D, R7D, and R14D-reloading for 3, 7, and 14 days following hindlimb suspension. Data are means ± SE of 3–4 mice from each time point and treatment. *P < 0.05 compared with control treatment at each time point; †P < 0.05 compared with the NS time point within the same treatment.
and IGF-IA-treated muscles showed significantly reduced median fiber area until R14D, the IGF-IS-treated muscles were able to regain the nonsuspended median fiber area at R7D and were significantly larger than control muscles at R14D. Additionally, there was no change in fiber type during suspension, presumably because of the brief period of unloading consistent with recent observations (19), but there was a fiber type shift toward more fast (MHC 2A) fibers during the reloading in control muscles (Fig. 3A) with increased fiber numbers (Fig. 3B), but IGF-IS treatment, and to a lesser extent IGF-IA treatment, were able to prevent the fiber type shift and maintain the fiber number throughout suspension and reloading.

**IGF-IS does not alter satellite cell activation during muscle reloading.** During reloading, atrophied muscles undergo remodeling to repair damage and to recover muscle size and strength. Although not required, activation and proliferation of satellite cells can contribute to this process (21), and so we determined the proportion of proliferating satellite cells in soleus muscles by immunohistochemistry (Fig. 5A). There was no difference in the total proportion of Pax7-positive cells in muscles from nonsuspended or suspended animals, and IGF-I treatment did not alter these numbers (Fig. 5B). However, with the exception of IGF-IA-treated muscles at 3 days of reloading, there were elevated numbers of satellite cells marked by Pax7 in all groups throughout reloading compared with nonsuspended control muscles (Fig. 5B). By 14 days of reloading, IGF-IA-treated muscles had significantly more satellite cells than in other conditions. This suggests that IGF-IA treatment may have caused a delay in the recruitment of satellite cells but that the pool of satellite cells was enhanced by IGF-IA later in reloading. Generalized cell proliferation indicated by Ki67-positive nuclei was elevated most extensively during the early phases of reloading, with IGF-IS treatment causing the greatest increase (Fig. 5C). Dually positive cells were considered to be activated satellite cells (Fig. 5D). Control and IGF-IS-treated muscles exhibited an approximately fivefold increase in satellite cell activation at R3D, with 60% of the identified satellite cells also labeled by Ki67. IGF-IA-treated muscles showed significantly lower activated satellite cell counts at R3D, where only 38% of their satellite cells were activated. By 7 days of reloading, all groups had equivalently elevated activation, and this returned to baseline levels by 14 days reloading. Thus, while we observed alterations in satellite cell activation during reloading, these changes were not specific to IGF-IS treatment, and so they did not appear to underlie the beneficial effects of IGF-IS on mass and function.

**IGF-I downregulates atrophy-related genes during reloading.** Several ubiquitin E3 ligases in skeletal muscle are important factors in driving atrophy and are regulated by IGF-I (10, 17, 28). Therefore, we investigated the gene expression levels of atrophy-related genes in gastrocnemius muscles after suspension and during reloading (Table 3). Fbxo40 targets IRS1 for degradation and reduces the efficiency of the IGF-I signaling pathway (29). There was no difference in Fbxo40 expression in untreated muscles at any time point. However, the IGF-IA- and IGF-IS-treated muscles exhibited differences during reloading where Fbxo40 expression was approximately fourfold more in the IGF-IS-treated gastrocnemius at R3D, and its expression was almost undetectable in the IGF-IA-treated muscles at R14D, indicating extreme downregulation of this gene.

Atrogin1 and Murf1 encode skeletal muscle specific ubiquitin-proteasome proteins that are elevated during catabolic processes (10). There was no significant change in Atrogin1 or Murf1 expression during suspension in control muscles, and levels of both genes were reduced during reloading of control muscles, consistent with the drive for regaining muscle mass. IGF-IA-treated muscles displayed a decrease in Atrogin1 in suspension and in reloading, where expression was virtually undetectable at R14D, yet no changes were observed at any time point for Murf1 expression in IGF-IA-treated muscles. A third pattern of expression was found in IGF-IS-treated muscles, where, like control muscles, both transcripts had reduced

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Fig. 5. Satellite cell activation in soleus muscles before and after hindlimb suspension and during muscle reloading following hindlimb suspension assessed via immunohistochemistry of Ki67 for proliferating cell marker, Pax7 for satellite cells, and DAPI for all nuclei. A: immunohistochemistry images of Ki67, Pax7, DAPI, and all three merged of IGF-IS-treated soleus at R3D. B: control and IGF-IS-treated muscles have elevated levels of Pax7-positive cells compared with nonsuspended muscle samples throughout reloading, yet IGF-IA-treated muscles have reduced satellite cells at 3 days of reloading, and increased Pax7-positive cells at 14 days of reloading compared with control muscles. C: the number of Ki67-positive cells normalized to 100 fibers showed that IGF-IS-treated solei had significantly higher numbers of proliferating cells in the early reloading process. D: the number of proliferating satellite cells was determined by colocalization of Ki67 and Pax7 immunostaining. IGF-IA-treated muscles did not display elevated proliferating satellite cells in the early recovery period, unlike IGF-IS and untreated samples. Data are means ± SE for n = 3–4 samples. \( ^* \) P < 0.05 compared with control treatment at each time point; \( ^{**} \) P < 0.05 compared with the NS time point within the same treatment; \( ^{***} \) P < 0.05 between IGF-IA and IGF-IS at the same time point.
expression during reloading, and this decrease was more extensive than that seen in IGF-IA-treated muscles. However, at R14D, Atrogin1 expression increased above the levels found in nonsuspended muscles.

Last, we measured myostatin gene expression, which is a negative regulator of growth in skeletal muscles. No changes in expression were found in suspended muscles, but, during reloading, there was strong downregulation of myostatin at 7 and 14 days of reloading. As with Murf1, IGF-IS muscles showed decreased gene expression prior to IGF-IA or untreated muscles. Thus increased IGF-I tends to counter the atrogeone program during recovery from disuse atrophy, and IGF-IS is more efficient at blocking this pathway with more robust and accelerated changes in expression.

**Gene expression of IGFBPs was altered in IGF-IA- and IGF-IS-treated muscles.** The bioavailability of IGF-I is regulated in part by IGF binding proteins (IGFBPs). IGFBPs can stabilize IGF-I in the circulation and in the extracellular matrix to prolong its half-life. The IGFBP affinity for IGF-I can differ across all treatment conditions. The IGFBP-3, IGFBP-4, and IGFBP-5 during suspension and reloading across different treatment groups to determine whether IGFBP expression changes could be responsible for the enhanced actions of IGF-IS on restoration of mass and function.

Expression of Igfbp3 differed across all treatment conditions. The IGF-IA-treated gastrocnemius showed an elevated Igfbp3 expression at all time points with significant increases in Igfbp3 expression in nonsuspended and suspended samples compared with control and IGF-IS-treated muscles (Table 4). In contrast, IGF-IS-treated muscles exhibited higher Igfbp3 expression only early in the reloading phase (R3D and R14D). Finally, untreated muscles showed a significant upregulation in Igfbp3 expression at R7D and R14D.

The untreated muscles exhibited no alteration in Igfbp4 expression throughout the time points, but the IGF-IA- and IGF-IS-treated muscles showed divergent changes in gene expression. IGF-IA-treated muscles had very low expression of Igfbp4 during reloading (R3D and R14D). However, even though IGFBP-3, IGFBP-4, and IGFBP-5 during suspension and reloading and across different treatment groups to determine

### Table 3. Fold change in gene expression of atrophy related genes

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Treatment</th>
<th>NS S R3D R7D R14D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbxb40</td>
<td>Control</td>
<td>1.06 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>1.87 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>1.11 ± 0.44</td>
</tr>
<tr>
<td>Atrogin1</td>
<td>Control</td>
<td>1.17 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>1.44 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>0.45 ± 0.29‡</td>
</tr>
<tr>
<td>Murf1</td>
<td>Control</td>
<td>1.14 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>0.67 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>1.39 ± 0.84</td>
</tr>
<tr>
<td>Myostatin</td>
<td>Control</td>
<td>1.09 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>0.92 ± 0.28</td>
</tr>
</tbody>
</table>

Data were collected from n = 3–4 mice for each treatment at each time point. All fold changes were relative to ΔCT values of NS control muscle group, and no fold change was represented as 1. Two-way ANOVA followed by Tukey’s multiple comparison test was utilized to determine significant difference. *P < 0.05 compared to control group at the same time point; †P < 0.05 compared to the NS time point within the group under same treatment; ‡P < 0.05 between IGF-IA and IGF-IS at the same time point.

### Table 4. Fold change in gene expression of IGFB binding proteins

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Treatment</th>
<th>NS S R3D R7D R14D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igfbp3</td>
<td>Control</td>
<td>2.04 ± 2.74</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>9.91 ± 2.53*</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>1.03 ± 0.27‡</td>
</tr>
<tr>
<td>Igfbp4</td>
<td>Control</td>
<td>1.10 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>0.73 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>0.008 ± 0.005‡‡</td>
</tr>
<tr>
<td>Igfbp5</td>
<td>Control</td>
<td>1.20 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>IGF-IA</td>
<td>1.23 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>IGF-IS</td>
<td>0.0009 ± 0.0003‡‡</td>
</tr>
</tbody>
</table>

Data were collected from n = 3–4 mice for each treatment at each time point. All fold changes were relative to ΔCT values of NS control muscle group, and no fold change was represented as 1. Two-way ANOVA followed by Tukey’s multiple comparison test was utilized to determine significant difference. *P < 0.05 compared to control group at the same time point; †P < 0.05 compared to the NS time point within the group under same treatment; ‡P < 0.05 between IGF-IA and IGF-IS at the same time point.
sion, then elevated during reloading. Thus the pattern of Igfbp expression differs greatly during suspension and reloading, and these changes are modulated in distinct ways by IGF-IA and IGF-IS treatment.

**DISCUSSION**

The overarching goal of this study was to compare the efficacy of IGF-IS and IGF-IA in preventing disuse atrophy and enhancing muscle recovery following loss of muscle mass and function. We found that neither IGF-I form attenuated the decrease in muscle mass in the hindlimb suspension model for disuse atrophy. However, IGF-IS treatment maintained normal force-generating capacity following 1 wk of suspension. Both IGF-I forms accelerated the return of muscle mass during reloading, although IGF-IS caused a more rapid increase in mass than IGF-IA. Importantly, IGF-IS treatment continued to maintain muscle strength throughout the 2-wk observation of recovery compared with both untreated or IGF-IA-treated muscles, where force output did not fully recover to initial levels even at 14 days of reloading. These results strongly support that mature IGF-I, which is the only IGF-I form produced by the IGF-IS construct, is superior to the IGF-IA construct, which produces predominantly glycosylated and nonglycosylated pro-IGF-I for promoting functional recovery from disuse atrophy.

In a previous study, we found that the form of IGF-I modulated IGF-I receptor phosphorylation in cell-based assays (12). Specifically, mature IGF-I and pro-IGF-I possessed equivalent activity, whereas glycosylation of pro-IGF-I reduced activity. This raised the possibility that, in vivo, the presence of glycosylation might also impair ligand-receptor interaction. Complementary to the activity of each IGF-I form is its ability to be stored in the extracellular matrix through association with IGFBPs or matrix proteins. Recent evidence suggests that the E-peptide is important for IGF-I storage (20), raising the alternative scenario where loss of the E-peptide might impair bioavailability of IGF-I. The current study addresses this issue by removing the COOH-terminal E-peptide from IGF-I, thereby preventing the occurrence of any N-glycosylation. If the absence of the glycosylated E-peptide increases activity, then one would predict that IGF-IS provides the best recovery from disuse atrophy. However, if the E-peptide were required for boosting storage in the matrix and improving bioavailability, then IGF-IA would be the optimal forms to deliver to aid in recovery from disuse atrophy. Our results demonstrate a clear advantage for mature IGF-I in promoting functional gains in mass following disuse atrophy, suggesting that, while glycosylation may be important for storing IGF-I in the matrix, it impairs activity. We suggest that the glycosylated E-peptide must be removed prior to efficient receptor binding through extracellular proteases. Consistent with this hypothesis, we found an accumulation of mature IGF-I during the reloading phase of our study in the IGF-IA-treated muscles, potentially arising from cleavage of the stored IGF-IA. Although we cannot confirm this is the case with the constructs utilized for this study, future experiments where pro-IGF-I cleavage and/or glycosylation is prevented would clarify whether additional forms can enhance recovery from disuse atrophy or whether mature IGF-I is the optimum ligand for these actions.

Additional storage of IGF-I is achieved through the IGFBPs, and we investigated expression changes in a subset of this family of proteins to determine whether differential regulation contributed to IGF-I actions in disuse or reloading. During suspension, IGF-IS treatment was associated with a dramatic reduction in Igfbp4 and Igfbp5 expression, whereas IGF-IA caused an increase in Igfbp3 expression. These results show that the benefits of IGF-IS to function during hindlimb suspension were not associated with an increase in IGFBP levels. During recovery, the most consistent and notable changes were in IGF-IA-treated samples, where Igfbp4 and Igfbp5 expression decreased substantially, and in IGF-IS-treated samples, where Igfbp4 increased at all time points. Free IGF-I is stable for only 15 min in the circulation (16), and requires association with IGFBP3 or IGFBP1 and the acid-labile subunit in a ternary complex to prolong half-life. In tissue, the job of stabilization falls to other members of the binding protein family, including IGFBP4–6. The differential regulation of expression during reloading, particularly of IGFBP4, suggests that it is needed to stabilize mature IGF-I, but not IGF-IA. Although the affinity of each IGF-I form for the IGFBPs is not known, this model is consistent with the recent findings described earlier, where the E-peptide might be sufficient to store IGF-I in the matrix without the aid of IGFBPs. Although beyond the scope of the current study, future experiments could incorporate IGF-I forms with reduced binding protein affinity, such as Long-R3-IGF-I (37), to determine the extent to which binding protein stabilization contributes to the enhanced recovery afforded by mature IGF-I.

In contrast to the progrowth effects of IGF-I, the atrogene expression differs greatly during suspension and reloading, although IGF-IS caused a more rapid increase in expression at IGF-I, we examined the proportion of activated satellite cells during reloading. In control and IGF-IS treated muscles that had not undergone suspension, the most consistent and notable changes were in atrogenes at all time points. Free IGF-I is stable for only 15 min in the circulation (16), and requires association with IGFBP3 or IGFBP1 and the acid-labile subunit in a ternary complex to prolong half-life. In tissue, the job of stabilization falls to other members of the binding protein family, including IGFBP4–6. The differential regulation of expression during reloading, particularly of IGFBP4, suggests that it is needed to stabilize mature IGF-I, but not IGF-IA. Although the affinity of each IGF-I form for the IGFBPs is not known, this model is consistent with the recent findings described earlier, where the E-peptide might be sufficient to store IGF-I in the matrix without the aid of IGFBPs. Although beyond the scope of the current study, future experiments could incorporate IGF-I forms with reduced binding protein affinity, such as Long-R3-IGF-I (37), to determine the extent to which binding protein stabilization contributes to the enhanced recovery afforded by mature IGF-I.

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In contrast to the progrowth effects of IGF-I, the atrogenes are known to be negative regulators of growth, acting to increase protein degradation and to counter IGF-I signaling (17). Surprisingly, we did not observe any significant changes in atrogenes expression associated with hindlimb suspension, even though previous reports clearly show upregulation of these proteins driving atrophy. This may be due to the relatively brief period in which hindlimb suspension was performed, or that the change in expression happens transiently within a few days of disuse, where we did not measure expression. However, a definitive downregulation of all atrogenes occurred in the IGF-IS- and IGF-IA-treated samples during reloading, compared with both control samples at the same time points, and treated muscles that had not undergone suspension. This suggests that IGF-I production combined with reloading turns off the atrogenes pathway, and this may be beneficial to accelerating recovery. Further, IGF-IS caused reduced expression of these genes at earlier time points than IGF-IA (R3D and R7D vs. R14D), which reflects the enhanced activity of IGF-IS during reloading.

IGF-I is known to act both on muscle fibers and on activated satellite cells to drive hypertrophy (8), and there is an ongoing debate as to whether either target is required for increasing muscle mass. For instance, in adult mice where satellite cell ablation was achieved through Pax7 Cre induction (24), hypertrophy following synergist ablation was not affected. Further, in mice harboring dominant negative IGF-IR specifically in striated muscle (14), muscles also respond to load normally and increase muscle mass following synergist ablation (30). To address the role of satellite cells in recovery from disuse atrophy in our model, we examined the proportion of activated and total satellite cells during reloading. In control and IGF-
IS-treated muscles there was a rapid and robust increase in proliferating satellite cells. IGF-IA-treated muscles also exhibited a similar increase, but the changes were delayed with respect to the other treatment groups. While this demonstrates an increase in satellite cell activation associated with the return of mass and function during reloading, it does not explain the enhanced actions of IGF-IS. This is because control and IGF-IS treatment groups exhibit virtually identical satellite cell activation patterns yet completely different rates of functional recovery. Therefore, we assert that satellite cells are not the cause of the enhanced recovery from disuse atrophy afforded by IGF-IS.

One weakness in this study is that not all measurements were performed on a single muscle group. Many of our measures were obtained from the soleus muscle, including muscle mass and function, fiber number, size and type, and satellite cell assessment. As a postural muscle, the soleus exhibits a strong response to disuse atrophy, and exhibits significant weakness upon reloading (e.g., see Refs. 31 and 34). However, because muscle stimulation can alter signaling pathways (4), and the use of the soleus muscle for morphological measurements precluded its use for biochemical assays, we relied on the TA and gastrocnemius muscles, which exhibited similar mass changes during unloading and reloading, to examine alterations in gene expression, IGF-I content, and downstream signaling pathways associated with IGF-I receptor activation. Therefore, these measurements reflect the processes associated with disuse atrophy and recovery in the entire hindlimb, but may not provide an absolute representation of the soleus response. For example, the TA muscle showed increased P-Akt levels with IGF-IA and IGF-IS treatments, but the signal did not diminish with suspension. While this result is consistent with the lack of increase in Fbxo40 levels found in the gastrocnemius, the maintenance of P-Akt could also be due to ongoing muscle activity in the TA muscle during suspension.

Ultimately, the primary goal of any rehabilitative therapy is to regain muscle strength. In the current study, IGF-IS protected the muscles from weakness not only during unloading, but also during reloading, which is when muscle succumbs to the greatest stress. This may be due to the increased availability of this form of IGF-I, where it lacks the capacity to be stored in the matrix. As a result, the well-characterized pathways driving cell survival and protein synthesis are more active and protect against the loss of contractile proteins. Alternatively, there may be distinct features of IGF-IA activation that may prevent full functional rescue of muscle, in contrast to the actions of IGF-IS, and these may be actions associated with the E-peptide portion of the growth factor. In our previous study, we found that the inclusion of the E-peptides regulates a subset of genes in an IGF-I receptor independent manner, including matrix-metalloproteinase 13, which can accelerate myoblast migration (23). In contrast, expression of other genes, such as the survival factor BclXL, were enhanced by the presence of the E-peptides, yet, without functional IGF-I receptors, the response was ablated regardless of which IGF-I form was expressed (7). Thus, if the functional improvement in the current study is IGF-I form specific rather than a matter of level of activity and/or bioavailability, then future studies could identify which differences are important for function. Because muscle function is modulated by many different factors, including calcium handling, contractile protein density, neuro-muscular junction patency, and extracellular matrix composition, it is challenging to know a priori which pathway may underly improved strength after IGF-IS treatment. However, the expression profiling data we previously collected may hold clues worth pursuing. For instance, even though 91 genes changed expression similarly in IGF-IS- and IGF-IA-treated muscles, only 7 transcript changes were unique to expression of IGF-IS, and an additional 52 genes were altered only by IGF-IA (7). Thus genes whose change in expression modulates function could be identified within the transcripts that are unique to each IGF-I treatment.

Disuse atrophy and recovery are two of many potential arenas where IGF-I can provide functional benefit for muscle. Whether mature IGF-I is the optimal form of this growth factor for all therapeutic applications is not clear. For instance, in our previous work, IGF-IA drove more hypertrophy both in young and mature animals than mature IGF-I or the alternative splice form, IGF-IB (6, 7). However, if IGF-I actions are the result of initial bioavailability and differential release of local stores, then several factors will ultimately contribute which form is best for any given context, including sarcopenia and genetic disease (35). Given the large number of IGF-I forms produced by alternative splicing and posttranslational processing, combined with multiple binding proteins for stabilization and proteases for release, there is a long road ahead to identify the ideal IGF-I agent for every muscle therapy.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.P., B.K.B., M.L., and J.M.S. performed experiments; S.P., B.K.B., M.L., and J.M.S. interpreted results of experiments; S.P. and E.R.B. analyzed data; S.P. and E.R.B. edited and revised manuscript; E.R.B. conceived and designed research.

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