Modulation of Insulin-like Growth Factor (IGF)-I and IGF-Binding Protein Interactions Enhances Skeletal Muscle Regeneration and Ameliorates the Dystrophic Pathology in *mdx* Mice

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Administration of recombinant human insulin-like growth factor-I (rhIGF-I) has beneficial effects in animal models of muscle injury and muscular dystrophy. However, the results of these studies may have been confounded by interactions of rhIGF-I with endogenous IGF-binding proteins (IGFBPs). To date, no study has examined whether inhibiting IGFBP interactions with endogenous IGF-I can improve muscle fiber regeneration or muscular pathologies. We tested the hypothesis that reducing IGFBP interactions with endogenous IGF-I would enhance muscle regeneration after myotoxic injury and improve the dystrophic pathology in *mdx* mice. We administered an IGF-I aptamer (NBI-31772; 6 mg/kg per day, continuous infusion) to C57BL/10 mice undergoing regeneration after myotoxic injury or to *mdx* dystrophic mice. NBI-31772 binds all six IGFBPs with high affinity and releases “free” endogenous IGF-I. NBI-31772 treatment increased the rate of functional repair in fast-twitch tibialis anterior muscles after notexin-induced injury as evidenced by an increase in maximum force producing capacity (P_0) at 10 days after injury. In contrast, NBI-31772 administration for 28 days did not alter P_0 of extensor digitorum longus (EDL) and soleus muscles or normalized force of diaphragm muscle strips from *mdx* mice. Although IGFBP inhibition reduced the susceptibility of the fast-twitch EDL and the diaphragm muscle to contraction-mediated damage, it increased muscle fatigability during repeated maximal contractions. Although the results in the myotoxic injury model suggest IGF-I signaling is important in this model, the results in the *mdx*

model are mixed. (Am J Pathol 2007, 171:1180–1188; DOI: 10.2353/ajpath.2007.070292)

Skeletal muscle injury and degeneration can result from trauma caused by mechanical (laceration, crush, strain), chemical (myotoxin), or metabolic (temperature) factors and is a major contributor to the etiology of myopathies, such as the muscular dystrophies. Although muscle fibers have an inherent capacity to regenerate when damaged, the regeneration process is often slow, inefficient, and incomplete with respect to functional restoration.¹ Treatment strategies to enhance the regenerative capacity of muscle are significant for patients suffering from muscle injuries and other muscle wasting conditions.

The muscular dystrophies define a group of genetic disorders characterized by progressive skeletal muscle wasting and weakness. The most severe and rapidly progressing of these conditions is Duchenne muscular dystrophy, affecting ~1 in 3500 males born worldwide.² The absence of a cytoskeletal protein, dystrophin, renders dystrophic muscles extremely fragile and easily damaged by everyday activities, especially by contractions where muscles are activated and then stretched forcibly.^{3,4} This increased susceptibility to contraction-mediated injury results in ongoing cycles of muscle fiber degeneration and less than adequate regeneration and contributes to an etiology of progressive muscle wasting and weakness.⁵

Although animal models can reproduce some (or most) of the pathologies associated with muscular dystrophies, models of muscle injury such as crush or strain

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injuries can be problematic especially for generating reproducible responses in animal models. On the other hand, injecting muscles with a myotoxic agent such as notexin or cardiotoxin results in complete and reproducible muscle fiber degeneration⁶ and the ability to monitor the events of and mechanisms controlling muscle fiber regeneration.

Regardless of the initial injury, effective muscle regeneration is dependent on the timed induction of myogenic regulatory factors and growth factors, including insulin-like growth factor-I (IGF-I).^{1,7} IGF-I activates both myoblast proliferation and subsequently differentiation, two processes that are crucial to muscle repair and regeneration.^{8,9} The importance of IGF-I in skeletal muscle regeneration has been demonstrated in transgenic mice, where overexpression of IGF-I localized to skeletal muscle maintained regenerative capacity in aged mice¹⁰ and reduced the dystrophic pathology in *mdx* mice, an animal model of Duchenne muscular dystrophy.^{11,12} In addition, we have shown previously that exogenous administration of recombinant human IGF-I (rhIGF-I) increased the rate of functional recovery after myotoxic injury¹³ and improved the dystrophic pathology in *mdx* mice.^{14–16} Although rhIGF-I administration and transgenic IGF-I overexpression have beneficial effects on skeletal muscle, their mechanism of action seems to be vastly different. For example in mice, transgenic IGF-I overexpression resulted in marked muscle hypertrophy,¹¹ an effect not observed following rhIGF-I administration.^{14–16} This discrepancy is likely due to different effects of IGF-binding proteins (IGFBPs) on IGF-I in the bloodstream (systemic delivery) compared with muscle-specific transgenic overexpression of IGF-I.

The biological actions of IGF-I *in vivo* are strongly mediated by IGFBPs, which bind ~99% of IGF-I in the circulation.¹⁷ This large reservoir of bound, biologically inactive IGF-I has a prolonged half-life and is protected from degradation.^{17–19} IGFBPs are believed to primarily transport IGF-I to target tissues, modulate IGF-I action, and prevent hypoglycemia.^{17–19} In general, IGFBPs are thought to inhibit IGF-I actions *in vitro* and *in vivo* by preventing IGF-I binding to cell surface receptors.^{20–22}

Although the effects of rhIGF-I administration and IGF-I overexpression on skeletal muscle regeneration have been well characterized,^{10–12,14–16} the role of IGFBPs in skeletal muscle regeneration remains poorly understood. Thus, the aim of the current study was to examine the effects of IGFBP inhibition on muscle regeneration after myotoxic injury and also in *mdx* dystrophic mice. We tested the hypothesis that inhibition of endogenous IGFBPs would enhance skeletal muscle regeneration and improve the dystrophic pathology in a manner similar to exogenous rhIGF-I administration.

Materials and Methods

Experimental Animals

All procedures were approved by the Animal Experimentation Ethics Committee of The University of Melbourne

and conformed to the Guidelines for the Care and Use of Experimental Animals as described by the National Health and Medical Research Council of Australia (Canberra, ACT, Australia). To examine the effect of IGFBP inhibition on muscle regeneration, male C57BL/10ScSn (BL/10) mice (12 to 14 weeks old, $n > 5$ per group) were used. To determine the role of IGFBPs in dystrophic skeletal muscles, male C57BL/10ScSn-*mdx*/J dystrophic (*mdx*) mice (8 to 10 weeks old, $n = 8$ per group) were used. All mice were obtained from the Animal Resource Centre (Canning Vale, WA, Australia) and housed in the Biological Research Facility at The University of Melbourne under a 12-hour light/dark cycle, with drinking water and standard chow provided *ad libitum*. For both experiments treated mice received continuous subcutaneous (s.c.) administration of the IGF-I aptamer NBI-31772 (6 mg/kg per day in 50% dimethyl sulfoxide and 50% polyethylene glycol), whereas control mice received vehicle alone (50% dimethyl sulfoxide and 50% polyethylene glycol). Continuous systemic delivery of NBI-31772 (or vehicle alone) was achieved by subcutaneous implantation of a micro-osmotic pump (Alzet model 1002; Alzet, Cupertino, CA) as described previously.¹⁴ NBI-31772 is a nonpeptide ligand that has high-affinity binding for all six IGF binding proteins,²³ and *in vitro* binding assays have revealed that NBI-31772 displaces IGF-I from its interaction with binding proteins, thus releasing “free” biologically active IGF-I.²⁴ BL/10 mice received NBI-31772 infusion for a period of 10, 14, or 21 days, whereas *mdx* mice received NBI-31772 for 28 days.

Myotoxic Injury of Tibialis Anterior Muscles

In BL/10 mice, tibialis anterior (TA) muscles were injured using the myotoxic agent notexin, as described previously.¹³ In brief, mice were anesthetized deeply with sodium pentobarbitone (60 mg/kg i.p., Nembutal; Rhone Merieux, Pinkenba, QLD, Australia), and an adequate depth of anesthesia was maintained, such that the mice were unresponsive to tactile stimulation. The right hindlimb was shaved, and a small portion of the anterior aspect of the TA muscle was surgically exposed. The muscle was injected with 40 μ l of notexin (10 μ g/ml in isotonic saline; Latoxan, Valence, France) with a 29-gauge fixed needle. Care was taken to prevent leakage of notexin from the muscle. An injection volume of 40 μ l was the maximum holding capacity of a TA muscle and reliably produced complete degeneration of all myofibers.^{6,13} After the intramuscular injection, the skin incision was closed with surgical (Michel) clips (Aesculap, Tuttlingen, Germany), and the mouse was allowed to recover on a heat pad.

Contractile Properties Measured In Situ

The methods for *in situ* contractile analysis of TA muscles from mice have been described in detail elsewhere.¹⁶ In brief, TA muscles from injured and control BL/10 mice were stimulated by supramaximal (10 V) 0.2-ms square wave pulses of 300 ms in duration delivered via two wire electrodes adjacent to the femoral nerve. Optimum mus-

Table 1. Morphological and Isometric Twitch Contractile Properties of Tibialis Anterior (TA) Muscles in NBI-31772-Treated and Untreated (Control) Mice at 10, 14, and 21 Days after Myotoxic Injury

	Day 10		Day 14		Day 21	
	Control	NBI-31772	Control	NBI-31772	Control	NBI-31772
Mass (mg)	33 ± 2	40 ± 3	51 ± 2	46 ± 2	56 ± 3	59 ± 4
P _t (mN)	125 ± 15	186 ± 29	275 ± 17	347 ± 9*	341 ± 10	427 ± 38
dP _{twitch} /dt (mN/ms)	22 ± 4	35 ± 4*	46 ± 3	56 ± 2*	60 ± 3	75 ± 4*
TPT (ms)	23 ± 1	19 ± 1*	19 ± 1	18 ± 1	17 ± 1	17 ± 1
½ RT (ms)	25 ± 3	18 ± 2*	19 ± 1	17 ± 1	15 ± 1	13 ± 1

dP_{twitch}/dt, maximum rate of force development during a twitch contraction; P_t, peak twitch tension; ½ RT, one-half relaxation time; TPT, time to peak twitch tension.

**P* < 0.05 control versus NBI-31772.

cle length (L_o) was determined from maximum isometric twitch force (P_t), and maximum isometric tetanic force (P_o) was recorded from the plateau of the frequency-force relationship.

Contractile Properties Measured In Vitro

The contractile properties of extensor digitorum longus (EDL) and soleus muscles and of diaphragm muscle strips from *mdx* mice were analyzed *in vitro*, as described previously.²⁵ In brief, EDL, soleus, and diaphragm muscle preparations were stimulated by supramaximal (40 V) 0.2-ms square wave pulses of 350-, 1200-, and 450-ms duration, respectively, delivered via platinum plate electrodes that flanked both sides of the muscle. L_o was determined at P_t and P_o was recorded from the plateau of the frequency-force curve. The EDL and soleus muscles from the right hindlimb, as well as a diaphragm muscle strip, were assessed for their susceptibility to contraction-induced injury, whereas the EDL and soleus muscles from the left hindlimb, as well as another diaphragm muscle strip, were assessed for their resistance to a fatiguing stimulation protocol.

The contraction-induced injury protocol used in this study was similar to others described previously.^{26,27} Muscles were lengthened at a velocity of two L_f /s at progressively increasing magnitudes of stretch beyond L_f (5, 10, 20, 30, 40, and 50%), with maximum isometric force being determined after each lengthening contraction. The "force deficit" after contraction-induced damage was determined by calculating the difference between the P_o measured 2 minutes after the lengthening contractions and P_o determined before any of the lengthening contractions and is expressed as a percentage of the maximum P_o determined before the protocol of lengthening contractions.^{16,26}

Muscle fatigue was assessed using a standard fatigue stimulation protocol we have described previously.²⁵ Muscles were stimulated maximally once every 4 seconds for 4 minutes, with maximum force recorded every minute. During recovery, maximum force was determined at 5, 10, and 15 minutes after completion of the fatigue protocol.

Skeletal Muscle Morphology

At the completion of all of the contractile function analyses, the muscles were mounted in embedding medium, frozen

in thawing isopentane, and stored at -80°C . A portion of each muscle was cryosectioned transversely (8 μm) through the midbelly region. Muscle sections were stained with hematoxylin and eosin (H&E) to determine general muscle architecture and to determine the cross-sectional area (CSA) of individual muscle fibers. Median values for CSA were calculated from at least 200 individual muscle fibers per cross section, as described previously.¹⁶

Figure 1. Mean data for maximum absolute tetanic force (P_o ; **A**) and specific or normalized force (sP_o ; **B**) of TA muscles from treated and untreated (control) mice measured *in situ* at 10, 14, or 21 days after notexin-induced injury. *Significantly higher tetanic force production following treatment with NBI-31772 compared with control (*P* < 0.05; main effect).

Myosin Heavy Chain Analysis

Myosin heavy chain (MyHC) isoform composition within TA muscles of BL/10 mice was determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Muscle samples were homogenized on ice in phosphate-buffered saline and centrifuged at $300 \times g$ for 2 minutes at 4°C . The pellet was resuspended in 100 μl of Guba-Straub buffer (300 mmol/L NaCl, 100 mmol/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 50 mmol/L NaH_2PO_4 , 1 mmol/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mmol/L $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, and 10 mmol/L ethylenediamine tetraacetic acid) and centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The supernatant was diluted with 1.5 ml of H_2O and refrigerated overnight. The following day, the supernatant was centrifuged again at $12,000 \times g$ for 15 minutes at 4°C , and the pellet was resuspended in 300 μl of Guba-Straub buffer containing 10% glycerol. Protein concentration was determined via the method of Bradford,²⁸ and all samples were equalized. Ten micrograms of protein was added to 90 μl of Guba-Straub buffer and 30 μl of bromophenol blue solution. Immediately before gel loading, samples were heated at 95°C for 5 minutes, after which 4 μl of sample was loaded per well. MyHC isoforms were separated on an 11% acrylamide-bis (50:1) gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were run at 70 V at 4°C for 36 hours. Protein bands were visualized using the InVitrogen Silver Stain kit (LC6100; Victoria, Australia). The gels were scanned and band densities analyzed using the image analysis software described previously.²⁹

Citrate Synthase Activity

Citrate synthase activity was determined in homogenates prepared from EDL, soleus, and diaphragm muscles using a citrate synthase assay kit (CS0720; Sigma-Aldrich, St. Louis, MO).³⁰ Total muscle protein was determined in triplicate by the method of Bradford,²⁸ and the protein concentration of all samples was equalized. Citrate synthase activity was determined in triplicate based on the formation of 2-nitro-5-thiobenzoic acid at a wavelength of 412 nm at 25°C on a spectrophotometer (Multiskan Spectrum; Thermo Electron Corporation, Waltham, MA). In each well, 8 μl of sample was added to a reaction medium containing 178 μl of assay buffer, 2 μl of 30 mmol/L acetyl coenzyme A, and 10 mmol/L 2-nitro-5-thiobenzoic acid. The baseline solution absorbance was recorded, reactions were initiated by the addition of 10 μl of oxaloacetic acid, and the change in absorbance measured every 15 seconds for 2 minutes.

Statistical Analysis

All values are expressed as mean $\pm$ SEM unless specified otherwise. Groups were compared using two-way analysis of variance where appropriate. The level of significance was set at $P < 0.05$ for all comparisons. Bonferroni's post hoc MCP was used to determine significant differences between groups. CSA data were analyzed using the Anderson-Darling Normality Test. Muscle fiber CSA was not normally distributed, and so the 95% con-

fidence interval of the median was used and differences were considered significant when no overlap existed between the 95% confidence interval of the median.

Results

IGFBP Inhibition Enhances the Functional Recovery

Administration of NBI-31772 did not alter the mass of regenerating TA muscles at 10, 14, or 21 days after injury (Table 1). Administration of NBI-31772 increased P_o by 34% at 10 days after injury ($P < 0.05$; Table 1, Figure 1). Administration of NBI-31772 also increased P_t by 26% and likewise increased sp_o by 24% compared with untreated muscles 14 days after injury ($P < 0.05$; Table 1). Administration of NBI-31772 hastened the time course of the twitch response throughout regeneration compared with untreated muscles, as evident from the 60, 23, and 26% increase in dP_{twitch}/dt at 10, 14, and 21 days, respectively ($P < 0.05$; Table 1). Furthermore, NBI-31772

Figure 2. Representative photomicrographs of H&E-stained transverse sections of TA muscle (left) and graphical representations depicting individual and median data of myofiber cross-sectional area (right) at 10, 14, or 21 days after notexin-induced injury (A, B, and C, respectively). *Significantly higher median CSA following treatment with NBI-31772 compared with control ($P < 0.05$).

administration decreased time-to-peak twitch tension (TPT) by 15% and one-half relaxation time ($\frac{1}{2}$ RT) by 29% compared with untreated TA muscles at 10 days after injury ($P < 0.05$; Table 1). It is worth noting that administration of NBI-31772 enhanced functional recovery as evident from the higher P_o values during regeneration ($P < 0.05$; main effect; Figure 1).

IGFBP Inhibition During Regeneration Increases Muscle Fiber CSA and the Proportion of Fast-Type IIb MyHCs

Representative H&E-stained transverse muscle sections and the median and individual CSA values for TA muscles 10, 14, and 21 days after myotoxic injury are presented in Figure 2. The median CSA of myofibers was 35, 24, and 16% greater following NBI-31772 administration at 10, 14, and 21 days after injury, respectively (Figure 2).

NBI-31772 administration altered the proportions of MyHC isoforms in regenerating TA muscles (Figure 3) with embryonic MyHC isoform expression transitioning to MyHC IIa/x and MyHC IIb as regeneration progressed from 10 to 21 days (Figure 3). Administration of NBI-31772 increased the relative proportion of MyHC IIb with a concomitant decrease of MyHC IIa/x compared with untreated muscles at 21 days after injury ($P < 0.05$; Figure 3).

Muscle Mass and Force Production in mdx Mice Are Not Altered by IGFBP Inhibition

EDL and soleus muscle mass and diaphragm muscle strip thickness in *mdx* mice were not different following NBI-31772 treatment (Table 2). Administration of NBI-31772 did not alter the TPT responses in the soleus or diaphragm muscles of *mdx* mice but hastened $\frac{1}{2}$ RT in EDL muscles of *mdx* mice by 17% ($P < 0.05$; Table 2). Administration of NBI-31772 induced a faster contractile phenotype in the EDL muscles as evident from the 26% increase in dP_{tetanic}/dt in treated compared with untreated *mdx* mice ($P < 0.05$; Table 2). NBI-31772 treatment also increased $-dP_{\text{tetanic}}/dt$ by 36% in diaphragm muscle strips compared with control *mdx* mice. In contrast to the data for regenerating TA muscles, NBI-31772 treatment did not alter P_o or sP_o of the EDL or soleus muscles or the sP_o of diaphragm muscle strips.

Inhibition of IGFBPs Reduces the Susceptibility of Dystrophic Muscles to Contraction-Induced Injury

EDL muscles from NBI-31772-treated *mdx* mice were less susceptible to contraction-induced damage compared with

Figure 3. Representative detection of myosin heavy chain (MyHC) protein isoforms in TA muscles at 21 days after injury following treatment with NBI-31772 or control (A). Relative isoform composition of TA muscles 10, 14, or 21 days following treatment with NBI-31772 or control (B). *Significantly lower MyHC IIa/x and higher MyHC IIb following treatment with NBI-31772 compared with control at 21 days after injury ($P < 0.05$).

Table 2. Morphological and Isometric Contractile Properties of EDL and Soleus Muscles and Diaphragm Muscle Strips from NBI-31772-Treated and Untreated (Control) *mdx* Mice

	Control	NBI-31772
EDL		
Mass (mg)	11 ± 1	12 ± 1
% of fibers centrally nucleated	66 ± 2	68 ± 2
P_t (mN)	120 ± 10	127 ± 12
dP_{twitch}/dt (mN/ms)	16 ± 1	17 ± 2
TPT (ms)	22 ± 1	20 ± 1
$\frac{1}{2}$ RT (ms)	24 ± 1	21 ± 1*
$-dP_{\text{tetanic}}/dt$ (mN/ms)	-12 ± 1	-15 ± 1*
P_o (mN)	372 ± 29	406 ± 22
sP_o (kN/m ²)	201 ± 12	196 ± 11
Soleus		
Mass (mg)	8 ± 1	8 ± 1
% of fibers centrally nucleated	66 ± 3	66 ± 4
P_t (mN)	50 ± 2	51 ± 3
dP_{twitch}/dt (mN/ms)	7 ± 1	7 ± 1
TPT (ms)	39 ± 1	39 ± 1
$\frac{1}{2}$ RT (ms)	53 ± 2	54 ± 2
$-dP_{\text{tetanic}}/dt$ (mN/ms)	-2.7 ± 0.2	-3.2 ± 0.5
P_o (mN)	222 ± 8	228 ± 11
sP_o (kN/m ²)	205 ± 7	214 ± 9
Diaphragm		
Thickness (μm)	514 ± 59	514 ± 57
% of fibers centrally nucleated	32 ± 3	36 ± 3
sP_t (kN/m ²)	47 ± 3	48 ± 3
dP_{twitch}/dt (mN/ms)	5 ± 1	5 ± 1
TPT (ms)	42 ± 1	40 ± 1
$\frac{1}{2}$ RT (ms)	47 ± 1	47 ± 3
$-dP_{\text{tetanic}}/dt$ (mN/ms)	-1.4 ± 0.1	-1.9 ± 0.1*
sP_o (kN/m ²)	145 ± 3	142 ± 8

dP_{twitch}/dt , maximum rate of force development during a twitch contraction; $-dP_{\text{tetanic}}/dt$, maximum rate of relaxation following a tetanic contraction; P_o , maximum isometric force; P_t , peak twitch tension; $\frac{1}{2}$ RT, one-half relaxation time; sP_o , specific force (force per muscle cross-sectional area); sP_t , specific twitch force (force per muscle cross-sectional area); TPT, time to peak twitch tension.

* $P < 0.05$ control versus NBI-31772.

muscles from untreated *mdx* mice ($P < 0.05$; Figure 4). The force deficits following active stretches of 30, 40, and 50% beyond L_f were reduced by 18, 24, and 19%, respectively ($P < 0.05$; Figure 4). Likewise, the diaphragm muscle strips from NBI-31772-treated *mdx* mice exhibited an overall protection from contraction-induced injury ($P < 0.05$, main effect; Figure 4). The soleus muscles of *mdx* mice are already far less susceptible to contraction-induced injury than the EDL or diaphragm muscles and therefore demonstrated no change in injury susceptibility after NBI-31772 treatment (Figure 4).

Increased Fatigability Associated with IGFBP Inhibition

EDL muscles from NBI-31772-treated *mdx* mice were more fatigable and exhibited an impaired recovery of force producing capacity after repeated contractions such that following the fatigue protocol EDL muscles from NBI-31772-treated *mdx* mice produced 7% less force than control *mdx* mice ($P < 0.05$; Figure 5). This NBI-

Figure 4. Susceptibility of EDL (A), soleus (B), and diaphragm (C) muscles of *mdx* mice to contraction-induced injury following treatment with NBI-31772. Muscles were subjected to a lengthening contraction protocol of progressive stretches (5, 10, 20, 30, 40, and 50% of L_f) with tetanic force reported after each lengthening contraction. *Significantly higher force production (ie, lower force deficit or reduced contraction-induced damage) in EDL muscles of *mdx* mice ($P < 0.05$). *Significantly higher force production (ie, reduced contraction-induced damage) in diaphragm muscles of *mdx* mice ($P < 0.05$; main effect).

31772-induced increase in EDL muscle fatigability was associated with a 14% decrease in citrate synthase activity ($P < 0.05$; Figure 5). The soleus muscles from NBI-31772-treated *mdx* mice were also more fatigable and had impaired recovery of their force producing capacity compared with muscles from untreated *mdx* mice ($P < 0.05$; main effect, Figure 5). Likewise, diaphragm muscle strips from NBI-31772-treated *mdx* mice produced 8% less force

Figure 5. EDL muscles from NBI-31772-treated *mdx* mice exhibited increased fatigability, reduced recovery of force production after fatigue, and lower citrate synthase activity (A). Likewise, soleus (B) and diaphragm (C) muscles from NBI-31772-treated *mdx* mice demonstrated increased fatigability and reduced recovery. *Significantly lower values for tetanic force production (P_o) or the maximum rate of citrate synthase activity after NBI-31772 treatment compared to control values ($P < 0.05$). #Significantly lower P_o in soleus muscles after NBI-31772 treatment ($P < 0.05$; main effect).

than untreated *mdx* mice after the fatigue protocol and also demonstrated an impaired functional recovery ($P < 0.05$; Figure 5). Soleus muscles and diaphragm muscle strips did not exhibit any change in citrate synthase activity after NBI-31772 treatment (Figure 5).

Muscle Morphology in *mdx* Mice

Representative H&E-stained transverse muscle sections and CSA data for the EDL, soleus, and diaphragm muscles are presented in Figure 6. The median CSA of myofibers from EDL muscles of *mdx* mice was 12% smaller after NBI-31772 treatment compared with *mdx* mice ($P < 0.05$; Figure 6). The median CSA of myofibers from soleus muscles of *mdx* mice was not different after treatment with NBI-31772 (Figure 6). Likewise, CSA of myofibers from the diaphragm muscles of *mdx* mice was not different after treatment with NBI-31772 (Figure 6). Administration of NBI-31772 did not alter the proportion of centrally nucleated fibers in the EDL, soleus, or diaphragm muscles from *mdx* mice (Table 2).

Discussion

The most important findings of this study were that IGFBP inhibition increased the rate of functional recovery after

Figure 6. Representative photomicrographs of H&E-stained transverse sections of EDL (A), soleus (B), and diaphragm (C) muscles (left) and graphical representations depicting individual and median data of myofiber cross-sectional area (right) from *mdx* mice. *Significantly lower median CSA following treatment with NBI-31772 compared with control ($P < 0.05$).

myotoxic damage and reduced the severity of contraction-induced injury in dystrophic *mdx* mice. These results support the hypothesis that abrogating IGFBP interactions with systemic IGF-I has significant therapeutic potential for enhancing muscle repair after muscle injury and for skeletal muscle pathologies such as the muscular dystrophies. To our knowledge, this is the first study to use long-term *in vivo* inhibition of IGFBPs in models of muscle regeneration and dystrophic muscle pathology.

In regenerating TA muscles IGFBP inhibition increased force production and hastened the rates of contraction and relaxation. These functional improvements were associated (at day 21) with an increased proportion of MyHC type IIb. During muscle regeneration, there is a progression in MyHC isoform expression from early embryonic and neonatal isoforms through to mature MyHC isoforms such as type IIb and IIa/x.³¹ In uninjured TA muscles, 80% of the MyHCs are of the type IIb isoform, with the remaining 20% being MyHC type IIa/x.¹⁶ NBI-31772 treatment facilitated a more rapid progression from these slower-contracting MyHC isoforms to the faster MyHC isoforms and so increased the overall rate of

regeneration. This is further evident from the increase in median fiber CSA observed in regenerating TA muscles after IGFBP inhibition.

Similar to its effects on regenerating TA muscles, IGFBP inhibition increased the speed of relaxation in EDL muscles from *mdx* mice, which was associated with a decrease in fatigue resistance, a reduced force producing capacity after fatigue, and a reduced citrate synthase activity. Taken together, these findings show that IGFBP inhibition caused a shift toward a faster, less oxidative muscle phenotype in *mdx* mice. This finding is in direct contrast to results obtained in previous studies where low-dose rhIGF-I administration resulted in a more oxidative, less fatigable muscle phenotype in *mdx* mice.^{14–16} However, following plasmid-based overexpression of IGF-I in rat skeletal muscle, a shift toward a more glycolytic phenotype has been observed,³² an effect also seen in transgenic mice over-expressing muscle-specific IGF-I.³³ The effects of IGF-I on skeletal muscle oxidative capacity appear to be strongly modulated by IGFBPs, and IGFBP inhibition promotes a glycolytic muscle phenotype similar to that observed with IGF-I overexpression. These results also suggest that the skeletal muscle response to exogenous administration of rhIGF-I is altered through interactions with IGFBPs. Interestingly, in EDL muscles from dystrophic *mdx* mice, median fiber CSA decreased with IGFBP inhibition. Together with the effects on the regenerating TA muscle, this indicates that although IGFBP inhibition increases early events of muscle fiber regeneration, including proliferation and differentiation,^{8,9,34,35} it does not alter the rate of muscle fiber degeneration in *mdx* mice. Thus, for dystrophic muscles, IGFBP inhibition may increase the proportion of smaller caliber regenerating muscle fibers.

Similar to findings from our other studies where we administered rhIGF-I to *mdx* mice,¹⁶ IGFBP inhibition in *mdx* mice reduced the susceptibility to contraction-induced injury in EDL muscles and diaphragm muscle strips. This finding has significant therapeutic implications as contraction-mediated injury is a contributing factor to the progressive muscle fiber breakdown in muscular dystrophy³ and further highlights the therapeutic potential of modulating the IGF-I signaling pathway for ameliorating muscle pathologies. It is interesting to note that studies demonstrating a reduced susceptibility of dystrophic skeletal muscles to contraction-induced injury have also reported a shift toward a slower muscle phenotype. It has been shown that fast-twitch muscles from dystrophic *mdx* mice are particularly susceptible to contraction-induced damage,^{26,36,37} a finding supported by studies on Duchenne muscular dystrophy patients where fast-twitch fibers were more susceptible to such damage than slow-twitch fibers.³⁸ From these studies, and from others by our laboratory,^{14–16} it had been hypothesized that the IGF-I-mediated protection from contraction-induced injury was due to the induction of a slower phenotype that renders muscles less susceptible to such injury. However, the results of the present study are the first to demonstrate protection from contraction-induced injury concomitant with a faster, less oxidative muscle phenotype. Although this increased fatigability may be

viewed as being detrimental, it is unlikely that such repeated maximal contractions would ever be performed by patients. Regardless, *in vivo* measures of strength, endurance, and longevity would help to resolve these issues. Under normal situations, the primary disability in dystrophic muscles is an inability to generate sufficient force to perform the tasks of daily living because of a lack of viable muscle tissue, rather than a lack of functional capacity to perform sustained maximal contractions. It is therefore likely that the IGF-I-mediated protection from contraction-induced injury occurs through an altogether different mechanism, potentially by altering specific parameters of excitation-contraction coupling.¹⁶ From the contrasting results obtained with NBI-31772 and rhIGF-I administration in *mdx* mice, it is clear that IGFBPs play a significant role in the determination of skeletal muscle phenotype, either independently or via their interactions with IGF-I.

We have demonstrated that systemic inhibition of IGFBPs has beneficial effects for muscle fiber regeneration and for the dystrophic pathology in *mdx* mice. One of the limitations of the present study was the use of a non-specific aptamer that inhibited the actions of all six IGFBPs. Previous studies have demonstrated that during regeneration skeletal muscle levels of IGFBPs are regulated differentially. For example, IGFBP3 expression is increased during early muscle regeneration following ischemic injury,³⁹ whereas IGFBPs 4, 5, and 6 are increased during the later stages of regeneration.^{39,40} Future studies should aim to inhibit specific IGFBPs to determine the exact role of each isoform in skeletal muscle regeneration. Identifying the specific roles for IGFBPs during muscle fiber regeneration will have important therapeutic implications for the muscular dystrophies and muscle wasting disorders.

In conclusion, this study examined the effect of modulating IGFBP interactions with IGF-I on the functional properties of regenerating skeletal muscle and on the dystrophic pathology in *mdx* mice. We have demonstrated an entirely novel way of manipulating IGF-I signaling for improving muscle fiber regeneration. We demonstrated that IGFBP inhibition significantly enhanced muscle fiber regeneration and increased the rate of functional recovery after myotoxic injury. We also showed that IGFBP inhibition in *mdx* mice reduced the susceptibility of dystrophic muscles to contraction-mediated injury, a contributing factor to the etiology of the dystrophic pathology. The findings highlight the therapeutic potential of IGF-I signaling for pathologies where muscle wasting and weakness are indicated.

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