Viral expression of insulin-like growth factor I E-peptides increases skeletal muscle mass but at the expense of strength

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Submitted 6 January 2014; accepted in final form 21 February 2014

Brisson BK, Spinazzola J, Park S, Barton ER. Viral expression of insulin-like growth factor I E-peptides increases skeletal muscle mass but at the expense of strength. Am J Physiol Endocrinol Metab 306: E965–E974, 2014. First published February 25, 2014; doi:10.1152/ajpendo.00008.2014.—Insulin-like growth factor I (IGF-I) is a protein that regulates and promotes growth in skeletal muscle. The IGF-I precursor polypeptide contains a COOH-terminal extension called the E-peptide. Alternative splicing in the rodent produces two isoforms, IA and IB, where the mature IGF-I in both isoforms is identical yet the E-peptides, EA and EB, share less than 50% homology. Recent in vitro studies show that the E-peptides can enhance IGF-I signaling, leading to increased myoblast cell proliferation and migration. To determine the significance of these actions in vivo and to evaluate if they are physiologically beneficial, EA and EB were expressed in murine skeletal muscle via viral vectors. The viral constructs ensured production of E-peptides without the influence of additional IGF-I through an inactivating mutation in mature IGF-I. E-peptide expression altered ERK1/2 and Akt phosphorylation and increased satellite cell proliferation. EB expression resulted in significant muscle hypertrophy that was IGF-I receptor dependent. However, the increased mass was associated with a loss of muscle strength.

EA and EB have similar effects in skeletal muscle signaling and on satellite cells, but EB is more potent at increasing muscle mass. Although sustained EB expression may drive hypertrophy, there are significant physiological consequences for muscle.

IGF-I; E-peptides; skeletal muscle; hypertrophy; adeno-associated viral vectors

Insulin-like growth factor I (IGF-I) has endocrine, autocrine, and paracrine activity through its release into the circulation by the liver, and its local production and secretion by many tissues, including skeletal muscle. Virtually all cells respond to IGF-I, and muscle is no exception. When the IGF-I receptor (IGF-IR) is specifically blocked in skeletal muscle, mass is 10–30% reduced (19), and increased IGF-I in muscle causes hypertrophy (1, 10, 15, 34). Muscle mass is modulated in the muscle fibers through protein synthesis and also through activation of quiescent stem cell-like cells called satellite cells. Signals for growth and repair cause satellite cells to proliferate, migrate to sites of damage, and differentiate by fusing with myofibers, leading to resolution of damage and improved muscle function (21).

The general consensus is that all IGF-I activity is mediated by mature IGF-I, but IGF-I is synthesized as a precursor protein (26). The full-length precursor, pro-IGF-I, contains a signal peptide, mature IGF-I, and the E-peptide (Fig. 1A). After signal peptide removal in the endoplasmic reticulum (ER), the polypeptide including IGF-I and the E-peptide is called pro-IGF-I. Proprotein convertases can cleave pro-IGF-I, resulting in mature IGF-I and a free E-peptide (17). Pro-IGF-I has also been found in cell culture media and in vivo in serum (9, 18, 39, 43), indicating that cleavage is not required for secretion. Furthermore, pro-IGF-I activates IGF-IR as well as mature IGF-I in cell-based assays (18). Thus, there are additional biologically active forms of IGF-I.

In addition to posttranslational processing producing different IGF-I forms, alternative splicing of Igf1 also generates distinct isoforms (Fig. 1A). In the rodent, there are two isoforms, IGF-IA (IA) and IGF-IB (IB) (11, 41) (Fig. 1A and B). Splicing alters only the E-peptide sequence, giving rise to EA and EB peptides from IA and IB. The predominant Igf1 mRNA isoform expressed is Igf1a, comprising 90–99% of the transcripts, and it is the most conserved across all species examined (28, 42).

Studies have investigated E-peptide activity in muscle cell culture, and EB has been shown to increase the proliferation and migration of myoblasts and has been implicated in satellite cell activation. Some of these activities were apparent even when a neutralizing antibody to IGF-IR was present (4, 25, 32, 44), suggesting that EB activity is IGF-I independent. However, our findings show that these activities depend on IGF-IR signaling and localization (13). Furthermore, a recent exhaustive investigation of EB activity in myoblast cultures found virtually no response to EB treatment (22). Accordingly, it is necessary to determine whether the E-peptides have activity independent of IGF-I and IGF-IR signaling or if they modify IGF-I activity.

E-peptide activity has not been extensively evaluated in vivo. Our laboratory has sought to compare the IGF-I isoforms in muscle and found that expressing IA, IB, or IStop (mature IGF-I only without E-peptide, ISt) via adeno-associated virus (AAV) vectors in mouse hindlimbs, causes different degrees of hypertrophy (7, 8). However, it is difficult to discern how much of that activity is due to the E-peptides, as the constructs produce IGF-I in addition to the E-peptides, and the activity of IGF-I could overshadow any E-peptide effects.

In this study, we directly compared EA and EB expression in vivo without expressing functional IGF-I. We took advantage of a naturally occurring mutation in humans [valine 44 to methionine (V44M)], which renders the IGF-I nonfunctional (16), but ensures normal production of the E-peptides at a heightened level. The primary goal of this study was to evaluate the physiological impact of E-peptide activity in skeletal muscle and to understand the mechanisms underlying these actions.

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Materials and Methods

IGF-I V44M plasmids. IGF-I isoform constructs in pRRES plasmids were previously described (36). The plasmids were mutated at valine 44 to methionine by site-directed mutagenesis (QuikChange II; Stratagene, La Jolla, CA). The IGF-I V44M constructs were then subcloned into pAAV-CB plasmids for vector generation (Fig. 2A).

Cell transfection. Reverse transient transfection of 3T3 cells (ATCC, Manassas, VA) with pRRES plasmids containing IGF-I isoform constructs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Controls included transfection of empty vector (GFP). Twenty-four hours after transfection, conditioned medium from transfected 3T3 cells was used to stimulate IGF-I isoform-overexpressing fibroblasts (P6 cells), and IGF-I phosphorylation was measured using a kinase receptor activation ELISA assay and normalized to GFP protein expression (immunoblot not shown) to control for transfection efficiency. GFP, transfection with empty plasmid. Bars represent means ± SE; n = 3 replicates. *P < 0.05 for comparisons between all IGF-I constructs and GFP only; †P < 0.05 wild-type vs. V44M constructs via 1-way ANOVA and Bonferroni post hoc test. D: conditioned medium was concentrated and subjected to immunoblotting for IGF-I.

Viral injections. All experiments were approved by the University of Pennsylvania animal care committee. AAV serotype 2/8 vectors expressing the IGF-I V44M isoforms were generated by the Children’s Hospital of Philadelphia Vector Core. Solutions containing 5 × 10^10 viral particles diluted in 50 μl of phosphate-buffered saline (PBS) were injected into the anterior compartment of the lower hindlimbs of anesthetized C57Bl/6j (C57) and Mkr (19) mice, targeting the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. For IA and IB V44M-injected mice, the right limb was injected with ISt V44M to serve as a contralateral control. In addition, a cohort of mice was injected with ISt V44M in one limb and needle prick only (no injection) in the other. After injection, mice were housed in the animal facility until time of analysis. They were euthanized 1 wk, 1 mo, or 3 mo after injection. The TA muscles were dissected and rapidly frozen in liquid nitrogen for biochemical analysis, while the EDL muscles were subjected to force measurements (described below).

Quantitative RT-PCR. Total RNA was isolated from frozen TA muscles using TRIzol (Invitrogen). Equal amounts of total RNA from each sample were subjected to single-strand reverse transcription (Applied Biosystems, Foster City, CA). The resultant cDNA was utilized for quantitative real-time PCR with oligonucleotides specific for genes listed in Table 1, using the Applied Biosystems 7300 Real-Time PCR System, and reagents (Power SYBR Green PCR Master Mix). All samples were loaded in duplicate in 96-well plates. Expression of 18S was used to control for cDNA content.

IGF-I ELISA. Total IGF-I content in muscle protein extracts was determined by a standard sandwich ELISA protocol using commercially available kit (MG100; R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations and as previously described (7, 36).

Immunoblotting. Tissues and cells were processed for immunoblotting as previously described (18). Media from transfected cells were concentrated 10-fold using microcentrifugal filters (Microcon; Millipore, Bedford, MA) supplemented with protease inhibitors (P8340; Sigma, St. Louis, MO) and then subjected to immunoblotting. The following primary antibodies were used for the immunodetection: IGF-I (AF791; R&D Systems); EA (13); EB (38); GAPDH (sc-22333; Santa Cruz Biotechnology, Santa Cruz, CA); p-Akt (no. 9271) total Akt (no. 2920), p-ERK1/2 (no. 9101), total ERK1/2 (no. 9102), and GAPDH (no. 2955) (Cell Signaling, Beverly, MA).

Immunohistochemistry. Frozen cross-sections (10 μm) from the midbelly of EDL muscles were stained for laminin (rabbit Ab-1; NeoMarkers, Fremont, CA) or dystrophin (VP-D505; Vectorlabs, Burlingame, CA) to outline the muscle fibers. Fibers were fiber typed with antibodies recognizing myosin heavy chain (MHC) 2A (SC-71), MHC 2B (BF-F3), and MHC 1/β (BAF-8) (Developmental Studies Hybridoma Bank, Iowa City, IA). Myofiber area was measured in EDL sections (all fibers measured, ~1,000 fibers per muscle) at ×100 magnification. For satellite cells, sections were first dried at room temperature and then fixed in 2% formaldehyde for 10 min. Slides underwent dehydration and rehydration in ethanol, permeabilization for 10 min in 0.5% Triton X-100, boiling for 20 min in 10 mM sodium citrate, followed by staining using the mouse-on-mouse staining kit (Vector Labs) and antibodies toward Pax7 (MAB1675, R&D Systems) and Ki67 (ab15580; Abcam, Cambridge, MA). Nuclei were counterstained with DAPI. Stained sections were visualized on a Leica DMR microscope, and digital images were analyzed using OpenLab software (Improvision; PerkinElmer, Waltham, MA).

Analysis of muscle contraction. EDL muscle strength was analyzed as previously described (33), and CSA was measured as in Ref. 14. Briefly, the EDL muscles were removed and placed in a bath of Ringer solution gas equilibrated with 95% O_2-5% CO_2 and subjected to isolated muscle mechanical measurements using the Aurora Scientific In Vitro Muscle Test System. After determination of optimum length by single supramaximal twitch stimulation, maximum isometric tetanus was measured using a series of three 120-Hz 500-ms stimulation trains separated by 5 min.

After force measurements were completed, the muscles were removed from the bath, blotted, weighed, pinned at optimum length, surrounded by OCT embedding compound (TissueTek; Sakura Finetek USA, Torrance, CA), and rapidly frozen in melting isopentane. Muscles were stored at −80°C for subsequent analysis.

Glucocorticoid atrophy. Two weeks after AAV injection, mice were treated with dexamethasone (DEX; D2915 Sigma) as described previously (6) for 14 days in drinking water. Effectiveness of treatment was verified by measurement of body and spleen weight, which decrease with glucocorticoid treatment.

Statistical analysis. All data were analyzed via Student’s t-tests (paired and unpaired), or one-way ANOVA followed by a Tukey post hoc test. Statistical significance was accepted at P < 0.05.
RESULTS

To express the E-peptides in their native form without increasing the levels of mature IGF-I, we generated IGF-I constructs harboring the V44M mutation (16), which is conserved in murine \( \text{Igf1} \). We mutated murine IGF-I expression vectors (36) to generate V44M forms of IA, IB, and ISt, where ISt lacked any E-peptide (Fig. 1, A and B). To confirm that rodent IGF-I V44M could not activate IGF-IR, we transfected all constructs into 3T3 cells and used the conditioned medium for IGF-IR-specific kinase receptor activation assays (13) (Fig. 1C) and immunoblotting to IGF-I (Fig. 1D). While wild-type IA, IB, and ISt constructs led to robust IGF-IR phosphorylation, the V44M constructs failed to activate IGF-IR, even though all constructs led to increased IGF-I in the medium (Fig. 1D). Thus, IGF-I V44M is effectively nonfunctional at IGF-IR activation.

To express the E-peptides in vivo, we generated self-complementing AAV (31) harboring the V44M cDNAs (Fig. 2A). Anterior hindlimbs of young (2–3 wk old) C57 mice were injected with AAV-IGF-I V44M isoforms IA, IB, or ISt, targeting TA and EDL muscles. Expression of the different isoforms was confirmed by quantitative real-time PCR, and the expression of endogenous \( \text{igf1} \) was not affected by the any of the V44M isoforms (Fig. 2, B–E, and Table 1). One month after injection, all three V44M IGF-I isoforms produced more IGF-I than uninjected limbs when measured by ELISA (Fig. 2F).

Table 1. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Location</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV IstFwd</td>
<td>5'-ctacaaagcagctaggagc-3'</td>
<td>Spans 3' end of mature ( \text{Igf1} ) coding sequence, stop codon, and insertion site in AAV plasmid</td>
<td>Igf1st isoform from AAV transgene</td>
</tr>
<tr>
<td>AAV IstRev</td>
<td>5'-aggtcaagggcgacag-3'</td>
<td>AAV plasmid poly(A)</td>
<td></td>
</tr>
<tr>
<td>AAV IaFwd</td>
<td>5'-agactcaagggagaaacac-3'</td>
<td>3' region in exon 6 exclusive to isoform ( \text{Igf1a} )</td>
<td>( \text{Igf1a} ) isoform from AAV transgene</td>
</tr>
<tr>
<td>AAV IaRev</td>
<td>5'-ggccagctgctgctgtaaa-3'</td>
<td>AAV plasmid poly(A)</td>
<td></td>
</tr>
<tr>
<td>AAV IbFwd</td>
<td>5'-ttcctagacaaacagagaaag-3'</td>
<td>Exon 5</td>
<td>( \text{Igf1b} ) isoform from AAV transgene</td>
</tr>
<tr>
<td>AAV IbRev</td>
<td>5'-gtgtagctagagtctagcag-3'</td>
<td>AAV plasmid poly(A)</td>
<td></td>
</tr>
<tr>
<td>Igf1 Fwd</td>
<td>5'-gaagttcagctagacag-3'</td>
<td>5' region in exon 6 common to ( \text{Igf1a} ) and ( \text{Igf1b} )</td>
<td>Endogenous ( \text{Igf1} )</td>
</tr>
<tr>
<td>Igf1 Rev</td>
<td>5'-gctagtttctgctgctgctgctg-3'</td>
<td>5'fl 3'-UTR</td>
<td></td>
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</tbody>
</table>
significant effect on the PI3K/Akt pathway, IB V44M expression resulted in a twofold increase in p-ERK1 and p-ERK2, respectively. Mutant IGF-I. However, IA V44M expression caused significant increases in p-Akt levels, further supporting the lack of activity of this mutant IGF-I in serum and tissues (9), which was not immunoreactive with anti-EA or anti-EB. IA V44M had multiple bands from 11–18 kDa detected by both anti-IGF-I and anti-EA. These bands represent pro-IA, and glycosylated pro-IA (5, 18). A single 14-kDa band in the IB V44M lane was detected with anti-IGF-I and anti-EB and represents pro-IB. Neither the ~3–6 kDa free E-peptides nor the mature IGF-I produced from cleavage of pro-IGF-I were detectable by immunoblotting, consistent with the normal pattern of IGF-I forms in skeletal muscle (18). Based on the sizes and antibody reactivity, AAV IGF-I V44M injections produced the correct IGF-I isoforms. Localization of E-peptides in injected muscles was determined by immunohistochemistry with anti-EA and anti-EB (Fig. 2F). EA and EB were apparent in IA V44M and IB V44M sections, respectively. Staining was restricted to within muscle fibers and was not found in the extracellular matrix (ECM). Staining of EA and EB was not detected in ISt V44M expressing muscles.

We previously reported (13) that the E-peptides modulate muscle cell signaling that relies on the IGF-IR. Namely, E-peptides increase MAPK signaling but do not alter the PI3K/Akt pathway in myoblast cell culture, which are both downstream from IGF-IR activation. To test whether the E-peptides also activated these pathways in vivo, muscles were harvested 1 wk after AAV injection (Fig. 3). Compared with sham-injected muscles, IST V44M did not alter p-ERK1/2 or p-Akt levels, further supporting the lack of activity of this mutant IGF-I. However, IA V44M expression caused significantly elevated p-ERK1/2 compared with IST V44M, with 8- and 3-fold increases in p-ERK1 and p-ERK2, respectively. Similarly, IB V44M expression resulted in a twofold increase in p-ERK1/2. Most interestingly, while IA V44M had no significant effect on the PI3K/Akt pathway, IB V44M expression resulted in a reduction of p-Akt compared with ISt V44M. Thus, both E-peptides modulated the MAPK and PI3K/Akt pathways in vivo.

To assess the physiological effects of E-peptide expression, the same study design was employed as with signaling measurements, but muscles were analyzed 1 mo postinjection. We examined all V44M constructs, where mass, cross-sectional area (CSA), and isometric force were analyzed in the EDL muscles (Table 2 and Fig. 4, A, E, and I). IA V44M expression did not increase muscle mass significantly, but improved muscle strength compared with ISt V44M-injected muscles in the same animal. In contrast, IB V44M expression resulted in a 5–6% increase in mass and CSA, with a commensurate increase in force production and similar specific forces to ISt V44M injected muscles. Thus, at 1 mo, both E-peptides were driving beneficial effects on mass and/or function. ISt V44M-injected limbs were not significantly different from un.injected limbs in any of the parameters analyzed, although wild-type ISt caused an ~16% increase in muscle mass (Fig. 5A), confirming that mature IGF-I V44M was nonfunctional in vivo.

To determine the persistence of these effects, we analyzed muscles 3 mo after injection (Table 2 and Fig. 4, B, F, and J). Here, ISt V44M-injected EDLs were larger than ISt V44M-injected contralateral muscles, with a significant increase in CSA (~6%; Fig. 6F). The effects of IB V44M expression also continued to build, with a 10% CSA and over 6% mass increase compared with ISt V44M (Fig. 4, B and F). However, IB V44M expression was associated with reduced function at this time point, where injected EDLs were weaker than controls in both tetanic and specific force (Table 2 and Fig. 4J). Thus, although the muscles were larger, sustained EB expression negatively affected muscle strength.

We had previously observed that mature murine muscle did not respond to viral expression of wild-type IGF-IB, which we presumed was due to the need for an active satellite cell pool for this isoform to promote hypertrophy (7). To address whether this was also required for the E-peptide actions, we injected aged wild-type mice (1 yr old) with the V44M AAV constructs. No isoform had an effect on any of the parameters measured (Table 2 and Fig. 4, C, G, and K), showing that only young muscle responded to E-peptides at 1 mo postinjection.

In cell culture, EB requires a functional IGF-IR to increase myoblast MAPK signaling, proliferation, and migration (13). Accordingly, we sought to determine if EB activity in vivo,
namely muscle hypertrophy, also requires IGF-IR. We injected young MKR mice, which express a dominant negative IGF-IR in mature skeletal muscle, for 1 mo (20). In muscle without functional IGF-IR, neither EA nor EB had any effects on muscle size or strength (Table 2 and Fig. 4, D, H, and L). Consistent with our culture data (13), E-peptide activity in vivo requires functional IGF-IR.

Muscle mass can increase either through changes in muscle fiber size, or in fiber number. To determine how the E-peptides affected mass, sections from EDL muscles were labeled with dystrophin to outline muscle fibers (Fig. 6A), allowing for total fiber number enumeration, and measurement of the cross-sectional area of each fiber. Neither ISt V44M (Fig. 5B) nor IA V44M affected fiber area, but IB V44M expression caused a rightward shift in the size distribution, resulting in a 20% increase in mean fiber size compared with ISt V44M-injected muscles (Fig. 6, B, C, and D). There was no apparent change in fiber number in any treatment group. To determine whether the change in fiber size was associated with a specific fiber type, the areas of MHC 1/β, 2A, and 2B fibers were measured. The average fiber size of 2B fibers was significantly larger following IB V44M expression than ISt V44M controls by 25%, but 1/β and 2A fibers were not significantly affected (Fig. 6E), indicating that EB-induced hypertrophy predominantly affects 2B fibers.

The fiber type-specific hypertrophy from IB V44M expression raised the possibility that the EB-peptide could also provide protection against MHC 2B fiber-specific atrophy. Glucocorticoids like DEX cause general atrophy in mice, but in muscle, 2B fibers are the most affected fiber type (2). Wildtype mice were injected with IB V44M in one limb and ISt V44M in the contralateral limb for 1 mo, and 2 wk before dissection, mice were treated with DEX or vehicle (Fig. 6F). DEX treatment caused a reduction in body, spleen, and EDL mass (31, 50, and 25%, respectively). IB V44M expression caused an ~5% increase in EDL mass in vehicle-treated mice. However, IB V44M expression did not increase mass of DEX-treated muscles. Both ISt- and IB V44M-injected limbs were smaller in DEX-treated mice; so, even though IB V44M can cause hypertrophy in healthy muscle, it cannot block glucocorticoid-induced atrophy.

EB has been reported to activate satellite cells and increase proliferation in culture (4, 25, 32, 44). To examine whether E-peptide expression altered satellite cell proliferation in vivo, satellite cell number (Pax7 positive), and proliferating satellite cells (Pax7 and Ki67 positive) were counted in EDL sections following IA, IB, or ISt V44M AAV injections (Fig. 7). ISt V44M expression did not affect satellite cell number or proliferation compared with un.injected controls. In contrast, both IA V44M and IB V44M caused an increase in the number of proliferating satellite cells 1 wk after injection (Fig. 7F). However, at 1 mo, there were fewer total satellite cells with no change in proliferation (Fig. 7C). By 3 mo of expression, satellite cell depletion was resolved, and no differences in satellite cell number or proliferation were observed (Fig. 7, D and G). Thus, satellite cells may contribute to the E-peptide effects on growth, but appear to do so only transiently.

**DISCUSSION**

Although it is straightforward to produce IGF-I in the absence of an E-peptide (8, 36), it has been more technically challenging to generate E-peptides in the absence of IGF-I so that their actions can be distinguished from that of the better-characterized growth factor. To overcome this hurdle, we utilized the V44M mutation to afford normal processing of the IGF-I precursor and block the activity of mature IGF-I, yet

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**Table 2. Masses, CSAs, and forces**

<table>
<thead>
<tr>
<th>AAV Injection</th>
<th>Uninjected</th>
<th>ISt V44M</th>
<th>IA V44M</th>
<th>ISt V44M</th>
<th>IB V44M</th>
<th>ISt V44M</th>
</tr>
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<tbody>
<tr>
<td><strong>Wild-type 1 mo</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>7.33 ± 0.38</td>
<td>7.44 ± 0.39</td>
<td>8.03 ± 0.43</td>
<td>8.10 ± 0.65</td>
<td>7.90 ± 0.24*</td>
<td>7.55 ± 0.26</td>
</tr>
<tr>
<td>CSA (mm²)</td>
<td>1.25 ± 0.02</td>
<td>1.20 ± 0.04</td>
<td>1.37 ± 0.06</td>
<td>1.41 ± 0.07</td>
<td>1.32 ± 0.05*</td>
<td>1.25 ± 0.05</td>
</tr>
<tr>
<td>Tetanic force (mN)</td>
<td>293.67 ± 7.43</td>
<td>319.26 ± 13.33</td>
<td>364.39 ± 29.45</td>
<td>361.96 ± 31.14</td>
<td>330.63 ± 21.47*</td>
<td>313.50 ± 19.06</td>
</tr>
<tr>
<td>Specific force (N/cm²)</td>
<td>23.32 ± 1.00</td>
<td>23.95 ± 0.66</td>
<td>26.02 ± 1.05*</td>
<td>25.06 ± 0.95</td>
<td>24.86 ± 0.84</td>
<td>25.04 ± 0.79</td>
</tr>
<tr>
<td><strong>Wild-type 3 mo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>ND</td>
<td>ND</td>
<td>9.61 ± 0.45</td>
<td>9.37 ± 0.42</td>
<td>9.43 ± 0.24*</td>
<td>8.85 ± 0.31</td>
</tr>
<tr>
<td>CSA (mm²)</td>
<td>ND</td>
<td>ND</td>
<td>1.58 ± 0.06*</td>
<td>1.50 ± 0.07</td>
<td>1.53 ± 0.05*</td>
<td>1.39 ± 0.06</td>
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<tr>
<td>Tetanic force (mN)</td>
<td>ND</td>
<td>ND</td>
<td>390.94 ± 22.41</td>
<td>388.78 ± 18.86</td>
<td>350.41 ± 17.57*</td>
<td>376.24 ± 18.24</td>
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<tr>
<td>Specific force (N/cm²)</td>
<td>ND</td>
<td>ND</td>
<td>24.63 ± 0.73</td>
<td>25.94 ± 1.01</td>
<td>22.94 ± 0.96*</td>
<td>27.07 ± 0.91</td>
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<tr>
<td><strong>Aged wild-type 1 mo</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>12.10 ± 0.45</td>
<td>12.17 ± 0.47</td>
<td>12.95 ± 0.44</td>
<td>12.97 ± 0.23</td>
<td>12.31 ± 0.40</td>
<td>12.14 ± 0.47</td>
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<tr>
<td>CSA (mm²)</td>
<td>ND</td>
<td>ND</td>
<td>1.89 ± 0.07</td>
<td>1.91 ± 0.04</td>
<td>1.79 ± 0.06</td>
<td>1.77 ± 0.05</td>
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<tr>
<td>Tetanic force (mN)</td>
<td>ND</td>
<td>ND</td>
<td>479.82 ± 6.54</td>
<td>481.55 ± 22.13</td>
<td>454.75 ± 10.18</td>
<td>450.55 ± 18.91</td>
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<tr>
<td>Specific force (N/cm²)</td>
<td>ND</td>
<td>ND</td>
<td>25.58 ± 0.95</td>
<td>25.23 ± 1.08</td>
<td>25.55 ± 0.52</td>
<td>25.57 ± 1.20</td>
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<tr>
<td><strong>MKR 1 mo</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>5.30 ± 0.99</td>
<td>5.38 ± 0.883</td>
<td>5.74 ± 0.44</td>
<td>5.83 ± 0.37</td>
<td>5.77 ± 0.31</td>
<td>5.70 ± 0.38</td>
</tr>
<tr>
<td>CSA (mm²)</td>
<td>ND</td>
<td>ND</td>
<td>0.94 ± 0.04</td>
<td>0.94 ± 0.05</td>
<td>1.01 ± 0.05</td>
<td>1.04 ± 0.09</td>
</tr>
<tr>
<td>Tetanic force (mN)</td>
<td>ND</td>
<td>ND</td>
<td>242.15 ± 20.60</td>
<td>247.79 ± 12.68</td>
<td>262.71 ± 21.09</td>
<td>255.10 ± 23.97</td>
</tr>
<tr>
<td>Specific force (N/cm²)</td>
<td>ND</td>
<td>ND</td>
<td>25.63 ± 0.95</td>
<td>26.43 ± 0.42</td>
<td>25.82 ± 0.83</td>
<td>24.44 ± 0.82</td>
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Data are means ± SE; n = 5–8 extensor digitorum longus (EDL) muscle pairs per injection. ND, not determined; CSA, cross-sectional area. *P < 0.05 for comparisons to ISt V44M contralateral control EDLs via paired t-tests.
Fig. 4. IA and IB V44M expression affects muscle size and strength. AAV IA or IB V44M EDL muscles were compared with IS V44M control EDLs. Percent change in mass (A–D), CSA (E–H), and Specific Force (I–L) is graphed. Young (2–3 wk old) wild type C57 mice dissected 1 (A, E, I) and 3 (B, F, J) mo after injection; Aged (1 yr old) wild-type mice dissected 1 mo after injection (C, G, K); MKR mice that do not express functional IGF-I in skeletal muscle, dissected 1 mo after injection (D, H, L). Bars represent means ± SE; n = 4–8 EDL muscle pairs per injection. *P < 0.05 vs. ISt V44M contralateral control EDLs via paired t-tests.

Fig. 5. ISt V44M does not affect muscle size. Uninjected EDLs were compared with IS V44M EDLs. A: percent change in mass. Bars represent means ± SE; n = 7–8 EDL muscle pairs per injection. *P < 0.05 vs. ISt V44M contralateral control EDLs via paired t-tests. B: sections of AAV V44M-injected EDLs were stained for dystrophin to outline muscle. Areas of all fibers in EDLs (~1,000 fibers on average per EDL) were measured, and histograms were generated representing the proportion of fibers at a certain size (n = 5 EDL muscle pairs).
found that the E-peptides accumulated inside the muscle fibers, not outside in the ECM. Because these sections are only a single time point, we cannot tell whether the intracellular localization represents a pool of the IGF-I forms that is destined for secretion from AAV transduced fibers or if it represents internalized E-peptide following binding to cell surface receptors. Our cell culture experiments confirm that the IGF-I forms, regardless of the mutation, can be secreted from cells. However, additional experiments are needed to clarify whether the same holds true in vivo.

We found that muscle hypertrophy by E-peptide expression relied on functional IGF-IR, similar to our results in vitro. How the E-peptides mediate their effects through this receptor is still unclear. Our recent work suggests that the E-peptides enhance the activity of IGF-I and that without any IGF-I present there are no E-peptide actions (13). Our results suggest that the E-peptides alter mature IGF-I binding to the receptor and do not affect the receptor in the absence of IGF-I. Although we only expressed the E-peptides, muscle stores IGF-I in the ECM, and there is also a constant source of IGF-I in the circulation. Thus, we cannot exclude the possibility that the E-peptides also rely on an endogenous source of IGF-I to mediate effects in vivo. Future studies could resolve this issue by combining viral expression of the E-peptides with animals lacking IGF-I (27).

The increase in mass after IB V44M expression was due to an increase in fiber size, and specifically in MHC 2B fiber size (Fig. 6). Even though average fiber size increased by 20%, whole muscle mass changed only by 5%. IGF-I treatment often has varying effects on mass vs. myofiber hypertrophy. While increased local expression of IGF-I leads to both enlarged muscle mass and fiber area (34), systemic delivery of recombinant IGF-I into wild-type mice leads to no significant change in muscle mass, but a 55% increase in average fiber area (29). The fiber type specificity of hypertrophy does not seem to depend on the exclusive expression of IGF-IR on 2B fibers, for the receptors are found on all fiber types (9). Rather, a simpler explanation is that the ability for MHC 2B fibers to change size more dynamically than other fiber types affords a greater response to pro-growth signals. Similarly, the fact that the EB expression failed to prevent glucocorticoid-induced atrophy also supports the notion that there is no fiber type specificity for EB activity.

Expression of the E-peptides also led to changes in the strength of the muscles. EDLs injected with AAV IA V44M were slightly stronger in specific force. IB V44M hypertrophy after 1 mo was coupled with a rise in maximum tetanic force, but after 3 mo, force-generating capacity was significantly reduced. This implies that long-term expression of EB has detrimental effects on muscle architecture, although there was still hypertrophy. The fiber type-specific hypertrophy combined with the loss of function is reminiscent of the effects of myostatin targeting, although to a lesser extent. Muscles from myostatin-null mice are much larger than wild-type muscles, but there is a reduction in muscle strength coupled by an increase in 2B fiber size and proportion and depletion of mitochondria (3). Although we did not see an increase in the proportion of 2B fibers, we did observe an increase in 2B fiber size.
size. One factor that may underlie diminished function in myostatin-null muscles is increased myonuclear domains. Diminished force may result when each myonucleus must support a greater region of the fiber accompanied by a proportional reduction in myosin content (40), although this is not always the case (12). In contrast, transgenic expression of wild-type IGF-1A exhibits hypertrophy but maintains myonuclear domain and myosin content. The possibility that IB V44M expression also led to increased myonuclear domains and reduced myosin content was not addressed in the current study. While the transient increase in proliferating satellite cells suggests that an early trigger for satellite cell activation occurs, this might not persist throughout the 3-mo duration of our study. If this is true, then the progressive hypertrophy promoted by IB V44M would not be associated with a supply of new myonuclei via satellite cell activation, resulting in increased myonuclear domains. Future studies of EB treatments at the single fiber level could help to determine if this mechanism is in play.

For almost all parameters, EB expression generated greater effects than EA, but both caused similar changes to the muscle. While we and others have shown that EB enhances MAPK signaling and proliferation in cell culture (13, 37, 44), there is contention in the field whether these results are reproducible (22, 30). One problem is that the effects are subtle, which is consistent with our results in this study, showing only a 5% increase in muscle mass in muscles expressing EB. To achieve this hypertrophy in vivo, we relied on viral production of this peptide, which affords very high levels of sustained expression after a single injection. Thus, whether the same mass increase can be achieved through other pharmacological strategies is not clear. However, an interesting point that is missed in many studies is the examination of the other rodent E-peptide, EA, which is rarely evaluated, even though Igf1a is the dominant Igf1 isoform. Whether the E-peptides have differential potency or completely different actions is still an open question. Although there is only 50% homology between the two peptides, a more significant difference may be the glycosylation of EA (5). We have recently reported that pro-IGF-IA is just as effective at receptor activation as mature IGF-I, but that glycosylation impairs its activity (18). Since viral expression of IA V44M produced both nonglycosylated and glycosylated EA, then it is quite possible that only the nonglycosylated peptide was acting in a similar manner to EB. The alternative splicing that occurs on the 3’ end of the Igf1 transcript may be a strategy to avoid E-peptide glycosylation. Future studies could test this through the removal of the glycosylation sites in the EA peptide.

In this study, we have extended our previous observations of E-peptide activity to address whether or not they have physiological significance for skeletal muscle. We found that the EB peptide, in particular, drives hypertrophy and that these pro-growth effects are dependent on the IGF-IR. Furthermore, only muscles from young growing mice respond to the E-peptides, suggesting that an active pool of satellite cells is also required their actions, since satellite cells in aged muscle, where the E-peptides had no effect, are mostly quiescent. If this is true, then future experiments should be performed that test this...
hypothesis. Muscles expressing the E-peptides could be injured to activate the satellite cell pool, or muscles could be reloaded after a period of disuse. Alternatively, satellite cell activation or proliferation could be inhibited in young mice expressing the E-peptides to confirm that the E-peptides act through affecting activated satellite cells. In this study, sustained expression of EB causes progressively more mass increase, and EA drives more modest changes in muscle size, but there is a loss of strength that appears 3 mo after viral injection of EB. Thus, the functional consequences of persistently increased EB expression may override the potentially beneficial pro-growth effects of this peptide. However, a possibility that has not been tested is the combined overexpression of EA and EB, where the negative impact of EB on function may be ameliorated in the presence of increased EA. Because both factors are normally present, the loss in force may be a consequence of losing the balance between these two peptides rather than an effect that is driven solely by EB. Additional combinations of E-peptide delivery with and without mature IGF-I will help to clarify their actions on mass and strength.

Based on our results, the therapeutic potential of E-peptides might be restricted to transient upregulation in muscle rather than continuous delivery. We show there is pro-growth potential, but there is ultimately compromised function. As such, strategies to boost E-peptides only during recovery from disuse atrophy or from damage could be beneficial. However, the focus on EB as a beneficial agent to prevent sarcopenia may be misplaced (23). Although wild-type IA expression can return normal function to aged muscle (10), we found that neither IB (7) nor EB expression leads to hypertrophy in aged muscles, and so they may require an activated satellite cell pool to have activity. In addition, we have recently shown that mature IGF-I aids in recovery after muscle reloading, more so than the IA isoform, further showing that the E-peptides may modulate IGF-I function (35). Thus, the clinical use of the E-peptides alone or in combination with IGF-I to boost muscle mass or strength should be carefully evaluated to ensure that the end effects are beneficial to patients.

ACKNOWLEDGMENTS

We are grateful to Zaozhen Tian for performing functional measurements.

GRANTS

This work was supported by National Institutes of Health Grants AR-057363 to E. R. Barton. B. K. Brisson was supported by a fellowship from the Pennsylvania Muscle Institute, U. of Pennsylvania, Philadelphia, PA (AR-053461).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B. K. B. and E. R. B. conception and design of research; B. K. B., J. M. S., and S. P. performed experiments; B. K. B., J. M. S., and S. P. analyzed data; B. K. B., J. M. S., and S. P. interpreted results of experiments; B. K. B., J. M. S., and S. P. prepared figures; B. K. B. drafted manuscript; B. K. B., J. M. S., and S. P. edited and revised manuscript.

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