Akt activation prevents the force drop induced by eccentric contractions in dystrophin-deficient skeletal muscle

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Skeletal muscles of the mdx mouse, a model of Duchenne Muscular Dystrophy, show an excessive reduction in the maximal tetanic force following eccentric contractions. This specific sign of the susceptibility of dystrophin-deficient muscles to mechanical stress can be used as a quantitative test to measure the efficacy of therapeutic interventions. Using inducible transgenesis in mice, we show that when Akt activity is increased the force drop induced by eccentric contractions in mdx mice becomes similar to that of wild-type mice. This effect is not correlated with muscle hypertrophy and is not blocked by rapamycin treatment. The force drop induced by eccentric contractions is similar in skinned muscle fibers from mdx and Akt-mdx mice when stretch is applied directly to skinned fibers. However, skinned fibers isolated from mdx muscles exposed to eccentric contractions in vivo develop less isometric force than wild-type fibers and this force depression is completely prevented by Akt activation. These experiments indicate that the myofibrillar-cytoskeletal system of dystrophin-deficient muscle is highly susceptible to a damage caused by eccentric contraction when elongation is applied in vivo, and this damage can be prevented by Akt activation. Microarray and PCR analyses indicate that Akt activation induces up-regulation of genes coding for proteins associated with Z-disks and costameres, and for proteins with anti-oxidant or chaperone function. The protein levels of utrophin and dysferlin are also increased by Akt activation.

INTRODUCTION

Eccentric exercise, such as downhill running, is known to cause prolonged weakness and muscle soreness resulting from stretch-induced muscle damage. Fast muscles are more susceptible than slow muscles and fast muscles of the dystrophin-deficient mdx mouse, a model of Duchenne Muscular Dystrophy, are especially sensitive to damage caused by eccentric contractions. Mild protocols of lengthening contractions, which do not affect force production in normal mouse muscles, cause a marked reduction in the maximal tetanic force produced by mdx tibialis anterior and extensor digitorum longus (EDL) but not by mdx soleus (1–3). A similar force drop has been observed in dystrophin-deficient canine muscles (4). The force deficit caused by eccentric contractions has been variably attributed to alterations at the plasma membrane (5), membrane systems involved in excitation–contraction coupling (6,7), myofibrils (8) and cytoskeleton (9); however, the problem is still unresolved.

A current view is that exercise-induced injury is due to plasma membrane disruptions that produce both efflux of cell constituents, such as creatine kinase (CK), into the blood and influx of serum components, such as albumin, into skeletal muscle fibers (10). Focal disruptions of the sarcolemma were first detected by electron microscopy in some non-necrotic fibers in Duchenne muscular dystrophy in 1975 (11). A primary function of dystrophin would be to provide mechanical reinforcement to the sarcolemma and thereby
Muscle force was likewise reduced in muscles by treatment with anti-myostatin antibodies (18) or in dystrophin-deficient skeletal muscle. Loss of myostatin, a protein that regulates muscle growth, reduces muscle force caused by eccentric contractions was similar in tamoxifen- and oil-treated mice. However, reduction in muscle force induced by lengthening contractions was not improved by myostatin blockade (18). Indeed, following two lengthening contractions, fast muscles from mdx mice are more susceptible to eccentric contractions when these are applied directly in vitro to skinned fibers, suggesting that dystrophic symptoms do not arise from factors within myofibrils (16). Stimulation of muscle growth has been explored as a strategy to prevent muscle wasting and increase muscle strength in dystrophin-deficient skeletal muscle. Loss of myostatin, a negative regulator of muscle growth that causes muscle hypertrophy, reduces muscle force caused by eccentric contractions (17). Reduced muscle strength was induced in mdx mice by treatment with anti-myostatin antibodies (18) or by pharmacological blockade with a myostatin propeptide (19). However, reduction in muscle force induced by lengthening contractions was not improved by myostatin blockade (18). Indeed, following two lengthening contractions, fast muscles from myostatin null mice had a greater force deficit compared to control mice (20). Muscle force was likewise increased in mdx muscles by IGF-1 treatment (21) or by crossing mdx mice with transgenic mice expressing a local form of IGF-1 (mIGF-1) in skeletal muscle (22). The decrement in muscle force caused by eccentric contractions was similar in mdx and mdx-IGF-1 transgenic mice when compared at the same stimulation frequency, whereas some protection against damage was afforded when comparisons were made at equivalent forces (different stimulation frequency) (22).

IGF-1 activates Akt/PKB kinase and the increased phosphorylation of Akt has been demonstrated in mdx-IGF-1 transgenic mice (22), suggesting that the Akt pathway may be involved in mediating the beneficial effects of IGF-1. Activated Akt induces muscle hypertrophy in vivo by stimulating protein synthesis via the kinase mTOR (23–25) and blocks protein degradation by inhibiting the transcription factor FoxO, which controls both the ubiquitin-proteasome pathway (26,27) and the autophagy-lysosome pathway (28,29). However, the effect of Akt activation in dystrophin-deficient muscles has not been explored. Here, we have used an inducible muscle-specific Akt transgenic model to determine whether Akt activation is able to reduce the decrease in force induced by lengthening contractions in mdx skeletal muscle. We find that activated Akt prevents both the force drop of whole muscles in vivo and also the force drop of skinned fibers isolated from mdx muscles exposed to eccentric contractions in vivo. The effect of Akt is not correlated with muscle hypertrophy and is not dependent on mTOR. Gene expression analyses show that activated Akt induces increased protein level of utrophin and dysferlin and up-regulation of genes coding for proteins associated with Z-disks and costameres, as well as proteins with anti-oxidant or chaperone function.

RESULTS

Generation of mdx mice bearing an inducible activated Akt in skeletal muscle

Mice expressing an Akt inducible transgene in skeletal muscle were obtained by crossing a transgenic line bearing a silent Akt-ER, containing Akt1 fused to a src myristoylation signal and a mutated estrogen receptor hormone binding domain, with a transgenic line expressing Cre recombinase under the control of a myosin light chain 1 fast promoter (28). These mice were crossed with mdx mice resulting in an inducible Akt transgenic line in a mdx background, hereafter referred to as Akt-mdx (Fig. 1A). Adult (about 3-month-old) Akt-mdx mice were treated for 3 weeks every other day either with tamoxifen, to induce Akt activation, or with oil vehicle as control. Akt activation following tamoxifen treatment was demonstrated using antibodies specific for phospho-Akt S473 (Fig. 1B). This activation occurs in both fast and slow skeletal muscles of Akt-mdx mice, since the myosin light chain 1 fast promoter driving the Cre recombinase is active in all murine skeletal muscles during early development (30).

Akt activation leads to muscle hypertrophy but does not affect the pathological phenotype of mdx muscles

As shown in Figure 1C, Akt activation for 3 weeks led to a variable increase in muscle weight in the gastrocnemius (+22 ± 9%), EDL (+84 ± 17%) and soleus muscles (+54 ± 17%), as well as other skeletal muscles examined, including the diaphragm (not shown). During this period, body weight increased by 20 ± 3% in the tamoxifen-treated group when compared with 7 ± 2% in control animals. Muscle fiber size was correspondingly increased in tamoxifen-compared to oil-treated mice; however, fiber size was even more variable compared to control due to the large hypertrophy of some fibers (Fig. 1D). The proportion of fibers with central nuclei was similar in tamoxifen- and oil-treated animals (Fig. 1E). After Evans blue injection, a wide variability in the distribution of positive fibers among different muscles and among different areas of the same muscle was observed in both experimental groups, similar to that described in mdx mice (31). However, we noted a consistent tendency for positive fibers to be grouped in clusters in oil-treated mice, as in mdx mice, whereas positive fibers were usually diffusely distributed in tamoxifen-treated mice (Supplementary Material, Fig. 1A). A similar staining pattern was observed using antibodies against IgG (Supplementary Material, Fig. 1A). The proportion of Evans blue positive fibers was similar in tamoxifen- and oil-treated mice.
No significant change was likewise seen in the proportion of regenerating fibers or type I and IIA fibers, as determined by immunocytochemistry with myosin heavy chain-specific antibodies (not shown). Serum creatine kinase levels were not significantly different between oil- and tamoxifen-treated mice (Supplementary Material, Fig. 1B).

Akt activation does not increase muscle force but prevents the force deficit induced in mdx muscles by eccentric contractions

Gastrocnemius muscles from tamoxifen- and oil-treated mice were stimulated through the nerve at different frequencies, ranging from a single twitch to a completely fused tetanus, to assess force production. As shown in Figure 2A, no significant difference in force was observed when force was normalized for muscle mass. Twitch properties (time to peak and half-relaxation) were also identical in the two groups (not shown).

Repeated lengthening (eccentric) contractions are known to lead to a decrease in isometric force in fast muscles, which is higher in dystrophic than in wild-type muscle. We used a protocol of 20 eccentric contractions applied in vivo, as described in the materials and Methods section, and assessed the force deficit, namely the decrease in maximum force expressed as a percentage of initial force. As shown in Figure 2B, this protocol led to a force drop of 38 ± 3% in the gastrocnemius muscles of mdx mice but only 14 ± 1% in control muscles (Fig. 2B). Representative records of the responses of control and mdx muscles are illustrated in Supplementary Material, Fig. 2. To ascertain that this force drop was not due to metabolic fatigue, we performed the same protocol without lengthening the muscle and found no significant decrease in isometric force (not more than 5%) either in control or dystrophic muscle (Fig. 2E).

Next, we repeated the eccentric contraction protocol in the Akt-mdx mice. Mice treated with oil vehicle showed a 34 ± 4% decrease in isometric force, a reduction comparable to that measured in mdx mice. In contrast, tamoxifen treatment completely prevented the additional force drop induced by
lengthening contractions in dystrophic muscles (Fig. 2C). In fact after eccentric contractions, the force fell by 16 ± 2% in tamoxifen-treated Akt- mdx mice, a value very close to that measured in wild-type C57 mice. This effect is not due to tamoxifen per se, because tamoxifen treatment of mdx mice did not affect their response to eccentric contractions (Fig. 2F). The effect of Akt activation was also apparently not dependent on muscle hypertrophy, because no significant correlation was seen between the muscle weight/body weight ratio of singles muscles and their residual isometric force after eccentric contractions (Supplementary Material, Fig. 3).

**Effect of lengthening contractions on skinned fibers**

The striking effect of Akt activation in preventing the force drop induced by lengthening contractions in mdx muscles raises the question of the mechanism of this protective effect. Sarcolemmal ruptures are usually considered as the main culprit; however, we did not find any significant difference in the percentage of Evans blue positive fibers after eccentric contractions between oil- and tamoxifen-treated Akt- mdx mice (Supplementary Material, Fig. 4). To determine whether the myofibrillar-cytoskeletal system is involved in the reduced force drop induced by Akt activation in mdx muscles, we examined single permeabilized muscles fibers (skinned fibers). It was previously reported that mdx muscle fibers are not especially susceptible to eccentric contractions when these are applied directly in vitro to skinned fibers (16). Indeed, we confirmed that after two stretches applied on maximally activated skinned fibers, force deficits do not differ among fibers from gastrocnemius muscles of C57, mdx, oil- and tamoxifen-treated Akt- mdx mice (Fig. 3A).

Next, we compared skinned fibers dissected from muscles previously subjected to eccentric contractions in vivo. As a control, we used fibers taken from the gastrocnemius muscle of the controlateral leg not exposed to eccentric contractions. Whereas muscle fibers from wild-type C57 mice did not show any significant decrease in force after eccentric contractions in vivo, mdx muscle fibers showed a 28 ± 13% lower force than controlateral fibers (Fig. 3B). Interestingly, we did not observe any bimodal distribution of force drop in mdx muscle fibers. These findings support the notion that the myofibrillar-cytoskeletal system of mdx muscles is altered when eccentric contractions are performed in intact muscle fibers in vivo. The same experiment was then performed in tamoxifen- and oil-treated Akt- mdx mice. As shown in Fig. 3C, fibers from oil-treated mice showed a significant force decrease of 35 ± 11%, similar to that of mdx fibers, which was completely prevented by Akt activation.

**The effect of Akt activation in preventing the force deficit induced in mdx muscles by eccentric contractions is not dependent on mTOR**

Activation of Akt induces activity of mTOR which mediates the hypertrophic effect of activated Akt in skeletal muscle. In fact rapamycin, a selective inhibitor of mTOR, blocks muscle hypertrophy induced by functional overload (23) and muscle growth of regenerating muscle (25). Therefore, we used rapamycin to determine whether the response of tamoxifen-treated Akt- mdx mice to eccentric contractions is dependent on mTOR. We observe that the ‘protective’ effect of Akt activation is readily reversible, so that if Akt- mdx mice treated with tamoxifen for 2 weeks are shifted to oil vehicle during the third week, their response to eccentric contractions is comparable to that of mdx mice. Therefore, we examined the response of mice treated for 2 weeks with tamoxifen and the third week with tamoxifen plus rapamycin (Fig. 4A). The efficacy of rapamycin in blocking the mTOR pathway was demonstrated by the complete inhibition of the phosphorylation of the mTOR effector S6 in tamoxifen-treated Akt- mdx muscles (Fig. 4B). As shown in Figure 4C and D, the response of muscles from rapamycin-treated mice to eccentric contractions was essentially similar to that of tamoxifen-treated mice, namely the force drop was completely prevented.

**Gene expression changes induced by Akt activation**

How does Akt activation affect the response to eccentric contractions? The finding that short-term tamoxifen treatment, even 1 week treatment, has no significant effect (our unpublished observations) suggests that reprogramming of gene expression is involved in this process. As an initial step towards identifying the mechanism responsible for the protective
effect of Akt activation, we performed whole genome microarray analysis, followed by quantitative RT–PCR of selected genes and western blotting of a few gene products. We especially focused on changes in components associated to the sarcolemmal and/or myofibrillar cytoskeleton. Gene expression profiling showed that Akt activation causes up-regulation of genes coding for proteins associated to the Z-disks, such as desmin and muscle LIM protein, or costameres, such as several integrins (Table 1). Also dysferlin, a protein involved in plasma membrane repair, showed a 1.9-fold increase (not shown). The up-regulation of many of these genes was confirmed by quantitative RT–PCR analysis (Fig. 5A) and an increased protein content of desmin and dysferlin was demonstrated by western blotting (Fig. 6). Next we examined the expression of utrophin, the autosomal homologue of dystrophin, since it is known that an utrophin trans-     
     
- **Table 1.** Genes up-regulated by Akt activation*  

<table>
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<tr>
<th>Gene Code</th>
<th>Symbol</th>
<th>Fold Δ</th>
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<tr>
<td>Keratin 8</td>
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<tr>
<td>Integrin α9</td>
<td>Itga9</td>
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<td>Muscle LIM protein (MLP)/cysteine and glycine-rich protein 3</td>
<td>Csrp3</td>
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<td>Itga5</td>
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<td>Ankrd2</td>
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<td>Integrin αV</td>
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<td>Itgb1</td>
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<td>Calsarcin 1</td>
<td>Myo2b</td>
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</tr>
<tr>
<td>Desmin</td>
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*Gastrocnemius muscles from Akt-*mdx* mice treated with tamoxifen for 3 weeks were compared with muscles from the silent Akt line not crossed with the mlc1f-Cre line but bred onto *mdx* background and treated with tamoxifen for 3 weeks.

**DISCUSSION**

Dystrophin-deficient muscles are unable to sustain the mechanical stress induced by eccentric contractions and show an
mediated IGF-1 overexpression in these conditions. Other studies have shown that virus- 

sion) against contraction-induced injury was observed under effect of muscle-specific IGF-1 expression (22) and myostatin 
motivating muscle growth was initially proposed based on the 

The notion that muscular dystrophy can be alleviated by pro-

tection prevents completely the exacerbated deleterious effect 
efficacy of therapeutic interventions, such as utrophin overe-

activation prevents completely the exacerbated deleterious effect of eccentric contractions on mdx muscle function. Several 

Aspects of these findings deserve discussion: (i) the protective 
effect of Akt is not correlated with muscle hypertrophy and is 

not mediated by the mTOR pathway; (ii) the protective effect 
of Akt is accompanied by changes in distribution but not in 

proportion of Evans blue positive fibers; (iii) Akt activation 

prevents the force drop in skinned fibers from muscles 
exposed to eccentric contractions in vivo; (iv) Akt activation 
causes an increase of utrophin, dysferlin, proteins associated 

with Z-disks and costameres, and proteins with anti-oxidant 
and chaperone function.

Akt activation, muscle hypertrophy and prevention of 
contraction-induced force deficit

The notion that muscular dystrophy can be alleviated by pro-
moting muscle growth was initially proposed based on the 
effect of muscle-specific IGF-1 expression (22) and myostatin 

inactivation or blockade (17,18). However, no protection 

(myostatin blockade) or limited protection (IGF-1 overexpres-
sion) against contraction-induced injury was observed under 

these conditions. Other studies have shown that virus-

mediated IGF-1 overexpression in mdx muscles led to 
increased muscle mass but no protection against mechanical 
j injury (35). On the other hand, systemic administration 
of low dose IGF-1 for 8 week, that causes a shift towards 
a more oxidative fiber-type profile but not hypertrophy, was 
found to partially reduce contraction-induced force drop in mdx muscles (36).

We report here that muscle-specific Akt activation in an 
inducible transgenic expression model is able to provide a 
complete protection against eccentric contraction-dependent 
injury, reducing the force drop to levels comparable to those 
seen in normal muscles. This effect is independent of hyper-
trophy, because we found no significant correlation between 
increase in muscle weight and degree of protection, and is 
not mediated via mTOR, a major Akt effector and determinant 
of Akt-dependent muscle hypertrophy (23), because it was not 
prevented by rapamycin treatment. Furthermore, we did 
not observe any change in the fiber-type profile during the 
3 week period of Akt activation. Clearly, other Akt targets 
must be involved in this effect.

The protective effect of Akt is not accompanied by changes 
in the proportion of Evans blue positive fibers

Previous studies on isolated mdx and normal muscles chal-

lenged by forced lengthening during tetanic contractions in 

vitro in the presence of procion orange showed that penetration 
of dye occurs in a greater number of fibers in mdx muscles 
(13,37). Infection of mdx muscles with an adenovirus contain-
ing a minidystrophin gene showed an intermediate number of 
labeled fibers and a correspondingly intermediate force drop 
after eccentric contraction, so that a linear correlation was 
observed between the force drop and the proportion of procion orange positive fibers (37). These findings supported 

the notion that the contraction-induced force deficit results 
from sarcolemmal ruptures. However, an alternative inter-

pretation is that both the force deficit and the fraction of 
procion orange positive fibers are simultaneously but indepen-
dently reduced by the successful minidystrophin transfection. 
However, different results were obtained when eccentric 
contractions were induced in vivo. The reduction in susce-
ptibility to contraction-induced damage in vivo after long-term 
low dose IGF-1 administration was not linked to changes in 
Evans blue uptake (36). Accordingly, the greater force drop 
observed in muscles from dystrophin-deficient canine muscles 
compared to control did not correlate with the proportion of 
fibers showing Evan blue penetration (4). We also find no sig-
nificant difference in the proportion of Evans blue positive 
fibers between tamoxifen-treated Akt-mdx muscles, which 
show complete protection against eccentric contraction injury, 
and oil-treated controls, which show a much greater force 
drop, comparable to that of mdx muscles. It remains to be seen 
whether the difference between results in isolated muscles and 
muscles in situ are due to the different tracer used. Procion 
orange is a low molecular weight molecule and might penetrate 
even slightly damaged muscle fibers, whereas Evans blue is 
known to bind albumin and thus act as macromolecular tracer, 
which may need larger sarcolemmal ruptures to penetrate 
muscle fibers. Anyway, our findings with skinned fibers indicate 
that the force drop induced by eccentric contractions is not 
explainable in terms of ‘injured’ versus ‘non-injured’ fibers, 
since we did not observe any bimodal distribution of force 
drop in mdx muscle fibers. Thus ‘sarcolemmal ruptures’ do not 
seem to be required to produce the eccentric functional damage.
Akt activation prevents the force drop in skinned fibers from muscles exposed to eccentric contractions in vivo

A major original finding of this study is that skinned fibers from mdx muscles exposed in vivo to eccentric contractions display a marked reduction in force. Interestingly, this effect is not seen when mdx skinned fibers are exposed to eccentric contractions directly in vitro, in agreement with the previous study (16). The decreased force production of skinned fibers likely reflects an alteration of myofibrillar or cytoskeletal proteins causing either dysfunction of the myofibrillar contractile machinery or uncoordinated contraction of the sarcomeres due to cytoskeletal disruption. In mdx fibers, prepared from muscles exposed in vivo to eccentric contractions, more severe and extensive contractile impairment are observed. This could be due to disruption of myofibrillar or cytoskeletal proteins by mechanical stress or degradation of structural proteins caused by some process that takes place when mdx muscle fibers are subjected to eccentric contractions in situ. Calpain activation or changes produced by reactive oxygen species have been considered in recent studies (38,39). Whatever the cause, the fact that all mdx skinned fibers showed a marked force drop is not compatible with the view that the reduction in force induced in mdx muscles by eccentric contractions is due to the presence of a proportion of ‘injured’ fibers with sarcolemmal disruption leading to dye uptake. The contractile impairment is apparently distributed amongmost mdx fibers dissected from muscles exposed in vivo to eccentric contractions. It was previously noted that the percentage of mdx muscle fibers penetrated by procion orange following eccentric contraction in vitro is lower than the percentage of the force drop, for example ~10% procion orange positive fibers were found in muscles showing a force drop of over 40% (5). It was suggested that this mismatch may be due to the restricted longitudinal diffusion of the dye and an underestimation of the number of sarcolemmal disruptions when only few cross sections of the muscles are examined (5). However, analyses of serial sections indicate that dye uptake is present ‘over long segments (up to a few mm) of the same fiber’ (40). The results of the present study suggest that most if not all mdx muscle fibers are damaged by eccentric contractions, including fibers not penetrated by the dye. We can speculate that wide sarcolemmal disruptions, responsible of dye penetration, are not the cause of force deficit but possibly a more severe consequence of the same process that leads to reduction of force after eccentric stress.

Mechanism responsible for the protective effect of Akt activation

Transgenic experiments showed that the dystrophin-related protein, utrophin, is able to functionally compensate for dystrophin and to prevent muscular dystrophy in mdx mice (32). Interestingly, high levels of utrophin expression are not required for recovery, a 2-fold or 3-fold increase being sufficient to ameliorate the dystrophic phenotype. We observed a 2-fold increase in utrophin protein levels after Akt activation, whereas the utrophin mRNA levels were unchanged, suggesting the existence of post-transcriptional control of utrophin gene expression (41). However, it is not clear whether the resistance to stretch induced by Akt activation can be accounted for by utrophin up-regulation, because studies using an inducible transgenic system showed that the force drop after eccentric contraction was significantly reduced when utrophin was induced at birth, while no recovery was observed when utrophin was induced at 30 days after birth (34). The increased dysferlin protein levels may be involved in the effect of Akt, given the role of dysferlin in plasma membrane repair (42), although it has not been established whether dysferlin up-regulation is able to compensate for dystrophin deficiency.

The up-regulation of genes coding for different components associated with the Z-disk may also be relevant to the effect of Akt. Z-disks are thicker in slow compared to fast muscles, and slow muscles are known to be less susceptible to the effect of eccentric contractions. Interestingly, some of the up-regulated genes are selectively expressed in slow-twitch-oxidative muscle: this is true for muscle LIM protein (43), αB-crystallin (44) and calcyrin 1 (45). Muscle LIM protein is up-regulated by eccentric contractions both in mouse (46) and human muscles (47), and the same is true for ankirin repeat domain 2 (46,48). The small heat shock proteins Hspb1/Hsp25 (homologous to Hsp27 in human) and αB-crystallin respond immediately to eccentric exercise by binding to cytoskeletal/myofibrillar proteins (49,50). In the heart, muscle LIM protein is essential in the adaptation to biomechanical stress (51). Up-regulation of integrins may also be relevant, as transgenic overexpression of integrin α7β1 was found to ameliorate the dystrophic phenotype in mdx mice (52).

Oxidative stress is increased in muscles from Duchenne muscular Dystrophy patients and mdx mice and various molecules associated with anti-oxidant function are increased in dystrophic muscle (53). Using cultured myotubes from mdx and normal mice, Rando et al. (54) found that dystrophin-deficient cells were more susceptible to oxidative stress. Evidence for oxidative stress in mdx muscles even prior to the onset of the degenerative process was supported by the finding that the expression of genes coding for antioxidant enzymes was higher in the pre-necrotic mdx muscles and the level of lipid peroxidation were greater (55). A recent study shows that the anti-oxidant N-acetylcysteine reduced the force deficit associated with stretch-induced muscle damage (38). Therefore, the up-regulation of several genes associated with anti-oxidant function induced by Akt activation could be involved in the protection against the effect of eccentric contractions.

CONCLUSION

In summary, we report here that Akt activation prevents completely the excessive force drop induced by eccentric contractions in dystrophin-deficient mdx muscles. Such complete protection was not observed in previous studies, except after gene replacement strategy with dystrophin or after utrophin overexpression since early developmental stages (32,56). On the other hand, other aspects of the dystrophic phenotype, such as high levels of serum creatine kinase or the appearance of Evans blue labeled fibers, are not prevented by Akt activation at least within the time frame considered in the present study. It is possible that some aspects of the dystrophin-deficient phenotype, such as susceptibility to mechanical stress, are directly
dependent on dystrophin deficiency, whereas other aspects of muscle pathology are dependent on sarcoglycan deficiency. This interpretation is consistent with the finding that γ-sarcoglycan deficiency causes muscle degeneration, but no decrease in muscle force after eccentric contractions (57). A recent study reported that five genes have greater than 2-fold higher expression in mouse models of sarcoglycanopathy compared with dystrophinopathy; these include calsarcin1/myozin 2, ankinrin repeat domain 2, heat shock protein 1-like, muscle LIM protein and connective tissue growth factor (58). Interestingly, all these genes are up-regulated by Akt activation; connective tissue growth factor shows a 2.9-fold increase and heat shock protein 1-like a 1.6-fold increase (for the other three genes, see Table 1). It was recently pointed out that ‘... different therapeutic strategies may be necessary to combat the mechanical, signaling, and immune related mechanisms that lead to dystrophy’ (59). Our study opens the way to identify potential targets to combat the mechanical dysfunction induced by dystrophin deficiency.

MATERIALS AND METHODS

Transgenic model

The muscle-specific inducible Akt transgenic mouse line was described elsewhere (28). In brief, this line was obtained by crossing a transgenic line expressing a silent Akt-ER, containing the Akt1 coding sequence containing a src myristoylation signal fused to a modified estrogen receptor hormone binding domain (silent Akt line), with a line expressing Cre recombinase under the control of a myosin light chain 1 fast promoter (mlc1f-Cre line). This muscle-specific inducible Akt transgenic line was bred onto the dystrophin deficient mdx background to produce the Akt-mdx line (Fig. 1A). Akt activation was induced by injecting 1.5 mg of tamoxifen i.p. every other day for 3 weeks. Tamoxifen, which binds the mutated estrogen receptor, induces Akt phosphorylation and activation. Control animals received the vehicle (sunflower oil) for the same period of time. Mdx and wild-type C57B16 mice were also used in some experiments.

Muscle histology and Evans blue uptake

Muscle histology was examined on cryosections stained with Hematoxylin and Eosin. As a measure for membrane permeability, the vital dye Evans blue was used. Oil- and tamoxifen-treated mice (n = 6 per group) were injected i.p. with a solution containing 2% Evans blue in phosphate-buffered saline (pH 7.5), the injection volume being 0.5% relative to body mass. The solution was sterilized by passage through membrane filters with a 0.2 mm pore size and muscles were removed 18 h after dye injection. Similar Evans Blue injections were given to mice subjected to eccentric contractions; in this case, muscles were examined 1 h after exercise.

Creatine kinase assay

To evaluate the amount of creatine kinase present in the blood, samples of blood were obtained by peri-orbital bleeding. Creatine kinase content was measured in the serum by an indirect colorimetric assay (Sentinel Diagnostics kit).

Western blotting and immunohistochemistry

We used antibodies specific for phospho-Akt1 (phosphorylated Ser47, 1:50; Cell Signaling), Akt, phospho-S6, pan-actin (all 1:1000; Cell Signaling), utrophin (1:50 Iowa Hybridoma bank), desmin (1:1000 Sigma), dysferlin (1:1000 Novoceastra) and dystrophin N-terminus (gift from J. Chamberlain). HRP-, FITC-, Cy3- and Cy2-conjugated secondary antibodies were obtained from Bio-Rad.

In vivo gastrocnemius mechanics

Gastrocnemius muscle contractile performance was measured in vivo using a 305B muscle lever system (Aurora Scientific Inc.) in mice anæsthetized with a mixture of Xilazine and Zoletil. Mice were placed on a thermostatically controlled table, the knee was kept stationary and the foot was firmly fixed to a footplate, which was connected to the shaft of the motor. Contraction was elicited by electrical stimulation of the sciatic nerve. Teflon-coated seven multi-stranded steel wires (AS 632, Cooner Sales, Chatsworth, CA, USA) were implanted with sutures on either side of the sciatic nerve proximally to the knee before its branching. At the distal ends of the two wires, the insulation was removed, while the proximal ends were connected to a stimulator (Grass S88). In order to avoid recruitment of the dorsal flexor muscles, the common peroneal nerve was cut. The torque developed during isometric contractions was measured at stepwise increasing stimulation frequency, with pauses of at least 30 s between stimuli to avoid effects due to fatigue. Duration of the trains never exceeded 600 ms. Force developed by plantar flexor muscles was calculated by dividing torque by the lever arm length (taken as 2.1 mm). Eccentric contractions were analysed to study muscle damage. Muscle lengthening was achieved by moving the foot backward at a velocity of 40 mm/s while the gastrocnemius was stimulated with a frequency sufficient to induce full tetanic fusion (100 Hz). The footplate was moved 200 ms after initiation of stimulation train, thus eccentric pull occurs during the isometric plateau of the tetanus. The range of movement during the pull was calculated to be 30°, clearly inside physiological limits of movement for the foot. Total duration of tetanic stimulation was limited to 600 ms, assuring no sag of force. This protocol was repeated 20 times taking the decrease in the isometric force plateau (before beginning of the stretch) as an indication for muscle damage, with pauses of 30 s.

In vitro mechanics on gastrocnemius skinned fibers

Samples dissected from the superficial layers of the gastrocnemius exposed to eccentric contractions or from the contralateral non-stimulated muscle were immersed in ice cold skinning solution. Single fibers were manually dissected from fiber bundles under a stereomicroscope (10–20 × magnification). At the end of the dissection, fibers were bathed for 30 min in skimming solution containing 1% Triton X-100 to ensure complete membrane solubilization. Segments of
1–2 mm length were then cut from the fibers and light aluminum clips were applied at both ends. In each fiber segment, isometric tension (Po) was measured during maximal activations at 20°C, pCa = 4.8, sarcomere length = 2.75 mm. Digitized images of each fiber were taken with a camera connected with the microscope at 360 × magnification. Cross-sectional area was calculated from the average of three diameters, spaced at equal intervals along the length of the fiber segment, assuming a circular shape. The compositions of the solutions used (skinning, relaxing, pre-activating and activating) were as described (60).

To study the effect of eccentric contraction applied directly to skinned fibers in vitro, the following protocol was adopted. In each fiber segment, isometric tension (Po) was measured during two maximal activations at 20°C, pCa = 4.8. Two eccentric contractions were induced by stretching the fiber segment at the speed of 0.15 l/s for 0.7 s during maximal activation. After this, two maximal activations were induced in isometric conditions. To determine the damage to the fiber, isometric tension generated in two activations after the two stretches was expressed in relation to isometric tension before the two stretches.

Microarray and PCR analyses
Gastrocnemius muscles from three Akt-mdx mice treated with tamoxifen for 3 weeks were used for these experiments. Akt activation in these samples was verified by immunoblotting. RNA was isolated from the three samples using Trizol followed by RNeasy (Qiagen) clean up and pooled at equimolar ratios. In preliminary experiments, we found that tamoxifen per se causes significant changes in gene expression in skeletal muscle. Therefore, we generated mice from the silent Akt line not crossed with the mlc1f-Cre line but bred onto mdx background and treated these mice with tamoxifen for 3 weeks. RNA was isolated from gastrocnemius muscles of these mice and a pool of three samples was used as control. Complementary RNAs, prepared and labeled according to standard Affymetrix protocols, were hybridized to Affymetrix Mouse Genome 430 2.0 Arrays. Expression values were summarized using MAS 5.0 and differentially expressed genes determined using Excel. To identify enriched gene groups, we utilized ErmineJ (61) and searched for enrichment in gene ontology terms of molecular component or biological function among the up- or downregulated genes.

Quantitative RT–PCR
For utrophin mRNA expression, total RNA was prepared from skeletal muscles using Promega SV Total RNA Isolation kit. Complementary DNA generated with Invitrogen SuperScript III Reverse Transcriptase was analysed by quantitative real-time RT–PCR using Qiagen QuantiTect SYBR Green PCR Kit on an ABI Prism 7000. Urophin expression was detected with the following primers: Fw TTCAGTGAATCATCAT-TAAAGTCCAGATCT, Rv TCTTGAGAACGACGTCTAT. Data were normalized to GAPDH expression. For genes selected from the microarray analysis, we used the same RNA that was isolated for the DNA microarrays and prepared cDNA using Invitrogen SuperScript III Reverse Transcriptase. Real-time PCR was performed using the PCR Master Mix (ABI) and ready-made TaqMan expression assays. Expression values were normalized to b-actin expression and showed as fold increase in Akt-mdx samples compared to mdx samples.

Statistics
All data are expressed as means ± SEMs. Differences between groups were assessed using student’s t-test. Significance was defined as a P-value of less than 0.05 (95% confidence).

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Supplementary Material
Supplementary Material is available at HMG Online.

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References


