

Increased Extraocular Muscle Strength with Direct Injection of Insulin-like Growth Factor-I

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PURPOSE. Previous work has demonstrated the effectiveness of insulin-like growth factor (IGF)-II in increasing force generation in extraocular muscle (EOM). Studies in the literature have suggested that IGF-I would be even more effective than IGF-II. This study was performed to assess the effects on muscle mass and force generation of IGF-I injection in adult rabbit superior rectus muscle.

METHODS. Adult rabbits received a single injection of IGF-I at one of several doses into one superior rectus muscle. One week after treatment, the rabbits were euthanized, and the superior rectus muscle from each orbit was removed. Force generation was measured using an *in vitro* apparatus, and injected muscles were compared with the contralateral control. A second group of animals were injected similarly, and the muscles were examined at 1 week for changes in cross-sectional area of individual myofibers.

RESULTS. EOMs demonstrate significant numbers of cells expressing the IGF receptor. After the EOMs were injected with IGF-I, there were significant increases both in muscle force generation and cross-sectional area at all doses tested in this study. Doses of 10 and 25 μg IGF-I were most effective.

CONCLUSIONS. Direct muscular injection of IGF-I effectively increases EOM force generation without the potential biomechanical hazards of surgery such as permanently altered muscle length or insertional position on the globe. (*Invest Ophthalmol Vis Sci.* 2006;47:2461-2467) DOI:10.1167/iovs.05-1416

The treatment of strabismus, a common disorder of binocular alignment, often entails surgery in which overacting muscles are weakened and underacting muscles are strengthened. The purpose of surgery, put most simply, is to reestablish binocular interaction and stereopsis. An extraocular muscle (EOM) may be weakened by recessing its insertion on the globe, thereby decreasing its lever arm. This often is combined with a "strengthening" procedure of the direct antagonist in which the muscle is permanently shortened by resection. Any true strengthening effect is likely to be short-lived¹ as the

muscle length adapts to some extent to optimize actin-myosin overlap.

Incisional surgery has significant potential drawbacks that may affect long-term success. These include scarring,² permanent biomechanical alterations in the EOMs and adnexa, and rotational changes in muscle-globe interaction that may adversely affect muscle function. In approximately 25% to 69% of patients, another surgery is necessary, particularly in those patients with a misalignment of greater than 30 prism diopters.^{3,4} For these reasons, there has been renewed interest in pharmacological agents that could modulate muscle force generation without permanently altering muscle length or insertional position on the globe.

Botulinum toxin type A, developed in the 1980s as an injectable drug, is administered directly into an overacting muscle to weaken it.⁵ Botulinum toxin use shows that such an approach is feasible. The application of botulinum toxin to strabismus, however, has been limited by a short duration of action and by the reality that it can only weaken muscle. Our laboratory has been expanding on the success of botulinum toxin by studying new, longer-lasting muscle weakening agents.⁶⁻⁸ Furthermore, we are beginning to investigate the ability of injected myogenic growth factors to strengthen EOM. Numerous agents could conceivably be used to weaken an EOM, but the development of drugs to increase EOM force generation will probably be critical to successful pharmacological treatment of strabismus.

In an earlier study, we showed that a single intramuscular injection of insulin-like growth factor (IGF)-II resulted in a significant, albeit short-lived, increase in EOM strength.⁹ Although muscle force generation increased after IGF-II injection, muscle mass did not significantly increase. The literature suggests that IGF-I would be a significantly more potent myogenic growth factor.^{10,11} This approach has been validated by a recent study in which both IGF-I alone and a cocktail of muscle-strengthening factors including IGF-I were injected into the orbit of developing chicks, resulting in significant increases in force generation.¹² The long-term goal is to develop sustained release approaches for strengthening the EOMs, and so it is essential to choose the most effective agent for these studies. In this study, we examine the efficacy of a single injection of one of several doses of IGF-I to increase force generation in the superior rectus muscles of adult rabbits.

MATERIALS AND METHODS

Adult New Zealand White rabbits were obtained from Bakkon Farms (Red Wing, MN) and housed with Research Animal Resources. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and were in compliance with the NIH and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rabbits were anesthetized by an intramuscular injection of 10 mg/kg ketamine and 2 mg/kg xylazine. IGF-I was injected intramuscularly into one superior rectus, randomized before surgery. Five doses were used: 1, 5, 10, 25, and 50 μg , in 100 μL sterile saline (R&D Systems, Minneapolis, MN); six rabbits were injected with each dose of

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Supported by Grants EY15313 and EY11375 from the National Eye Institute, the Minnesota Medical Foundation, the Minnesota Lions and Lionesses, a Research to Prevent Blindness (RPB) Lew Wasserman Mid-Career Development Award (LKM), and an unrestricted grant to the Department of Ophthalmology from RPB.

Submitted for publication November 2, 2005; revised January 19, 2006; accepted March 22, 2006.

Disclosure: **B.C. Anderson**, None; **S.P. Christiansen**, None; **S. Grandt**, None; **R.W. Grange**, None; **L.K. McLoon**, None

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IGF-I. The contralateral superior rectus muscle received an injection of an identical volume of sterile saline to serve as a control.

One week after injection, the rabbits were anesthetized with ketamine and xylazine. The chest was opened surgically, and the animals were euthanized by thoracotomy followed by exsanguination. The superior rectus muscles of both orbits were removed from scleral insertion to their origin in the orbital apex. The muscles were placed immediately into oxygenated Ringers solution at 30°C. The muscles were carefully pinned to their in situ length, based on previous measurements in situ, and 4-0 silk was tied to the end of each muscle. Loops of suture were used to suspend the muscles in the in vitro incubation chambers, with the upper loop attached to a lever arm and force transducer. The muscles were continually bathed in oxygenated Ringers at 30°C for the duration of the experiment. Generated force in grams was recorded on a computer (model 1205 Intact Muscle Test System and Dynamic Muscle Control software; Aurora Scientific, Aurora, Ontario, Canada). Force produced by a muscle is directly related to the size of the muscle (i.e., a bigger muscle will produce more force than a smaller muscle) but may also be due to intrinsic changes in the muscle independent of size. To account for force generation due to differences in muscle size, we calculated stress, which is force divided by muscle cross-sectional area. After muscle length and mass were obtained, muscle cross-sectional area was determined by dividing muscle mass (grams) by the product of muscle length (centimeters) times a muscle density of 1.056 g/cm³. This yielded stress in grams per square centimeter, which was then converted to millinewtons per square centimeter. If differences in stress are evident between the injected and noninjected muscles, it suggests that increased force generation was due to factors other than just increased muscle size.

Both control and IGF-treated superior rectus muscles were tested simultaneously. Supramaximum stimulation intensity was determined by increasing voltage until maximum contraction was achieved using square-wave pulses of 0.5-ms duration (model 701B bi-phase current stimulator; Aurora Scientific) and delivered to the muscles via flanking platinum electrodes. Isometric length-tension curves were determined by stimulating each muscle at supramaximum intensity (500 mA, 0.5 ms), while varying the preload (resting length) over a range of 0.5 to 10.0 g. The optimal preload was determined by incrementally increasing the resting muscle length to achieve maximum isometric twitch force, allowing 60 seconds of rest between stimuli. All further tests were performed with supramaximal stimulus intensities at optimal preload. After two stabilizing tetanic stimulations (150 Hz, 500 mA, 0.5 ms) with 2 minutes rest between stimuli and 5 minutes rest after two consecutive stimulations, force development was determined for single, double, and triple pulses (0.5-ms pulse duration) with 2 minutes of rest between stimuli. Muscles were stimulated at frequencies of 10, 20, 40, 100, 150, and 200 Hz at a train duration of 500 ms with a 2-minute rest between each stimulation. After 2 minutes rest, the muscles were subjected to a fatigue protocol as follows: A tetanic stimulus was delivered every 2 seconds, consisting of a 1-second train at 150 Hz. The muscles were stimulated for 600 seconds or until there was a 50% reduction in generated muscle force. Data from treated and control muscles were pooled at each postinjection interval and compared with the paired *t*-test. *P* ≤ 0.05 was considered statistically significant.

In a second set of 12 rabbits, 10, 25, or 50 μg IGF-I in 100 μL isotonic saline was injected into one superior rectus muscle, whereas the contralateral muscle was injected with an equal volume of saline only. One week after injection, these rabbits were euthanized with an overdose of barbiturate anesthesia, and both superior rectus muscles were removed, embedded in tragacanth gum and frozen in methybutane that had been chilled to a slurry on liquid nitrogen. Muscles were sectioned serially at 12 μm and processed for immunohistochemical visualization of the fast, slow, developmental, and neonatal myosin heavy chain isoforms (MyHC). For pan-fast and -slow MyHC (Novocastra, Newcastle, UK) the unfixed sections were incubated with antibody at a 1:40 dilution. For developmental and neonatal MyHC, the antibody (Novocastra) was used at 1:20 dilution. The sections were blocked for nonspecific binding with horse serum and avidin-biotin blocking re-

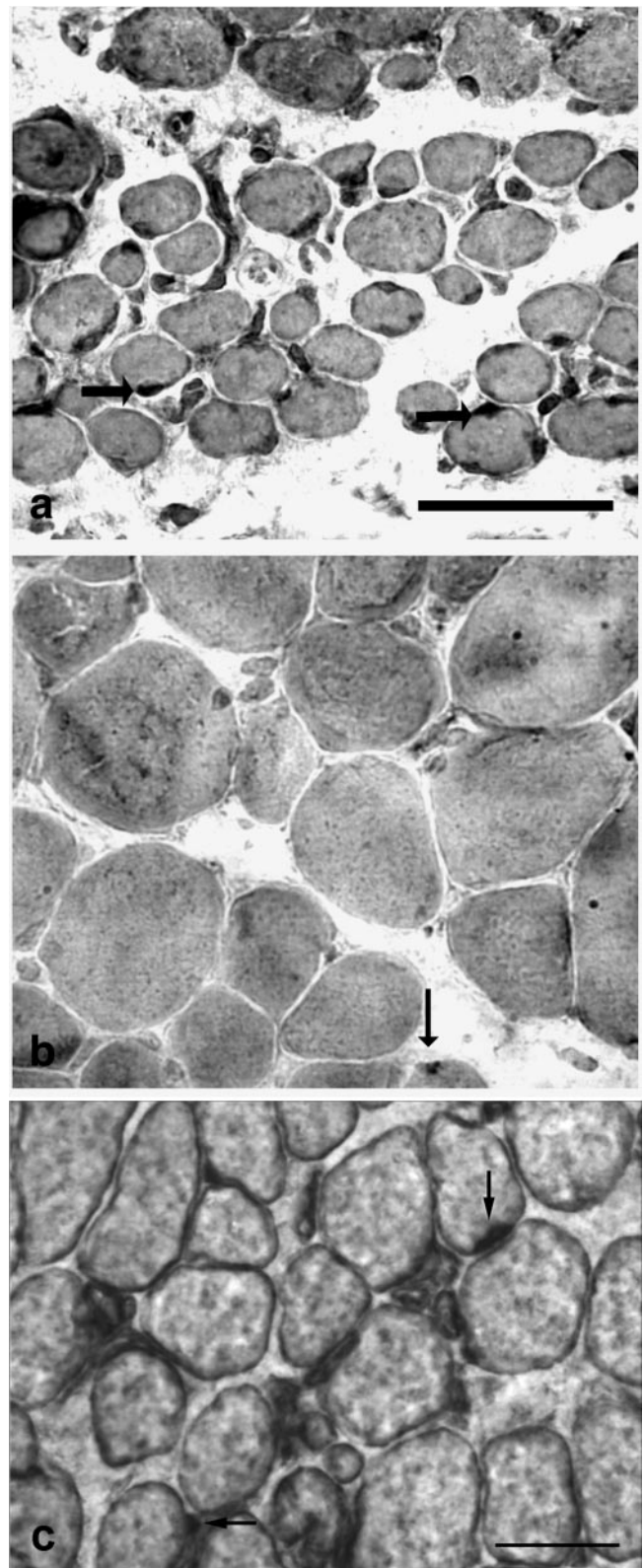


FIGURE 1. (a) Orbital layer of normal superior rectus muscle and (b) normal tibialis anterior muscle, both immunostained for expression of the insulin-like growth factor receptor, and (c) global layer of normal superior rectus muscle double stained for IGF-R and laminin. *Arrows*: indicate cells positive for the IGF-receptor. *Bar*: (a, b) 50 μm; (c) 20 μm.

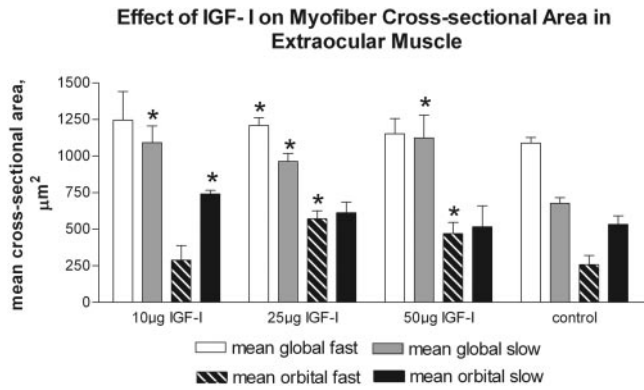


FIGURE 2. Effect of a single injection of 10, 25, or 50 μg IGF-I on myofiber cross-sectional area 1 week after treatment. *Significant difference from control.

agents (Vector Laboratories, Burlingame, CA), and incubated for 1 hour with the primary antibody. The sections were rinsed and incubated with the ABC reagents (Vectastain Elite; Vector Laboratories, Burlingame, CA). The reacted tissue sections were processed using the heavy metal intensified diaminobenzidine procedure.

At least four cross sections from the midbelly of each muscle were analyzed for individual myofiber cross-sectional area by manual tracing under bright-field microscopy. Between 200 and 400 myofibers from a minimum of four fields were analyzed. Mean cross-sectional areas for the myofibers were determined (Bioquant Nova Prime image analysis system; Bioquant Inc., Nashville, TN). Histograms were constructed from the cross-sectional area measurements of the individual control and IGF-treated muscles. The cross-sectional areas were divided into bins of 200- μm increments. The percentage of myofibers positive for the neonatal and developmental MyHC was determined in muscles treated with either 25 or 50 μg IGF-I. These represent the most effective doses and the doses where force began to decrease experimentally, although still significantly greater than control. Care was taken to analyze sections from midbelly region and at the tendon end, since the percentage of myofibers positive for neonatal and developmental MyHC changes along the tendon-to-tendon muscle length.¹³ In a second group of control rabbits, normal superior rectus, and tibialis muscles were obtained as previously described and immunostained with an antibody to insulin-like growth factor receptor (IGF-R; 1:10; Abcam, Cambridge, MA) or double immunostained for IGF-R and laminin (1:40; Vector Laboratories) as published previously.¹⁴ All data were analyzed for statistical significance using analysis of variance (ANOVA) and the Dunn multiple comparison tests (Prism and Statmate software; GraphPad, San Diego, CA, or SigmaStat 2.03; SPSS Science, Chicago, IL). An F-test was used to verify that the variances were not significantly different. Data were considered significantly different if $P \leq 0.05$.

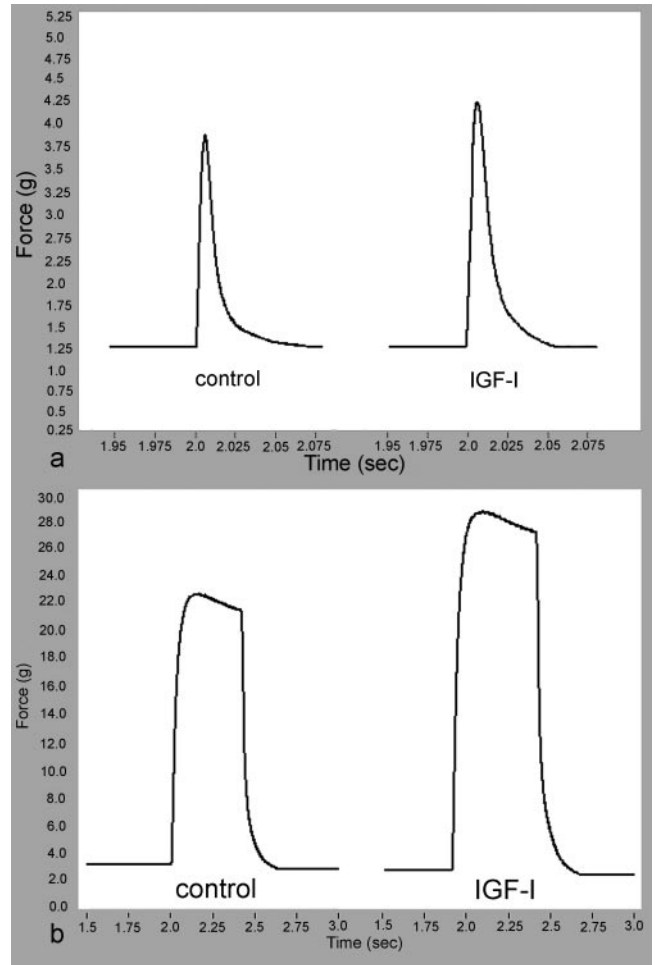


FIGURE 3. Representative records of twitch (a) and tetanic (b) contractions of control and IGF-I-treated superior rectus muscles 1 week after injection.

RESULTS

Normal adult EOMs express insulin-like growth factor receptor, which is normally downregulated in adult limb skeletal muscle (Fig. 1). Most of these positive cells were in the satellite cell position, deep to the basal lamina; however, there were IGF-R positive cells in the interstitial space as well. One week after a single injection of IGF-I into the superior rectus muscles of adult rabbits, a population of hypertrophic myofibers was present. Average myofiber cross-sectional areas were signifi-

TABLE 1. Stress in Superior Rectus Muscles, mN/cm²

Stimulus Frequency (Hz)	IGF-I					
	Control	1 μg	5 μg	10 μg	25 μg	50 μg
Twitch	36.2 \pm 1.9	63.9 \pm 11.1	50.8 \pm 2.3	79.4 \pm 6.9	86.1 \pm 9.9	61.5 \pm 3.1
10	44.5 \pm 2.5	69.0 \pm 12.9	49.6 \pm 1.5	84.9 \pm 11.9	87.0 \pm 11.1	61.9 \pm 4.1
20	51.2 \pm 2.8	77.9 \pm 14.1	56.2 \pm 2.1	96.3 \pm 12.8	100.9 \pm 11.8	68.8 \pm 4.8
40	78.2 \pm 3.2	120.1 \pm 18.4	86.6 \pm 5.1	146.7 \pm 18.8	152.7 \pm 17.6	101.8 \pm 9.5
100	249.9 \pm 14.0	402.0 \pm 74.8	375.0 \pm 30.5	457.6 \pm 57.9	501.3 \pm 50.8	328.7 \pm 23.6
150	303.8 \pm 18.1	504.5 \pm 97.2	478.4 \pm 27.2	591.5 \pm 79.7	632.5 \pm 64.0	411.3 \pm 28.9
200	309.4 \pm 18.0	519.8 \pm 100.5	500.0 \pm 27.9	642.8 \pm 88.5	655.5 \pm 61.4	429.4 \pm 31.2

Data are expressed as mean millinewtons per square centimeter \pm SEM. All IGF-I-treated muscles are significantly different from control values for each set of experimental animals at $P \leq 0.05$. $n = 6$ for each dose of IGF-I.

cantly increased in IGF-I injected superior rectus muscles compared to control (Fig. 2).

Direct injection of IGF-I into the superior rectus muscles resulted in a significant increase in muscle force and stress at all five doses of IGF-I compared with contralateral control muscles (Table 1, Figs. 3, 4, 5). In addition, a single injection of either 10 or 25 μg of IGF-I resulted in increased stress generation compared to 1, 5, or 50 μg . Interestingly, whereas the injection of 50 μg IGF-I resulted in a significant difference compared with the contralateral control muscles, it resulted in significantly less force generation than the 10- or 25- μg dose. (Fig. 5). No side effects of the localized injections of IGF-I were noted.

The twitch characteristics of the IGF-I and control muscles were compared (Table 2). The shorter total contraction time combined with a greater peak force suggested that the injected muscles both contracted and relaxed faster than the control

muscles, but this difference was subtle. For example, the time to peak force and half relaxation times (HRTs) were not different for the injected and control muscles. However, $\text{Max } df/dt$ was significantly greater in the injected muscles, indicating that the peak rate of force development was greater after IGF-I treatment, and peak force (stress) was also increased (Table 2). These data suggest that compared with the control, IGF-I-treated muscles generate more force.

The IGF-I-treated and control EOMs showed similar fatigue properties (Fig. 6), as was seen with those treated with IGF-II.

The percentage of myofibers positive for neonatal MyHC (Fig. 7a) and developmental MyHC (Fig. 7b) showed different expression patterns in muscles treated with 25 and 50 μg IGF-I. The percentage of myofibers positive for neonatal MyHC expression decreased in the global layers, particularly at the tendon end, for both doses of IGF-I. Of note, at 50 μg IGF-I, all

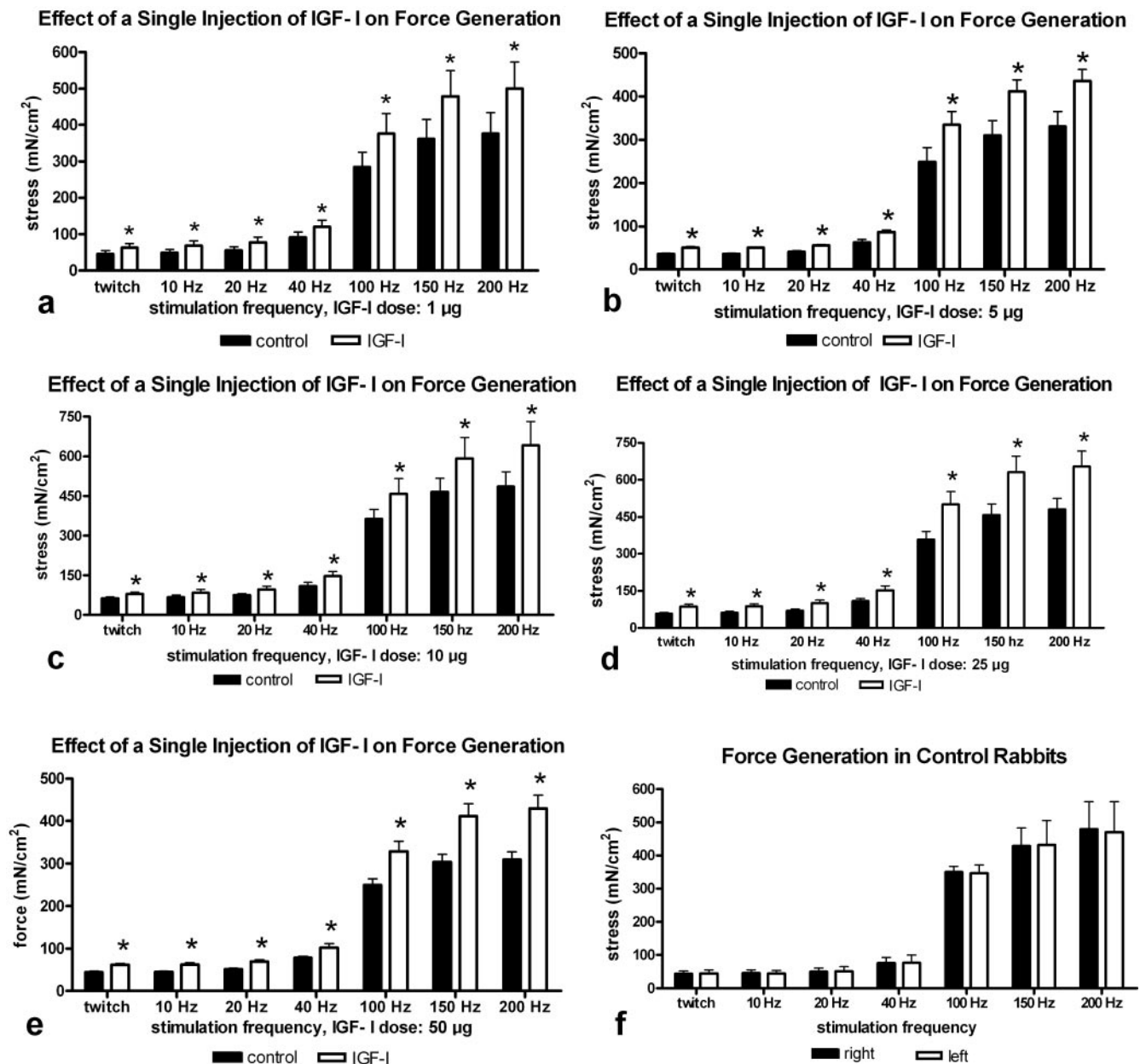


FIGURE 4. Force generation, expressed as stress, of superior rectus muscles 1 week after a single injection with 1 (a), 5 (b), 10 (c), 25 (d), or 50 (e) μg IGF-I at twitch and 10-, 20-, 40-, 100-, 150-, and 200-Hz stimulation frequencies. (f) Control rabbits injected with saline only. n equals six for control and treated muscles. *Significant difference from control.

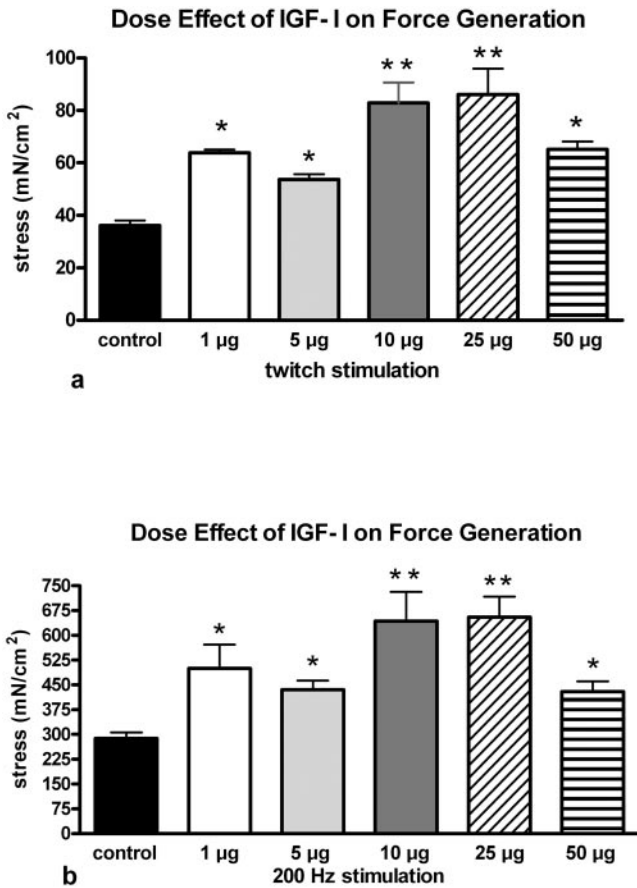


FIGURE 5. Effect of dose of IGF-I on force generation, expressed as stress, at both twitch and tetanic stimulation (200 Hz). All doses resulted in significantly increased force generation compared with control muscles (*). Both 10 and 25 µg resulted in significantly greater force generation than 1, 5, or 50 µg (**).

regions of the muscles showed a decrease in neonatal MyHC expression compared with the control. In the global layer in the midbelly region of the muscle there was a significant increase in myofibers positive for developmental MyHC, and in the global layer of the tendon region there was a significant decrease in the percentage of myofibers positive for developmental MyHC.

DISCUSSION

The presence of cells positive for insulin-like growth factor receptor supports the hypothesis that adult EOMs have the cellular machinery that would enable a rapid response to IGF-I treatment. Indeed, we found that a single injection of IGF-I into the superior rectus muscles of adult rabbits results in a significant increase in both cross-sectional area and force generation

TABLE 2. Twitch Characteristics of the Injected and Control Rabbit Superior Rectus Muscles

Properties of Single Twitch	Injected	Control
Total contraction time (ms)	132.1 ± 13.4	235.0 ± 113.8
Time to peak force (ms)	13.6 ± 3.0	16.3 ± 3.3
Half-relaxation time (ms)	10.9 ± 0.5	11.3 ± 0.6
Max <i>df/dt</i> (g/s)	414.6 ± 26.4*	332.8 ± 34.01
Time to max <i>df/dt</i> (ms)	6.7 ± 1.1	8.9 ± 3.2

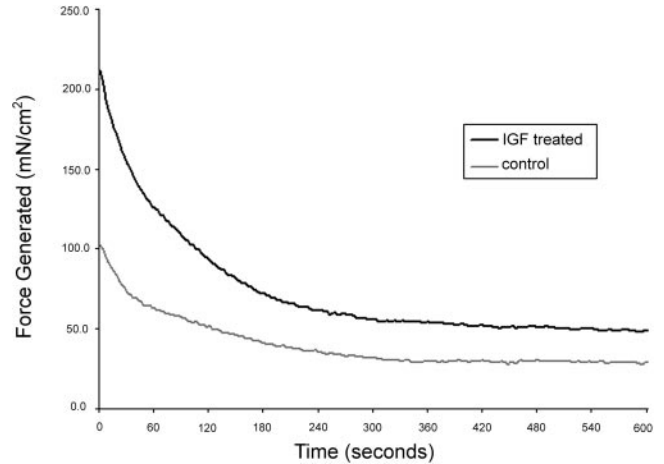


FIGURE 6. Fatigability was determined by stimulation of control and IGF-I-treated superior rectus muscles at 150 Hz every 2 seconds for 600 seconds or until there was a 50% reduction in generated muscle force. This is a representative trace from one experiment, in which 10 µg IGF-I was injected in the left superior rectus and saline only in the right superior rectus muscle.

compared with control muscles at all doses. This increase was greatest at both the 10- and 25-µg doses. At the lowest and highest doses tested, not only was the response to IGF-I injection less robust, but interanimal variability was greater.

Many studies using different animal models have demonstrated that IGF-I is a potent myogenic growth factor. IGF-I prevents muscle atrophy in mouse models of both aging¹⁰ and

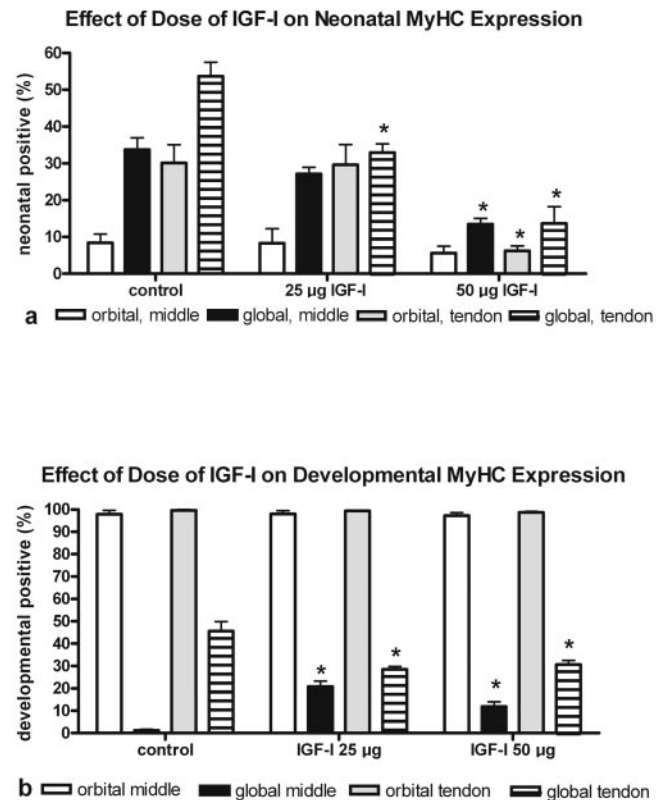


FIGURE 7. Effect of 25- and 50-µg IGF-I injections on the percentage of myofibers positive for neonatal MyHC expression (a) and developmental MyHC expression (b) compared with control superior rectus muscles. *Significant difference from controls.

muscular dystrophy.¹¹ Transgenic mice that overexpress IGF-I display significant muscle hypertrophy.¹⁵ On a metabolic level, localized infusion of IGF-I results in increased protein synthesis, which in turn results in increased muscle mass.¹⁶ Exogenously administered IGF-I in skeletal muscle results in increased satellite cell proliferation and differentiation^{17,18} as well as increased protein synthesis.¹⁹ The results of the present study appear to recapitulate these findings in EOM, raising the possibility that growth factors such as IGF-I may play a future role in the pharmacological treatment of ocular motor anomalies.

In previous work, we have demonstrated increased EOM force generation 1 week after single injections of IGF-II into rectus muscles.⁹ Although the total muscle mass of the treated compared with the control rectus muscles was greater after the IGF-II injections, there was no significant difference in mean cross-sectional area.⁹ This differential response of muscle to IGF-I and -II suggests that these myogenic growth factors act on different types of growth pathways within muscle cells.²⁰ In the present study, IGF-I appears to be a more potent myogenic growth factor for superior rectus muscles, based on increased mean muscle cross-sectional area as an index of muscle growth.

In addition to increased mass, a single injection of IGF-I into superior rectus muscles resulted in increased force generation. Even when force was normalized to muscle cross-sectional area, stress was still greater than in the noninjected muscles. There are few studies in which force generation has been specifically examined as a result of IGF-I treatment. Four weeks of subcutaneous delivery of IGF-I to skeletal muscles of *mdx* mice resulted in increased maximum force in the leg muscles examined.²¹ After 8 weeks of systemic IGF-I administration to *mdx* mice, force per cross-sectional area of diaphragm muscle was significantly elevated compared with untreated controls.²² However, in this dystrophic mouse model, decreased fatigue resistance was also noted,²³ which did not occur in our eye muscle studies. Sustained overexpression of IGF-I in transgenic mice prevented the age-related decreases in specific muscle force in flexor digitorum brevis muscle.²⁴

Although the systemic application of large doses of IGF-I resulted in increased muscle force generation in these animal models, in human studies systemic IGF-I injections were ineffective, and other growth factors tested in humans led to unacceptable side effects such as injection site pain and weight loss.²⁵ The muscle-specific approach to IGF-I treatment would reduce concerns about whole-body exposure to this growth factor, increasing safety without reducing efficacy. The lack of orbital or systemic side effects in treated animals in this study suggests that the targeted injection of IGF-II⁹ or IGF-I may minimize toxicity.

MyHC isoform diversity in skeletal muscles is associated with the diverse physiological properties of single myofibers, including maximum shortening velocity and power.²⁶ The patterns of expression of MyHC isoforms in EOMs in particular are extremely complex.²⁵ Expression patterns of these isoforms are highly adaptable and appear to change with most perturbations of the muscle.²⁷⁻²⁹ Injection of IGF-I resulted in decreased expression of neonatal MyHC, and this was most pronounced at the highest dose of IGF-I tested. Developmental MyHC expression increased in the global layer, but only in the midbelly region of the treated muscles. MyHC isoform expression affects contraction velocity,²⁸ and these changes must, in part, be responsible for the changes in twitch characteristics seen in this study.

Numerous myogenic growth factors are involved in muscle development and regeneration. These include, but are not limited to, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), epidermal growth factor, cardiotrophin-1

(CT-1), glial cell derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF). Treatment of denervated or atrophic muscles by one or more of these factors restored muscle size and/or force generation.³⁰ Individual myogenic growth factors or a cocktail of several growth factors, including CT-1, IGF-I, glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF), were injected retrobulbarly into the orbits of chicks, and this resulted in increased force generation of the EOMs examined.¹⁰ Treatment of the EOMs with various myogenic growth factors directly in the orbit is effective in increasing the muscle force generation of the treated muscles. Preliminary studies currently under way indicate that methods that allow for sustained release of IGF-I maintain this muscle force increase up to 3 months.

In summary, direct muscular injection of IGF-I effectively increases EOM myofiber size and force generation and appears to do so without local or systemic toxicity. If methods can be developed to prolong duration of exposure, this growth factor may be used alone or in conjunction with other force-modulating agents to alter rotational position of the eyes and do so without the potential adverse biomechanical hazards of standard strabismus surgery.

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