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A growth stimulus is needed for IGF-1 to induce skeletal muscle hypertrophy in vivo

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Summary

Here, we characterise new strains of normal and dystrophic (*mdx*) mice that overexpress Class 2 IGF-1 Ea in skeletal myofibres. We show that transgenic mice have increased muscle levels of IGF-1 (~13-26 fold) and show striking muscle hypertrophy (~24-56% increase in mass). Adult normal muscles were resistant to elevated IGF-1; they reached adult steady state and maintained the same mass from 3 to 12 months. By contrast, dystrophic muscles from *mdx*/IGF-1(C2:Ea) mice continued to increase in mass during adulthood. IGF-1 signalling was evident only in muscles that were growing as a result of normal postnatal development (23-day-old mice) or regenerating in response to endogenous necrosis (adult *mdx* mice). Increased phosphorylation of Akt at Ser473 was not evident in fasted normal adult transgenic muscles, but was 1.9-fold higher in fasted normal young transgenic muscles compared with age-matched wild-type controls and fourfold higher in fasted adult *mdx*/IGF-1(C2:Ea) compared with *mdx* muscles. Muscles of adult *mdx*/IGF-1(C2:Ea) mice showed higher $p70^{S6K}$(Thr421/Ser424) phosphorylation and both young transgenic and adult *mdx*/IGF-1(C2:Ea) mice had higher phosphorylation of rpS6(Ser235/236). The level of mRNA encoding myogenin was increased in normal young (but not adult) transgenic muscles, indicating enhanced myogenic differentiation. These data demonstrate that elevated IGF-1 has a hypertrophic effect on skeletal muscle only in growth situations.

Key words: IGF-1, Skeletal muscle, Growth

Introduction

Insulin-like growth factor-1 (IGF-1) is essential for normal growth and development. The mammalian gene that encodes IGF-1 (*Igf1*) consists of six exons and gives rise to several mRNA variants that are produced by differential promoter usage and alternative splicing. These transcript variants code for different precursor IGF-1 isoforms, that after cleavage of the leader (signal) and C-terminal E domains, yield a common 70 amino acid peptide required for activating IGF-1 receptors (reviewed by Shavlakadze et al., 2005b). Transcripts that initiate from exon 1 or exon 2 are termed Class 1 or Class 2 variants, respectively. Furthermore, in rodents, alternative splicing of exons at the 3′-end of the mRNA yields Ea and Eb variants (reviewed by Shavlakadze et al., 2005b). The mRNAs encoding exon 1 and Ea are expressed ubiquitously, whereas those encoding exon 2 and Eb are predominately expressed in the liver (Adamo et al., 1991; Butler et al., 1994). It is thought that Class 2 transcripts contribute to the release of IGF-1 into circulation, because their appearance coincides with that of circulating IGF-1 (Adamo et al., 1991). Liver expresses Class 1, Class 2, Ea and Eb transcripts (Adamo et al., 1991; Butler et al., 1994) and contributes to 75% of the circulating IGF-1 level (Le Roith et al., 2001). Various other tissues also produce IGF-1, where it can act in an autocrine or paracrine manner (Yakar et al., 2005) or can be released into the bloodstream (Naranjo et al., 2002). In skeletal muscle, IGF-1 transcripts initiate from exon 1 (Class 1 variants) (Butler et al., 1994; Shemer et al., 1992) and in rodents they can be spliced to carry Ea or Eb termination sequences (Barton, 2006; Hameed et al., 2003; Shavlakadze et al., 2005b). Whether different IGF-1 isoforms exert different biological functions, or whether existence of these isoforms reflects the mechanism for tissue (or environment) specific regulation of IGF-1 expression remains unclear.

IGF-1 acts via a transmembrane tyrosine kinase receptor and exerts an anabolic effect on skeletal muscle. Studies in cultured myotubes suggest that IGF-1 promotes muscle hypertrophy by activating PI3K/Akt signalling (Chakravarthy et al., 2001; Rommel et al., 2001), which leads to activation of mTOR and its downstream targets $p70^{S6K}$ and 4E-BP1 (Rommel et al., 2001). The hierarchy of the signalling events in this pathway was established by using inhibitors that were specific to various components of the pathway: the PI3K inhibitor blocked activation of Akt, mTOR and phosphorylation of its downstream targets, whereas the mTOR inhibitor did not affect Akt, but blocked phosporylation of $p70^{S6K}$ and 4E-BP1 (Rommel et al., 2001).

In vivo infusion of IGF-1 into muscles of ~10-week-old mice increases protein synthesis (Bark et al., 1998) and the muscle-specific overexpression of IGF-1 results is muscle hypertrophy (Barton, 2006; Fiorotto et al., 2003; Musaro et al., 2001). Although the hypertrophic effect of IGF-1 in live animals has been shown by various studies, very limited data are available concerning the mechanism of such hypertrophy in vivo. Since the Akt/mTOR pathway is activated in cultured myotubes that undergo hypertrophy, there has been much focus on this pathway, although in vivo the situation appears more complicated. It is noted that myotubes in vitro represent growing or differentiating muscle cells and are not equivalent to mature myofibres of adult muscle.

The role of IGF-1 signalling, specifically of Akt and mTOR activation for the maintenance of muscle mass in adult animals, is not clear, because pharmacological blockade of either of these

molecules in rats and mice does not affect muscle mass (Bodine et al., 2001). However, overexpression of constitutively active Akt in adult rat muscles results in myofibre size increase of ~60% (Bodine et al., 2001). Akt and mTOR signalling becomes activated in muscles that undergo compensatory hypertrophy in response to increased loading and in muscles that recover from atrophy caused by limb suspension (Bodine et al., 2001). In these models, blockade of mTOR prevents muscle mass increase (Bodine et al., 2001); however, it is not clear whether in overloaded muscles mTOR is activated downstream from Akt, because Akt-independent activation of mTOR has been shown in stretched muscles ex vivo (Hornberger et al., 2004).

In addition, it has not been conclusively established whether elevated IGF-1 increases Akt/mTOR signalling in vivo to result in muscle hypertrophy. Increased phosphorylation of Akt and p70^{S6K} has been shown in skeletal muscles after intra-peritoneal injection of des IGF-1 (truncated variant of IGF-1 with a reduced affinity to IGF-1-binding proteins) into mice (Li et al., 2002; Li et al., 2003) and Akt was also hyper-phosphorylated in skeletal muscles of mice following viral delivery of IGF-1 Ea isoform (Barton, 2006). By contrast, increased phosphorylation of mTOR(Ser2448) without increased phosphorylation of Akt was reported in skeletal muscles of adult transgenic mice with muscle-specific overexpression of IGF-1 Ea (Song et al., 2005). These mice also showed increased phosphorylation of p70^{S6K} on Thr421 and Ser424, but not on Thr389, which represents a major target site for the activated mTOR (Song et al., 2005).

A confounding factor in studies that examine the effect of IGF-1 on skeletal muscle hypertrophy is the age of the animals. With respect to the impact of IGF-1 on hypertrophy, studies in humans and mice suggest that adult muscle seems to be resistant to elevated levels of IGF-1. This is exemplified by studies in humans where administration of growth hormone to increase levels of circulating IGF-1 failed to ameliorate age-related muscle wasting (sarcopaenia) (Adams, 2000; Lynch, 2001). Furthermore, transgenic SK733 IGF-1 3′SK mice that overexpress the human IGF-1 peptide (driven by a chicken skeletal α-actin promoter) show that IGF-1-enhanced muscle growth and the increased rate of protein accretion are evident only until the mice reach adulthood (up to 10 weeks) (Fiorotto et al., 2003). In IGF-1(C1:Ea) mice (or mIGF-1 mice), the percentage increase in muscle mass is the same in transgenic and wild-type mice from 2 to 6 months (Musaro et al., 2001). A later study, in which IGF-1 Ea was virally delivered to skeletal muscles also showed that the responsiveness of muscle is blunted with age (Barton, 2006): 2 months after viral delivery of IGF-1 Ea to 2- to 3-week-old mice, the Extensor digitorum longus muscle mass increased by approximately 12%; however, by 4 months following the delivery, the difference in muscle mass between control mice and mice with overexpression of IGF-1 Ea was halved (Barton, 2006).

The present study used novel transgenic mice generated to constitutively overexpress the murine Class 2 IGF-1 Ea isoform in skeletal myofibres under the *Mlc1/3* promoter [IGF-1(C2:Ea) mice]. The Class 2 isoform has not been overexpressed in skeletal muscle previously and indeed Class 2 transcripts are not normally expressed in skeletal muscles (Shemer et al., 1992). To address the question of whether IGF-1 isoforms with different signal sequences (Class 1 and Class 2) have different abilities to cause skeletal muscle hypertrophy, the phenotypes of these novel IGF-1(C2:Ea) mice were compared with the well-described IGF-1(C1:Ea) strain, which overexpresses the Class 1 IGF-1 Ea isoform (Musaro et al., 2001; Shavlakadze et al., 2005a).

Phosphorylation of the signalling molecules (Akt, mTOR and p70^{S6K}) downstream from the IGF-1 receptor was also measured in muscles overexpressing IGF-1(C2:Ea), because activation of these kinases is implicated in the increased protein synthesis associated with hypertrophy. The signalling analysis was conducted in young growing (23-day-old) and fully adult IGF-1(C2:Ea) and wild-type muscles for both normal and dystrophic (*mdx*) transgenic and control mice. This allowed comparison of the response of muscles in vivo to elevated transgenic IGF-1 during the growth phase (postnatal development), in mature adult normal muscle and in regenerating dystrophic muscles.

Results

Total IGF-1 levels in skeletal muscle and blood

Transgenic non-dystrophic and dystrophic *mdx* mice were generated to express the Class 2 Ea isoform of IGF-1 (Fig. 1). Skeletal muscle and blood levels of IGF-1 protein were measured in wild-type, IGF-1(C2:Ea), *mdx* and *mdx*/IGF-1(C2:Ea) mice aged 3 and 12 months (Fig. 2A,B). Three-month-old IGF-1(C1:Ea) mice and their respective wild-type littermates were also included for comparison. All mice overexpressing the Class 2 or Class 1 IGF-1 Ea isoforms had significantly higher content of IGF-1 (~13-26-fold increase) in gastrocnemius muscles compared with non-transgenic littermates (Fig. 2A). There was a trend (P=0.54) towards increased circulating IGF-1 only in 3-month-old male IGF-1(C2:Ea) mice compared with wild-type mice and significantly increased IGF-1 (~1.4-fold) was seen in 12-month-old *mdx*/IGF-1(C2:Ea) males compared with

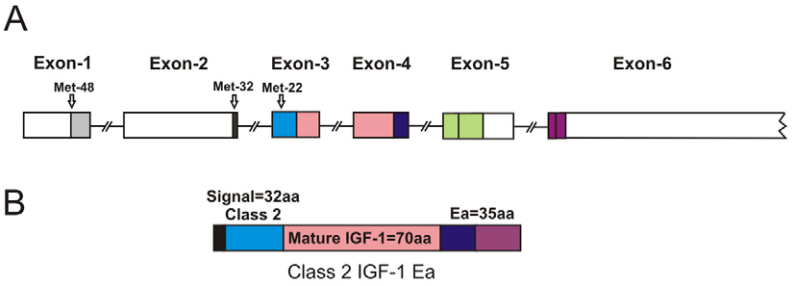

Fig. 1. Structure of the rodent gene encoding IGF-1 and the Class 2 IGF-1 Ea isoform used in the transgenic IGF-1(C2:Ea) mice. (**A**) Exon 1 and exon 2 of *Igf1* encode distinct signal peptides of the IGF-1 precursor. 70 amino acids of the mature IGF-1 are encoded by exons 3 and 4. Exon 4 also encodes the N-terminal portion of the extension peptide (E-peptide). Exons 5 and 6 each code for distinct portions of the E-peptides, termination codons and 3′-untranslated regions. (**B**) Class 2 IGF-1 Ea includes a signal sequence coded by exon 2 and exon 3 (32 amino acids), a sequence for mature IGF-1 (70 amino acids) and the Ea termination sequence (35 amino acids) generated by splicing of exon 4 to exon 6.

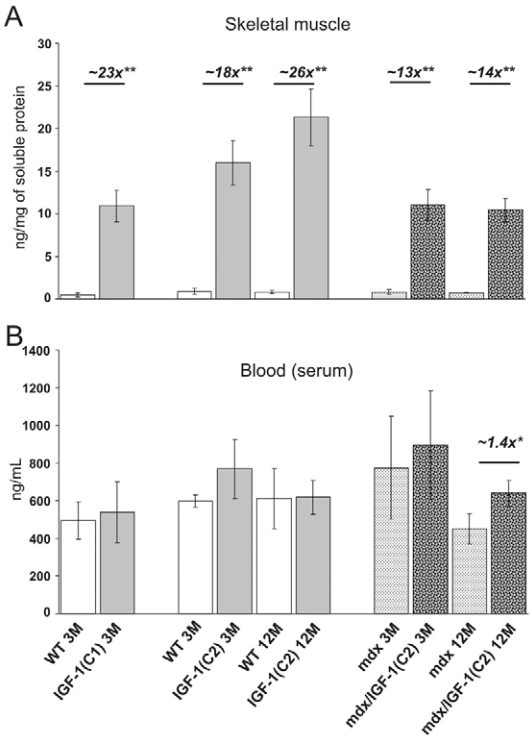

Fig. 2. Total IGF-1 protein levels in skeletal muscle and blood serum. Protein levels in (**A**) skeletal muscle and (**B**) blood from male IGF-1(C1:Ea), IGF-1(C2:Ea) and *mdx*/IGF-1(C2:Ea) mice and their respective non-transgenic littermate controls [wild-type (WT) or *mdx*] aged 3 and 12 months. n=4-8 mice for each group. **P<0.005; *P<0.05. Significant increases relative to controls are indicated above bars.

control littermates (Fig. 2B). There was no difference in the circulating IGF-1 levels between *mdx*/IGF-1(C2:Ea) and *mdx* mice at 3 months (Fig. 2B).

Linear growth, body and muscle mass in non-dystrophic IGF-1(C2:Ea) and dystrophic *mdx*/IGF-1(C2:Ea) mice

Tibia length, body mass and quadriceps muscle mass were compared between wild-type and IGF-1(C2:Ea) littermate males and females aged 3 and 12 months (Table 1, Fig. 3A,B). Measurements from 3-month-old IGF-1(C1:Ea) and their respective wild-type littermates were included for comparison. The tibia length was significantly increased at 12 months, but not at 3 months, for male and female IGF-1(C2:Ea) mice compared with age- and sex-matched wild-type littermates, and this increase was more pronounced in males than in females (Table 1). Similarly, the tibia length increase was not significant in 3-month-old IGF-1(C1:Ea) mice compared with their respective wild-type littermates. Body mass, normalised to tibia length was significantly increased for 3-month-old transgenic IGF-1(C1:Ea) males, for 3- and 12-month-old transgenic IGF-1(C2:Ea) males, and for 3-month-old IGF-1(C2:Ea) females compared with age- and sex-matched wild-type littermates (Table 1).

The quadriceps muscle mass was also standardised to the tibia length (Fig. 3A,B). The standardised quadriceps muscle mass was significantly increased in all male IGF-1(C2:Ea) and IGF-1(C1:Ea) mice, compared with their respective age-matched wild-type littermates (Fig. 3A). There was no difference in the quadriceps mass increase between IGF-1(C2:Ea) and IGF-1(C1:Ea) mice compared with the wild-type controls, which indicates that Class 1 or Class 2 IGF-1 Ea isoforms produce similar muscle hypertrophy (Fig. 3A). In female IGF-1(C2:Ea) mice, the quadriceps muscle mass was significantly increased at 12 months but not at 3 months compared with non-transgenic controls (Fig. 3B). The absence of the significant muscle hypertrophy in 3-month-old females demonstrates a different gender response to the increased IGF-1 level.

Tibia length, body mass and quadriceps muscle mass were compared between *mdx* and *mdx*/IGF-1(C2:Ea) littermate males and females at 3 and 12 months (Table 2, Fig. 3A,B). Tibia length was significantly increased only in 12-month-old *mdx*/IGF-1(C2:Ea) males compared with age- and sex-matched *mdx* littermates (Table 2). Increased body mass (normalised to tibia length) was seen for all male *mdx*/IGF-1(C2:Ea) transgenic mice relative to non-transgenic controls (Table 2). In *mdx*/IGF-1(C2:Ea) females, the body mass increase was significant at 12 months, but not at 3 months (Table 2). Quadriceps muscle mass was significantly increased for all male *mdx*/IGF-1(C2:Ea) mice and this increase was more pronounced at 12 (~56%) compared with 3 months (~24%) (Fig. 3A). In female *mdx*/IGF-1(C2:Ea) mice, the quadriceps muscle mass

Table 1. Tibia length and standardised body mass of wild-type and IGF-1(C2:Ea) mice

Genotype	Sex	Age (months)	No.	Tibia length (cm)	Comparison with wild type (P)	Body mass standardised to tibia length (g/cm)	Comparison with wild type (P)
Wild type	Male	3	9	1.74±0.07	–	15.92±0.79	–
IGF-1(C1:Ea)	Male	3	6	1.81±0.04	+4.0% (NS)	17.33±1.2	+8.8% (P<0.05)
Wild type	Male	3	4	1.71±0.09	–	15.94±0.34	–
IGF-1(C2:Ea)	Male	3	6	1.77±0.05	+3.5% (NS)	16.89±0.26	+5.9% (P<0.005)
Wild type	Female	3	6	1.72±0.04	–	12.00±0.41	–
IGF-1(C2:Ea)	Female	3	4	1.75±0.06	+1.7% (NS)	14.49±0.67	+20.7% (P<0.005)*
Wild type	Male	12	8	1.74±0.05	–	17.75±0.99	–
IGF-1(C2:Ea)	Male	12	7	1.82±0.03	+4.6% (P<0.05)	19.49±0.96	+9.8% (P<0.01)
Wild type	Female	12	7	1.80±0	–	15.14±1.21	–
IGF-1(C2:Ea)	Female	12	8	1.81±0.02	+0.5% (P<0.05)	16.16±1.77	+6.7% (NS)

Parameters for 3-month-old male IGF-1(C1:Ea) mice and their respective wild-type littermate controls are included for comparison. The relative difference is indicated before the statistical significance. NS, not significant.

*A large increase in the body mass of 3-month-old female IGF-1(C2:Ea) mice compared with the wild-type littermates was partly due to significantly increased abdominal fat deposition. Such increase in the abdominal fat pad mass was transient and was no longer detected at 12 months (data not shown). staining. NS, not significant.

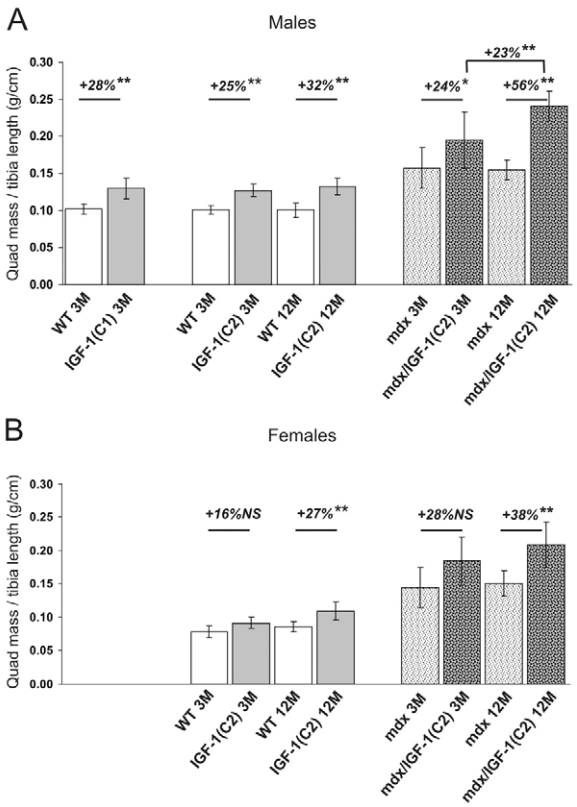

Fig. 3. Quadriceps muscle mass standardised to tibia length. Male (**A**) and female (**B**) IGF-1(C2:Ea) and *mdx*/IGF-1(C2:Ea) mice and their respective non-transgenic littermate controls [wild type (WT) or *mdx*] aged 3 and 12 months. Standardised quadriceps muscle mass from 3-month-old IGF-1(C1:Ea) mice and their wild-type littermates are also included for males (A) but not females (B). n=6-15 mice for each group. **P<0.005; *P<0.05; NS, not significant. Increases relative to controls are indicated above bars.

was significantly increased at 12 months (~38%), but not at 3 months (Fig. 3B).

Myofibre CSA in non-dystrophic IGF-1(C2:Ea) and dystrophic *mdx*/IGF-1(C2:Ea) muscles

The cross-sectional area (CSA) of different myofibre types was determined in quadriceps muscle sections stained with NADH-TR from non-dystrophic mice to differentiate fast (type 2B), intermediate (type 2 A,X) and slow (type 1) myofibre types (Shavlakadze et al., 2005a) (Table 3). At 3 months, the CSA of the fast (type 2B) myofibres was significantly increased by ~10% in male IGF-1(C2:Ea) quadriceps compared with controls: these fast myofibres manifest the highest expression of the *Mlc1/3* promoter, which drives the expression of the transgenic IGF-1(C2:Ea). The intermediate myofibres (type 2 A,X) also showed a trend towards increased CSA in the quadriceps of the male IGF-1(C2:Ea) mice, however this trend was not statistically significant. In quadriceps of 12-month-old males, all three types of myofibres were significantly increased in size: the CSA of fast, intermediate and slow myofibres was increased by ~21%, ~14% and ~21%, respectively.

In 3-month-old females, the CSA of the fast, intermediate and slow myofibres was similar between wild-type and IGF-1(C2:Ea) quadriceps (Table 3), in accordance with no significant increase in the quadriceps muscle weight of 3-month-old IGF-1(C2:Ea) females (Fig. 3B). Quadriceps of female IGF-1(C2:Ea) mice aged 12 months had significantly larger (~16%) fast myofibres and there was a trend towards increased CSA of intermediate and slow myofibres (Table 3).

Myofibre CSA was also measured on haematoxylin-and-eosin-stained sections of the quadriceps muscles from 3- and 12-month-old male *mdx* and *mdx*/IGF-1(C2:Ea) mice (Fig. 4A,B). Myofibre types were not distinguished with NADH-TR, since this staining does not yield reliable results in regenerating (*mdx*) muscles (T.S., unpublished observations). The mean myofibre CSA was significantly larger (by ~30%) in 3-month-old transgenic *mdx*/IGF-1(C2:Ea) quadriceps compared with control *mdx* quadriceps (Fig. 4Aa,b,B). However, there was no difference in the myofibre CSA between 12-month-old *mdx* and *mdx*/IGF-1(C2:Ea) quadriceps (Fig. 4Ac,d,B). The reduced average myofibre CSA in 12-month-old *mdx*/IGF-1(C2:Ea) muscle (compared with 3 months) might be due to myofibre splitting and/or branching; supporting this explanation, myofibres with the appearance of splitting or branching were present in both *mdx* and *mdx*/IGF-1(C2:Ea) muscles at 12 months (Fig. 4C).

Akt and mTOR phosphorylation in adult IGF-1(C2:Ea) muscle

Since fully adult IGF-1(C2:Ea) mice manifest increased muscle mass, we looked at activation of the Akt/mTOR signalling, which is involved in muscle hypertrophy. We first compared phosphorylation levels of Akt(Ser473) and mTOR(Ser2448) in gastrocnemius muscles of 3-month-old wild-type and IGF-1(C2:Ea) mice after an overnight fast. Fasting is important for analyses of IGF-1 signalling, because insulin and amino acids that rapidly increase after feeding activate signalling molecules involved in this pathway (Suryawan et al., 2007). No difference was seen in the

Table 2. Tibia length and standardised body mass of dystrophic *mdx* and *mdx*/IGF-1(C2:Ea) mice

Genotype	Sex	Age	No.	Tibia length (cm)	Comparison with *mdx* (*P*)	Body mass standardized to tibia length (g/cm)	Comparison with *mdx* (*P*)
mdx	Male	3 months	15	1.72±0.08	–	18.88±1.84	–
mdx/IGF-1(C2:Ea)	Male	3 months	9	1.77±0.09	+3% (NS)	19.66±1.72	+10% (P<0.05)
mdx	Female	3 months	6	1.72±0.04	–	14.81±2.17	–
mdx/IGF-1(C2:Ea)	Female	3 months	6	1.77±0.07	+3% (NS)	16.48±1.50	+14% (NS)
mdx	Male	12 months	6	1.78 ±0.041	–	19.3±1.3	–
mdx/IGF-1(C2:Ea)	Male	12 months	10	1.84±0.05	+3% (P<0.05)	22.96±1.38	+19% (P<0.005)
mdx	Female	12 months	5	1.80	–	16.42±2.63	–
mdx/IGF-1(C2:Ea)	Female	12 months	9	1.83±0.04	+2% (NS)	19.52±1.72	+19% (P<0.05)

The relative difference is indicated before the statistical significance. NS, not significant.

Table 3. Cross-sectional area of fast, intermediate and slow myofibres*

Genotype	Sex	Age	No.	Fast (μm^2)	Comparison with wild type (*P*)	Intermediate (μm^2)	Comparison with wild type (*P*)	Slow (μm^2)	Comparison with wild type (*P*)
Wild type	Male	3 months	6	4.042±200	–	2.359±206	–	1.622±113	–
IGF-1(C2:Ea)	Male	3 months	8	4.438±81	+10% (*P*<0.005)	2.640±125	+12% (NS)	1.653±138	+2% (NS)
Wild type	Female	3 months	6	4041±348	–	2.579±181	–	1.752 ±176	–
IGF-1(C2:Ea)	Female	3 months	4	4058±447	+0.4% (NS)	2.312±589	–10% (NS)	1.765±217	+1% (NS)
Wild type	Male	12 months	8	4.031±280	–	2.481±253	–	1.565±185	–
IGF-1(C2:Ea)	Male	12 months	5	4.858±338	+21% (*P*<0.005)	2.826 ±185	+14% (*P*<0.005)	1.895±159	+21% (*P*<0.005)
Wild type	Female	12 months	7	3.768±279	–	2.556±245	–	1.677±243	–
IGF-1(C2:Ea)	Female	12 months	7	4.367±580	+16% (*P*<0.05)	2.978±325	+16% (NS)	1.932±209	+15% (NS)

*Myofibres from the quadriceps of male and female wild-type and IGF-1(C2:Ea) mice at 3 and 12 months were differentiated using NADH-TR staining. NS, not significant.

phosphorylation levels of Akt(Ser473) or mTOR(Ser2448) in wild-type and IGF-1(C2:Ea) muscles (Fig. 5). These data demonstrate that although adult IGF-1(C2:Ea) transgenic mice have significantly increased muscle mass, they do not manifest increased Akt/mTOR signalling.

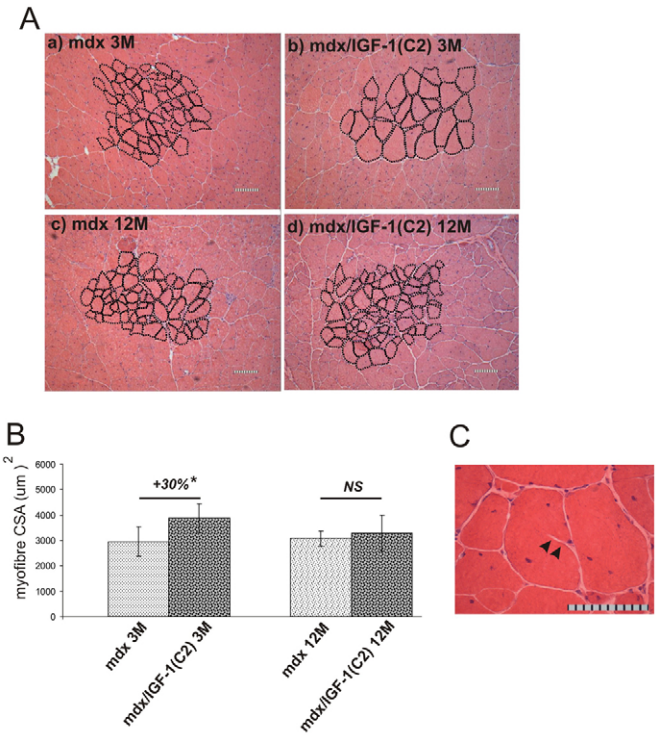

Fig. 4. Myofibre CSA in quadriceps muscles of 3- and 12-month-old male *mdx* and *mdx*/IGF-1(C2:Ea) mice. (**A**) H&E-stained sections of *mdx* (a,c) and *mdx*/IGF-1(C2:Ea) (b,d) quadriceps muscles with outlined examples of myofibres. At 3 months (3M), myofibre CSAs are visibly larger in *mdx*/IGF-1(C2:Ea) compared with *mdx* muscles (a,b). However, at 12 months (12M), no difference in the myofibre CSA is evident between *mdx* and *mdx*/IGF-1(C2:Ea) muscles (c,d). (**B**) For each individual quadriceps, 500 myofibres were measured from five randomly selected fields. (**C**) An example of a splitting or branching myofibre in the 12-month-old *mdx*/IGF-1(C2:Ea) quadriceps. Arrowheads indicate partial segmentation of the myofibre sarcoplasm characteristic of the initial splitting or branching process. Such myofibre division results in more myofibres with smaller CSA profiles. *n*=4 for each animal group. *P<0.05; NS, not significant. Scale bar: 100 μm.

Akt/mTOR/p70^{S6K} signalling in growing 23-day-old wild-type and IGF-1(C2:Ea) muscles

We hypothesised that the failure of IGF-1(C2:Ea) to increase Akt and mTOR phosphorylation in adult IGF-1(C2:Ea) muscle was due to the fact that the adult muscles had reached a stable state (minimal or no growth) and the signalling pathways responsible for protein synthesis were no longer significantly responsive to the elevated IGF-1. Thus, we next compared the phosphorylation levels of Akt(Ser473) and mTOR(Ser2448) in overnight-fasted young 23-day-old male IGF-1(C2:Ea) mice and their wild-type littermates (Fig. 6); day 23 was selected because mice undergo intensive growth up to 4 weeks of age (Davis and Fiorotto, 2009; Grounds et al., 2008). Young IGF-1(C2:Ea) muscles had 1.9-fold higher Akt(Ser473) phosphorylation compared with age-matched wild-type muscles (Fig. 6A,B); however, higher phosphorylation of Akt(Ser473) did not result in a higher phosphorylation of mTOR(Ser2448) in IGF-1(C2:Ea) muscles (Fig. 6A). As expected, Akt(Ser473) phosphorylation levels were high in fed mice and similar levels were seen for young wild-type and transgenic muscles (data not shown).

We next compared phosphorylation of mTOR and p70^{S6K} between freely fed and overnight-fasted 23-day-old mice (Fig. 7A,B), on the basis that feeding and amino acid availability is essential for activation of mTOR signalling and overnight fasting causes dephosphorylation of its downstream target p70^{S6K} (Sans et al., 2004). No difference was seen in mTOR(Ser2448) phosphorylation between wild-type and IGF-1(C2:Ea) muscles and these levels were not affected by fasting (Fig. 7A). Note that this is an additional set of data to that shown in Fig. 6A. Thr389 and Thr421/Ser424 phosphorylation was conspicuous in freely fed wild-

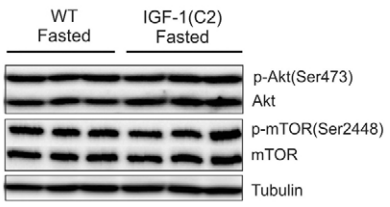

Fig. 5. Phosphorylation of Akt and mTOR in skeletal muscles of adult wild-type and IGF-1(C2:Ea) mice. Protein extracted from gastrocnemius muscles of 3-month-old wild-type (WT) and IGF-1(C2:Ea) mice fasted overnight was immunoblotted with antibodies against phosphorylated Akt(Ser473), total Akt, phosphorylated mTOR(Ser2448) and total mTOR. α-tubulin is shown as a control for loading.

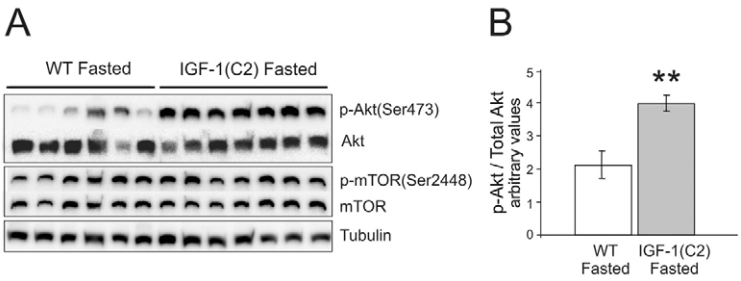

Fig. 6. Phosphorylation of Akt and mTOR in skeletal muscles of young wild-type and IGF-1(C2:Ea) mice. (**A**) Protein extracted from gastrocnemius muscles of 23-day-old wild-type (WT) and IGF-1(C2:Ea) mice fasted overnight was immunoblotted with antibodies against phosphorylated Akt(Ser473), total Akt, phosphorylated mTOR(Ser2448) and total mTOR. α-tubulin is shown as a control for loading. (**B**) Densitometric quantification of Akt(Ser473) to total Akt is based on n=10 for each wild-type and IGF-1(C2:Ea) group. $^{**}P<0.005$.

type and transgenic mice (Fig. 7B). However, these sites dephosphorylated to the same extent in fasted wild-type and IGF-1(C2:Ea) muscles (Fig. 7B).

Ribosomal protein S6 (rpS6) is a downstream target of p70^{S6K} (Ruvinsky and Meyuhas, 2006), therefore, we used rpS6 as a readout for the activation of p70^{S6K} and compared phosphorylation of rpS6 between freely fed and overnight-fasted 23-day-old mice (Fig. 7B). Phosphorylation of rpS6(Ser235/236) was high in all fed mice (Fig. 7B). After an overnight fast, phosphorylation of rpS6 was still easily detectable in IGF-1(C2:Ea) muscles, whereas it was greatly reduced in wild-type muscles (Fig. 7B).

Akt/mTOR/p70^{S6K} signalling in adult *mdx* and *mdx*/IGF-1(C2:Ea) muscles

Because we detected higher Akt(Ser473) phosphorylation in young growing, but not adult IGF-1(C2:Ea) muscles compared with age-matched wild-type muscles, we hypothesised that IGF-1 acts in a synergistic manner with the growth stimulus and that IGF-1 activation of the Akt signalling pathway is conspicuous only when the growth stimulus is present. In the 23-day-old immature muscles, normal postnatal maturation and increase in muscle mass served as the growth stimulus. We proposed that a similar hypertrophic response to elevated IGF-1(C2:Ea) would also be apparent in dystrophic muscles of adult *mdx* mice, because dystrophic skeletal muscles undergo persistent cycles of muscle necrosis and regeneration, followed by ongoing growth and maturation of the newly formed myotubes and young myofibres throughout adult life (in marked contrast to stable myofibres of normal adult mice). Therefore, immunoblots were performed on skeletal muscles from male adult 3-month-old overnight-fasted *mdx* and *mdx*/IGF-1(C2:Ea) mice to detect phosphorylated Akt(Ser473), mTOR (Ser2448), p70^{S6K}(Thr389), p70^{S6K}(Thr421/Ser424) and rpS6(Ser235/236) (Fig. 8).

Phosphorylation of Akt(Ser473) was fourfold higher in *mdx*/IGF-1(C2:Ea) compared with *mdx* muscles, which confirmed enhanced signalling in response to elevated IGF-1 in the regenerating muscles of adult *mdx* mice (Fig. 8A,B). Phosphorylation of mTOR(Ser2448) was similar between genotypes (Fig. 8A). Phosphorylation of p70^{S6K}(Thr389) was readily detectable by immunoblotting in overnight-fasted adult dystrophic muscles, and these levels were similar for *mdx* and *mdx*/IGF-1(C2:Ea) (Fig. 8A). Phosphorylation of p70^{S6K}(Thr421/Ser424) and rpS6(Ser235/236) was also detectable in fasted dystrophic muscles and was elevated in *mdx*/IGF-1(C2:Ea) compared with *mdx* (Fig. 8A) mice, indicating an effect of IGF-1.

Myogenin mRNA content

Activation of Akt upon ligand binding to the IGF-1R has been associated with an increased expression of the skeletal muscle transcription factor myogenin (Musaro and Rosenthal, 1999) and therefore *Myog* mRNA levels were analysed in the present study (Fig. 9). *Myog* mRNA content was much lower in 3-month-old compared with 23-day-old wild-type muscles (–68%) and IGF-1(C2:Ea) muscles (–74%) (P<0.005). There was no difference between wild-type and transgenic muscles at 3 months. However, at 23 days, IGF-1(C2:Ea) muscles showed significantly higher levels (52% increase, P<0.05) of *Myog* mRNA compared with age-matched wild-type muscles, indicating an IGF-1-mediated increase in *Myog* transcript in growing (but not adult) muscle. No difference in *Myog* mRNA content was apparent between *mdx* and *mdx*/IGF-1(C2:Ea) muscles at 3 months.

Discussion

The phenotype of the novel transgenic mice generated to constitutively overexpress the murine IGF-1(Class2:Ea) isoform in skeletal myofibres under the *Mlc1/3* promoter was described. To assess whether the IGF-1 Ea isoforms initiated from the distinct exon 1 or exon 2 had different biological effects on skeletal muscle phenotype, the phenotype of the IGF-1(Class2:Ea) strain was compared with mice that overexpress the IGF-1(Class1:Ea) isoform (i.e. IGF-1 Ea isoform with a leader sequence that initiates from exon 1) under the same promoter (Musaro et al., 2001). No

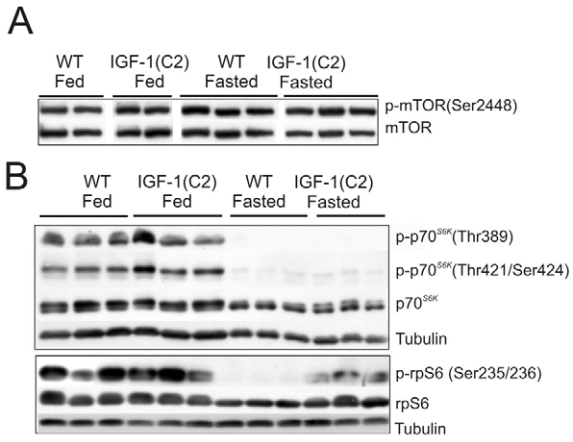

Fig. 7. Phosphorylation of mTOR, p70^{S6K} and ribosomal protein S6 in skeletal muscles of young wild-type and IGF-1(C2:Ea) mice. (**A**,**B**) Protein extracted from gastrocnemius muscles of freely fed and overnight-fasted 23-day-old wild-type (WT) and IGF-1(C2:Ea) mice was immunoblotted with antibodies against phosphorylated mTOR(Ser2448), total mTOR (A), p70^{S6K}(Thr389), p70^{S6K}(Thr421/Ser424), total p70^{S6K}, phosphorylated ribosomal protein S6(Ser235/236) and total ribosomal protein S6. α-tubulin is shown as a control for loading (B). For each group n=3-5 (representative blots are shown).

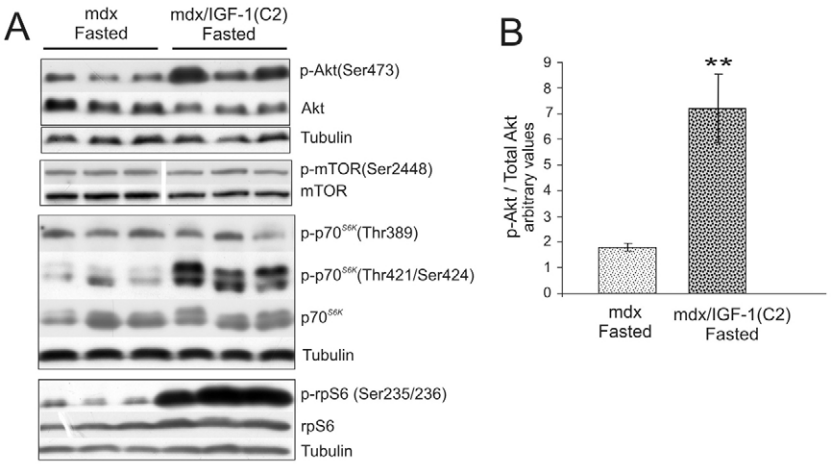

Fig. 8. Phosphorylation of Akt, mTOR, p70^{S6K} and ribosomal protein S6 in skeletal muscles of adult *mdx* and *mdx*/IGF-1(C2:Ea) mice. (**A**) Protein extracted from gastrocnemius muscles of 3-month-old *mdx* and *mdx*/IGF-1(C2:Ea) mice fasted overnight was immunoblotted with antibodies against phosphorylated Akt(Ser473), total Akt, phosphorylated mTOR(Ser2448), total mTOR, p70^{S6K}(Thr389), p70^{S6K}(Thr421/Ser424), total p70^{S6K}, phosphorylated ribosomal protein S6(Ser235/236) and total ribosomal protein S6. α-tubulin is shown as a control for loading. (**B**) Densitometric quantification for Akt(Ser473) to total Akt is based on n=10 for each wild-type and IGF-1(C2:Ea) group. $^{**}P$<0.005.

difference was found between the isoform specific IGF-1 transgenics and a striking association of IGF-1-mediated hypertrophy (phenotype and signalling) was demonstrated in vivo only in situations of muscle cell growth.

Expression of the *Mlc1/3* promoter is restricted to differentiated skeletal muscle cells with high level of expression in the fast-twitch myofibres (in the order 2B, 2X and 2A) and low levels in the slow-twitch (type 1) myofibres (Donoghue et al., 1991). Such an expression pattern of the transgene is reflected by a non-uniform increase in size for different type myofibres: this is especially evident in young adult 3-month-old IGF-1(C2:Ea) muscles (Table 3), where only type 2B myofibres show significant hypertrophy. Selective hypertrophy of the predominately fast muscles has been previously shown in Class 1 IGF-1 Ea mice (Musaro et al., 2001; Shavlakadze et al., 2005a). At 12 months, we saw a pronounced and significant size increase in all myofibre types in the IGF-1(C2:Ea) mice, which indicates that although IGF-1 expression is high in fast type myofibres, it has also affects slow type 1 myofibres.

In the present study, quadriceps muscles were used for morphometric analyses and gastrocnemius muscles were used for protein and mRNA expression analyses, because both of these muscles manifest the highest level of the *Mlc1/3* promoter (Musaro et al., 2001). For molecular analyses the entire gastocnemius muscle was used; however, because the 2B myofibre type predominates (representing 90% of total myosin heavy chain content) in gastrocnemius muscle of mice (Hamalainen and Pette, 1993), we extrapolated the effect of IGF-1 to this entire muscle.

The leader sequence does not affect the release of IGF-1 into circulation or the muscle phenotype

mRNA transcripts encoding Exon 2 (Class 2) IGF-1 are expressed in abundance in liver; however, they can also be detected at much lower levels in testes, lung, kidney and stomach (Shemer et al., 1992). They are undetectable in heart, brain and muscle (Shemer et al., 1992). Expression of transcripts encoding exon 2 is more dependent on growth hormone (compared with expression of exon 1 transcripts) and they are considered to contribute to the circulating levels of IGF-1 (Adamo et al., 1989; Adamo et al., 1991; Lowe et al., 1987). This assumption is based on the fact that transcripts encoding exon 2 appear later in postnatal development than exon 1 transcripts, increasing especially at the onset of GH-dependent linear growth, and their appearance coincides with that of circulating IGF-1 (Adamo et al., 1991).

We were interested to see whether overexpression of IGF-1(Class2:Ea) in skeletal muscles would result in increased blood levels of IGF-1 and how circulating IGF-1 levels would compare in Class 2 and Class 1 IGF-1 Ea mice. It has been assumed that IGF-1(Class1:Ea) mice do not have increased IGF-1 in blood; however, the actual measurements have not been done (Musaro et al., 2001). Comparison of blood (serum) levels of IGF-1 in male 3- and 12-month-old mice showed a trend towards increased circulating IGF-1 in 3-month-old IGF-1(Class2:Ea), but not IGF-1(Class1:Ea) mice compared with wild-type littermates. Only 1-year-old *mdx*/IGF-1(Class2:Ea) mice had significantly increased circulating IGF-1 compared with non-transgenic littermate controls.

These results indicate that muscle-specific overexpression of IGF-1 Ea isoforms that contain signal sequences from exon 1 or exon 2 does not significantly affect the circulating levels of IGF-1. Interestingly, overexpression of IGF-1(Class2:Eb) in liver elevates circulating IGF-1 by about 70% (Liao et al., 2006), which, together with our observations, suggests that the release of IGF-1 into blood is not dependent on the leader sequence, but instead depends on the tissue in which it is expressed. It is however noted that the mice generated by Liao and co-workers carry the IGF-1 isoform with

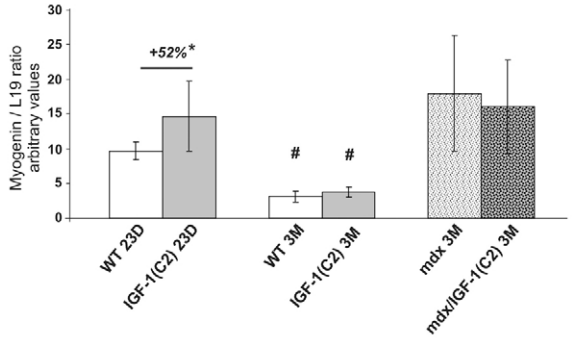

Fig. 9. *Myog* mRNA expression in the muscles of non-dystrophic and dystrophic mice. *Myog* mRNA expression standardised relative to *Rpl19* (L19) mRNA was quantified in gastrocnemius muscles of wild-type (WT) and IGF-1(C2:Ea) mice aged 23 days (23D) or 3 months (3M), and *mdx* and *mdx*/IGF-1(C2:Ea) mice aged 3 months. n=6 per each group. *Significant difference between 23-day-old WT and IGF-1(C2:Ea) (P<0.05). $^{\#}$Significant difference between 23-day-old and 3-month-old WT and IGF-1(C2:Ea) (P<0.005).

the Eb termination sequence (Liao et al., 2006): whether the Eb termination sequence contributes to the release of IGF-1 into the bloodstream is not known.

Although Class 1 or Class 2 IGF-1 Ea isoforms overexpressed in skeletal muscle do not markedly elevate IGF-1 in blood, they could still be released into the circulation. Transgenic mice used in the present study overexpress rodent (rat and mouse) IGF-1 isoforms, and we were not able to distinguish between transgenic and endogenous (mouse) IGF-1. Detectable transgenic human IGF-1 in the circulation released from skeletal muscles has been previously reported for SK733 IGF-1 3′SK mice (in which transgenic IGF-1 is overexpressed from the avian α-actin promoter), although the overall circulating IGF-1 level was not elevated (Fiorotto et al., 2003).

A slight elevation of IGF-1 protein in the circulation (demonstrated in the present study) was presumably sufficient to increase the linear body growth in transgenic IGF-1(Class2:Ea) and *mdx*/IGF-1(Class2:Ea) mice (as seen in Table 1). Circulating IGF-1 is an endocrine regulator of somatic growth and elongation of bones is a hallmark of such growth (Liao et al., 2006). There was a trend for increased tibia length in all male and female transgenic mice and this increase was significant in 12-month-old IGF-1(Class2:Ea) males and females and in *mdx*/IGF-1(Class2:Ea) males.

The hypertrophic effect of IGF-1 is evident in growing muscle

Our study suggests that a sustained overexpression of IGF-1(Class2:Ea) in myofibres has a hypertrophic effect on skeletal muscle during active growth (during postnatal development or adult regeneration). The detailed phenotypic characterisation of the IGF-1(C2:Ea) strain shows that although fully adult IGF-1(C2:Ea) quadriceps muscles were significantly heavier compared with age-matched wild-type muscles, there was no muscle weight accretion between 3 to 12 months for either genotype (wild-type or transgenic). By striking contrast, adult muscle hypertrophy was evident in dystrophic *mdx*/IGF-1(C2:Ea) mice (but not in *mdx* control littermates), where muscles continued to increase in size from 3 to 12 months. Muscle mass data from these IGF-1(C2:Ea) and dystrophic *mdx*/IGF-1(C2:Ea) mice indicate that muscles with sustained overexpression of IGF-1 have a superior weight gain only during the growth phase. In the present study, such growth was associated either with developmental postnatal growth or muscle regeneration that is an ongoing feature of dystrophic *mdx* mice. In our study, fully adult muscles of non-dystrophic mice were not sensitive to the hypertrophic effect of elevated IGF-1. A similar lack of response in adult mice, despite the sustained muscle-specific overexpression of IGF-1 is evident from two earlier reports (Fiorotto et al., 2003; Musaro et al., 2001).

Two other widely studied situations that involve muscle cell growth are myotubes in tissue culture and exercise-induced hypertrophy of adult muscles. Myogenesis in tissue culture that results from fusion of myoblasts to form myotubes is widely used for cell and molecular studies of muscle signalling: often these myotubes are studied at only 4 days post fusion and this relatively early stage represents a myotube that is still growing and differentiating (Burattini et al., 2004; Tolosa et al., 2005). Such myotubes are not a model for stable adult myofibres. Instead, the wealth of data derived from such cultured myotubes is more closely related to growing muscle; this applies to studies that investigate IGF-1 signalling in vitro. In adult muscles, growth of muscle cells occurs only in response to regeneration (resulting from myofibre necrosis) or from exercise (increased mechanical loading). Studies where IGF-1 was overexpressed specifically in skeletal muscles in mice and rats show that such localised elevation of IGF-1 does not augment exercise-induced muscle hypertrophy after wheel running (Paul and Rosenthal, 2002) or after resistance training (Lee et al., 2004). Similarly, administration of growth hormone with the aim of increasing systemic levels of IGF-1 does not enhance protein synthesis in exercising elderly humans (Yarasheski et al., 1995). Thus, whether elevated IGF-1 has an additive effect on exercise-induced muscle hypertrophy has not been conclusively established.

IGF-1 signalling is evident in muscles that grow owing to postnatal development or regeneration caused by necrosis

We were interested in the signalling events that are responsible for the hypertrophic action of IGF-1(C2:Ea). To date, three transgenic mouse strains with sustained muscle-specific overexpression of IGF-1 have been described (Fiorotto et al., 2003; Musaro et al., 2001; Shavlakadze et al., 2006). Two of these strains exhibit muscle hypertrophy (Fiorotto et al., 2003; Musaro et al., 2001); however, very little is known about the in vivo mechanism of muscle growth mediated by IGF-1 overexpression.

Studies in cultured muscle cells and in vivo support a central role for the PI3K/Akt pathway in skeletal muscle differentiation (Musaro and Rosenthal, 1999) and muscle growth (Bodine et al., 2001). Activation of Akt downstream from the IGF-1R is associated with increased expression of myogenin, a skeletal-muscle-specific transcription factor considered to be a marker for differentiation (Musaro and Rosenthal, 1999). Akt signalling also regulates protein synthesis and degradation. Phosphorylation of $p70^{S6K}$ and 4E-BP1 downstream of Akt via mTOR results in stimulation of translation initiation and protein synthesis. In addition to promoting protein synthesis, activated Akt might inhibit protein degradation, because activated Akt phosphorylates FOXO transcription factors and causes their exclusion from the nucleus, leading to transcriptional inactivation of the atrophy related genes (reviewed by Glass, 2003; Shavlakadze and Grounds, 2006).

Whether muscle hypertrophy in transgenic mice with sustained overexpression of IGF-1 involves Akt activation has not been resolved. One study carried out phospho-site screening in skeletal muscles of 5-month-old IGF-1(C1:Ea) mice, and their age-matched wild-type littermates and reported that the transgenic IGF-1(C1:Ea) muscle is characterised by hyper-phosphorylation of mTOR (Ser2448) and $p70^{S6K}$(Thr421/Ser424), without involvement of Akt (Song et al., 2005).

We first compared the levels of Akt(Ser473) and mTOR(Ser2448) phosphorylation between skeletal muscles of overnight-fasted 3-month-old wild-type and IGF-1(C2:Ea) mice. There was no difference in the phosphorylation level of Akt(Ser473) or mTOR(Ser2448) between adult (3 months) wild-type and IGF-1(C2:Ea) muscles. Absence of elevated mTOR phosphorylation on the Ser2448 site in adult IGF-1(C2:Ea) mice contradicts an earlier report on mice with muscle specific elevation of IGF-1(C1:Ea) (Song et al., 2005). We also analysed phosphorylation of Akt(Ser473) and mTOR(Ser2448) in adult 3-month-old IGF-1(C1:Ea) mice and similarly found no difference between transgenic and wild-type muscles (data not shown). Failure of IGF-1 Ea (C2 or C1) to activate Akt in adult muscle was unexpected, because the IGF-1 receptor β-subunit is hyper-phosphorylated at tyrosine residues in skeletal muscles of the fully

adult IGF-1(C2:Ea) and IGF-1(C1:Ea) mice (N.W. and N.R., unpublished results). However, the minimal growth of adult IGF-1(C2:Ea) muscles (between 3 and 12 months) shown in the present study indicates that these mature myofibres are resistant to the elevated IGF-1 stimulation.

Neonatal muscle has a heightened capacity to activate signalling cascades that promote protein translation. A study in neonatal pigs showed that the sensitivity of signalling molecules leading to translation initiation is developmentally regulated and their downregulation parallels the developmental decline in protein synthesis in skeletal muscle (Suryawan et al., 2007). Since young growing muscle is more responsive to growth stimuli, we compared Akt(Ser473) phosphorylation between 23-day-old (2 days following weaning) skeletal muscles of IGF-1(C2:Ea) and wild-type littermates. In laboratory mice, intensive muscle growth occurs up to 26 days of age and the rate of muscle growth declines with the increasing muscle mass (Davis and Fiorotto, 2009). In the fasted state, 23-day-old muscles of IGF-1(C2:Ea) mice displayed higher Akt(Ser473) phosphorylation compared with age-matched wild-type muscles. Phosphorylation of Akt(Ser473) was also higher in skeletal muscles of fasted 23-day-old IGF-1(C1:Ea) mice compared with their wild-type littermates (data not shown). Taken together, these data strongly suggest that in transgenic muscle, elevated IGF-1 Ea isoforms (Class 2 or Class 1) signal via Akt and that activation of Akt(Ser473) is conspicuous in the growing, but not in the adult state. These conclusions were supported by our further observation that Akt(Ser473) phosphorylation was increased in adult dystrophic *mdx*/IGF-1(C2:Ea) muscles compared with *mdx*. As shown in this study, owing to the continuous cycles of myofibre necrosis and regeneration, *mdx*/IGF-1(C2:Ea) muscles maintain sensitivity to elevated IGF-1 and continue to hypertrophy during adult life. Overall, our observation that in growing skeletal muscle IGF-1 Ea signals via Akt, is in agreement with a previous study where viral delivery of IGF-1(C1:Ea) construct results in increased Akt phosphorylation that was detected 4 weeks after transgene delivery (Barton, 2006).

Both insulin and IGF-1 regulate mTOR signalling through the PI3K/Akt pathway (Lee et al., 2007). It is suggested that Akt activates mTOR by destabilising the TSC1-TSC2 complex, which is an indirect negative regulator of mTOR (Inoki et al., 2002). Amino acids (Corradetti and Guan, 2006) and mechanical stretch (Hornberger et al., 2004) can activate the mTOR pathway independently of PI3K/Akt. P70^{S6K} is a downstream target of mTOR and phosphorylation of its rapamycin (a specific mTOR inhibitor)-sensitive Thr389 site is used as a functional readout of mTOR activity (Lee et al., 2007; Pearson et al., 1995).

The mTOR(Ser2448) site has become a widely used biomarker to detect activation of mTOR downstream from Akt and in general to assess the activity state of this kinase in skeletal muscles (Hornberger et al., 2004; Reynolds et al., 2002; Song et al., 2005; Suryawan et al., 2007), which was the reason why we selected this site for analyses in the present study. It was initially suggested that activation of Akt leads to phosphorylation of mTOR at a Ser2448 residue (Sekulic et al., 2000). However, later studies (in non-muscle cells) show that regulation of Ser2448 phosphorylation is complex and not necessarily linked to Akt activation (Chiang and Abraham, 2005) and mTOR(Ser2448) does not appear to regulate p70^{S6K}activity (Kuemmerle, 2003). Studies in HEK293 and HeLa cells suggest that Ser2448 phosphorylation is a consequence rather than a cause of p70^{S6K} activation (Chiang and Abraham, 2005). The functional significance of Ser2448 phosphorylation with respect to mTOR signalling is not known, because mutation of this site does not affect mTOR kinase activity. Furthermore, whether phosphorylation of mTOR(Ser2448) by p70^{S6K} amplifies or dampens mTOR signalling is not clear (Chiang and Abraham, 2005). In our study, the level of mTOR(Ser2448) phosphorylation was not linked to activation of Akt(Ser473) in young muscle or adult dystrophic muscle. In addition, mTOR(Ser2448) phosphorylation was not affected by the activity level of p70^{S6K}: high levels of p70^{S6K} phosphorylation on Thr389 and Thr421/Ser424 seen in freely fed young mice as well as high levels of p70^{S6K} phosphorylation on Thr421/Ser424 seen in *mdx*/IGF-1(C2:Ea) mice were not correlated with changes in mTOR(Ser2448) phosphorylation. It is also possible that in skeletal muscles in vivo the phosphorylation of mTOR(Ser2448) is more stable than p70^{S6K}, which would explain the persisting high level of mTOR(Ser2448) in fasted 23-day-old muscles with strikingly reduced p70^{S6K} phosphorylation. Overall, our data suggest that mTOR(Ser2448) phosphorylation is not a useful measure for mTOR activation in response to IGF-1 in skeletal muscle and that analysis of the downstream targets from mTOR are more informative.

IGF-1 phosphorylates p70^{S6K} on Thr389 as well as Thr421/Ser424 sites in cultured intestinal smooth muscle cells (Kuemmerle, 2003) and des IGF-1 increases phosphorylation of p70^{S6K} at Thr421/Ser424 site within 20 minutes after in vivo intra-peritoneal injection into adult C57BL/6 mice fasted for 12 hours (Li et al., 2002). In our study, the baseline (fasted) levels of phosphorylated Thr389 as well as Thr421/Ser424 sites were decreased dramatically in 23-day-old wild-type and also IGF-1(C2:Ea) mice, indicating that p70^{S6K} activity, and presumably activity of the protein synthesis machinery in young muscle, is highly dependent on feeding. High sensitivity of the protein synthesis rate on feeding has also been demonstrated in skeletal muscles of very young pigs (Davis et al., 1996).

By contrast, the baseline (fasted) level of p70^{S6K} phosphorylation was readily detectable in adult dystrophic muscles, presumably reflecting the fact that the dystrophic muscle is a mixture of mature and regenerating muscle (whereas the young myofibres are all at a similar stage of growth), and Thr421/Ser424 but not Thr389 phosphorylation was higher in the presence of IGF-1(C2:Ea). High levels of Thr421/Ser424 but not Thr389 phosphorylation have also been reported in normal adult muscle that overexpresses IGF-1(C1:Ea) (Song et al., 2005). With respect to p70^{S6K} phosphorylation, our data in adult transgenic *mdx* mice agree with the study by Song and colleagues (Song et al., 2005) but we never saw the increased mTOR(Ser2448) phosphorylation that they report (Song et al., 2005).

P70^{S6K} is an important serine/threonine protein kinase that enables efficient protein translation. Activation of p70^{S6K} depends on the sequential phosphorylation of serine/threonine (Ser/Thr) sites by several independently regulated kinases (Weng et al., 1998). It is suggested that phosphorylation of Ser/Thr residues in the autoinhibitory domain, such as at Thr421 and Ser424, is required for altering the conformation of p70^{S6K} and making Thr389 and Thr229 available for phosphorylation, thereby fully activating p70^{S6K} (Price et al., 1991; Weng et al., 1998). The Thr389 site has been shown to be rapamycin sensitive in cultured muscle cells (Rommel et al., 2001; Valentinis et al., 2000; Kuemmerle, 2003), as well as in skeletal muscle in vivo and ex vivo (Bodine et al., 2001; Hornberger et al., 2004), which places this site downstream of mTOR.

Kinases that phosphorylate p70^{S6K} at the Thr421/Ser424 site are not known. Some tissue culture (Dennis et al., 1996; Pearson et al.,

1995) and also ex vivo (Hornberger et al., 2004) studies suggest that the Thr421/Ser424 residues are rapamycin insensitive, whereas others show that Thr421/Ser424 phosphorylation can be blocked by rapamycin in tissue culture (Kuemmerle, 2003; Valentinis et al., 2000). In addition, a study in cultured smooth muscle cells using pharmacological inhibitors showed that the IGF-1 induced phosphorylation of p70^{S6K}(Thr421/Ser424) is dependent on activation of PI3K and independent of PDK-1(Kuemmerle, 2003), which transmits signal from PI3K to Akt (Hemmings, 1997).

In the present study, we used the Thr389 residue of p70^{S6K} as a readout for mTOR activation and did not detect differences between skeletal muscles with and without IGF-1(C2:Ea). The mTOR signalling detected by p70^{S6K}(Thr389) phosphorylation was strikingly diminished in skeletal muscles of 23-day-old mice after the overnight fast, which agrees with the view that young muscle is very sensitive to nutrient availability. Muscles from fasted 23-day-old mice were also characterised by p70^{S6K}(Thr421/Ser424) dephosphorylation, which indicates that either this site is also regulated via mTOR, or that nutrient availability (possibly via mTOR) has a permissive role for phosphorylation of this site. The latter notion is supported by the fact that the p70^{S6K}(Thr421/Ser424) site is hyper-phosphorylated in *mdx*/IGF-1(C2:Ea) muscle, which in the fasted state maintains phosphorylation of p70^{S6K}(Thr389). We cannot say what lies upstream of p70^{S6K}(Thr421/Ser424) in the IGF-1(C2:Ea) muscles. However, activation of the PI3K pathway is a good candidate, as this pathway has previously been implicated in cultured smooth muscle cells (Kuemmerle, 2003). In the IGF-1(C2:Ea) muscles, activation of the PI3K pathway is also supported by hyper-phosphorylation of Akt.

To identify whether IGF-1(C2:Ea)-induced muscle hypertrophy was dependent on activation of p70^{S6K} signalling, we looked at the phosphorylation of rpS6, which lies downstream from p70^{S6K} (Ruvinsky and Meyuhas, 2006). Higher phosphorylation of rpS6(Ser235/236) was indeed detected in young fasted IGF-1(C2:Ea) muscles compared with the wild-type muscles, as well as in the fasted dystrophic *mdx*/IGF-1(C2:Ea) muscles compared with muscles of *mdx* mice. A higher level of rpS6 phosphorylation in dystrophic *mdx*/IGF-1(C2:Ea) muscles compared with *mdx* muscles is consistent with the higher p70^{S6K} phosphorylation, which is an upstream kinase for rpS6 (Ruvinsky and Meyuhas, 2006). Although p70^{S6K} phosphorylation was diminished following the overnight fasting in 23-day-old muscles [both wild-type and IGF-1(C2:Ea)], we were able to detect higher levels of rpS6(Ser235/236) phosphorylation in IGF-1(C2:Ea) compared with wild-type muscles. This might be because phosphorylation of rpS6 is more stable than that of p70^{S6K}. Higher rpS6(Ser235/236) phosphorylation in transgenic muscle supports the proposal that IGF-1(C2:Ea) causes muscle hypertrophy (at least in part) via increasing protein synthesis following activation of the p70^{S6K} pathway. Although rpS6 is a useful marker to support activation of p70^{S6K} signalling, the correlation between activation of rpS6 and protein synthesis is less clear (Ruvinsky and Meyuhas, 2006).

Myogenin expression was quantified as an approach to measure myogenesis. Myogenin is expressed in differentiating muscle precursor cells and Northern blot analyses show that in normal (non-dystrophic) muscle, myogenin expression is high at birth and falls to a very low level by 24 days after birth (Beilharz et al., 1992). By contrast, adult dystrophic *mdx* muscle maintains a relatively high level of myogenin expression (compared with normal adult muscle), reflecting the repeated cycles of muscle necrosis and regeneration (Beilharz et al., 1992). We hypothesised that in young growing muscle (in vivo) the elevated IGF-1 promotes myogenic differentiation associated with muscle growth: accordingly we demonstrated that young 23-day-old IGF-1(C2:Ea) muscles have a higher myogenin expression compared with age-matched wild-type muscles. This observation is consistent with an in vitro study showing induction of myogenin downstream of PI3K signalling in muscle cell cultures that overexpress IGF-1 Ea (Musaro and Rosenthal, 1999). This idea is further supported by prolonged proliferation (and delayed differentiation) of myogenic cells in mice with muscle specific inactivation of the IGF-1 receptor (Fernandez et al., 2001).

In conclusion, these data show that adult non-growing skeletal muscles are refractory to hypertrophy in response to the elevated IGF-1. By contrast, growing muscles respond by activating signalling downstream from the IGF-1 receptor (demonstrated by phosphorylation of Akt, p70^{S6K}) to increase protein accretion by the myofibres. Thus, the IGF-1-mediated hypertrophy evident in adult transgenic muscles results from enhanced increase in muscle mass mainly during the postnatal growth phase. No differences were seen between hypertrophy mediated by the Class 2 or Class 1 IGF-1 Ea isoforms, indicating that these signal sequences do not influence the hypertrophic response or normal development of muscles.

The mechanism involved in IGF-1-mediated muscle hypertrophy in vivo is very complex and requires further investigation. A precise understanding of the responsiveness of adult muscle to IGF-1 is central to the considerable interest in potential therapeutic administration of skeletal muscle specific IGF-1 to increase postnatal muscle mass and especially to reduce muscle atrophy that occurs in many clinical situations.

Materials and Methods

Transgenic mice

Transgenic IGF-1(C2:Ea) mice were generated at the European Molecular Biology Laboratory, Rome, Italy. The IGF-1(C2:Ea) expression construct was generated by cloning the mouse IGF-1(C2:Ea) cDNA sequences into the previously described skeletal muscle-specific expression cassette containing the *Mlc1/3* promoter, a SV40 polyadenylation signal, followed by the *Mlc1/3* enhancer sequence (Musaro et al., 2001; Rosenthal et al., 1989). FVB mice were used as embryo donors. Positive founders were bred to FVB wild-type mice and positive transgenic mice were selected by PCR from tail digests. The IGF-1(C2:Ea) (FVB background) heterozygous colony was established at the Animal Resource Centre in Perth, Western Australia under pathogen-free conditions. IGF-1(C2:Ea) male mice were bred with dystrophic *mdx*/*mdx* females (C57BL/10Sncn background) to establish an *mdx*/IGF-1(C2:Ea) colony, as described previously (Shavlakadze et al., 2004). Mice were screened for exon 4 sequence: 5′-ACT GAC ATG CCC AAG ACT CAG-3′ and for SV40 sequence: 5′-ATT CCA CCA CTG CTC CCA TTC-3′ (product size 283 kb). The study also used the transgenic IGF-1(C1:Ea) mice and their respective wild-type littermates: these mice were genotyped according to the previously described protocol (Shavlakadze et al., 2004). Experimental animals were housed in standard animal cages and maintained at a 12 hour light, 12 hour dark regime with free access to standard chow and drinking water. For overnight fasting (8 hours), food was removed at 11 pm and tissue was collected the following morning. All experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council, Australia.

Quantification of the total IGF-1 protein in serum and skeletal muscle

The amount of total IGF-1 protein in serum was measured using mouse IGF-1 ELISA (IDS, AC-18F1) and in skeletal muscle using IGF-1 HS ELISA (IDS, AC-42F1). To measure the muscle levels of IGF-1, snap-frozen gastrocnemius muscle tissue was ground in liquid nitrogen with a mortar and pestle, the muscle powder was weighed, dissolved in five volumes of lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol, protease inhibitor cocktail tablet (Roche)], briefly homogenised with a Polytron homogeniser and incubated on ice for 20 minutes. Muscle lysates were centrifuged at 12,000 ***g*** and supernatant was stored at –80°C until used for analyses. Protein was quantified using Bradford assay (Bio-Rad). IGF-1 ELISA was carried out according to the manufacturer's (IDS) protocol using 30 μg extracted muscle protein per well.

Tissue collection for histological analyses and tibia length measurement

Mice were anesthetised with a gaseous mixture of 1.5% isoflurane (Merial), N_2O and O_2 and euthanised by cervical dislocation. Gastrocnemius muscles were removed first and snap frozen in liquid nitrogen for protein extraction. For histological analyses, quadriceps muscles were excised, cut transversely in the central area, mounted onto tragacanth gum, and frozen in isopentane quenched in liquid nitrogen. 8 μm cryo-sections were collected onto glass slides and stored at –20°C until used for histological or immunohistochemical analyses. Haematoxylin and eosin (H&E) staining was performed on all muscles to assess general architecture or to perform morphometric analyses. Tibia bone length was measured and the values were used to normalise body and muscle mass.

Histochemistry with nicotinamide adenine dinucleotide nitro-blue tetrazolium

Histochemistry with NADH-TR was used to differentiate fast, intermediate and slow myofibres (Shavlakadze et al., 2005a). Muscle sections were incubated in NADH-TR (1:1) solution for 30 minutes at 37°C, followed by three washes in distilled deionised water (DDW) and three exchanges of increasing (30%, 60%, 90%) and decreasing acetone solutions (30 seconds each). The slides were rinsed with DDW and mounted with polyvinyl acetate (PVA) aqueous mounting medium.

Morphometric analysis

The entire digital image of the transverse muscle section was reproduced by tiling non-overlapping individual images acquired using a Leica DM RBE microscope, a Hitachi HV-C20M digital camera and Image Pro Plus 4.5.1 software, which were used for all morphometric analyses. The myofibre cross sectional area (CSA) was measured by tracing each individual myofibre. Care was taken to exclude any myofibres cut obliquely (i.e. with one axis being markedly longer than the other), because this would distort the CSA. NADH-TR-stained sections were used to measure myofibre CSA in non-dystrophic muscles and H&E-stained sections were used to measure myofibre CSA in dystrophic (*mdx*) muscles. For every non-dystrophic muscle stained with NADH-TR, the CSA of fast, intermediate and slow myofibres (250 myofibres for each type) was measured and averaged. For every *mdx* muscle stained with H&E, CSA of a total of 500 myofibres was measured.

Protein extraction and immunoblotting

Gastrocnemius muscles were ground in liquid nitrogen and lysed in five volumes of PhosphoSafe reagent (Novagen) supplemented with a protease inhibitor cocktail tablet (Roche 04693159001), followed by a brief homogenisation with a Polytron homogeniser and incubation on ice for 20 minutes. Muscle lysates were centrifuged at 12,000 ***g*** and supernatant was stored at –80°C until used for analyses. Protein samples were resolved by SDS-PAGE, and blotted on PVDF membrane (Amersham). Western blotting was performed with phospho-Akt (Ser473) (9271), Akt (9272), phospho-mTOR(Ser2448) (2971), mTOR (2972), phospho-p70^{S6K}(Thr389) (9205), phospho-p70^{S6K} (Thr421/Ser424) (9204), p70^{S6K} (9202), phospho-ribosomal protein S6 (Ser235/236) (4858) and total ribosomal protein S6 (2217) all from Cell Signaling, and mouse anti-α-tubulin (Sigma T5169). Control cell extracts (Cell Signaling 9273 and 9203) were used to test the quality of the antibodies for phosphorylated and total Akt, mTOR and p70^{S6K}. Donkey anti-rabbit and donkey anti-mouse HRP-conjugated secondary antibodies (Pierce) were used to detect primary antibody binding.

RNA extraction and RT-PCR

RNA was extracted from gastrocnemius muscles using TRI Reagent (Sigma) and reverse transcription performed using M-MLV reverse transcriptase (Promega). The RT-PCR was performed on a Rotor Gene 6000 real-time rotary analyser using QuantiFast SYBR Green RT-PCR Kit and QuantiTec primers for *Myog* (QT00112378) and *Rpl19* (QT00166145) (Qiagen).

Statistical analyses

Values in the text, tables and figures are reported as mean ± s.d. Variables within the same strain were compared using the Student's *t*-test. Variables between Class 1 and Class 2 IGF-1 Ea strains were compared using two-factor analysis of variance.

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