Altered Activity of Signaling Pathways in Diaphragm and Tibialis Anterior Muscle of Dystrophic Mice

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Duchenne muscular dystrophy is a musculoskeletal disease caused by mutations in the dystrophin gene. The purpose of this study was to use the mouse model of muscular dystrophy (*mdx*) to determine if the progression of the dystrophic phenotype in the diaphragm (costal) versus limb skeletal muscle (tibialis anterior) is associated with specific changes in extracellular regulated kinase (ERK1/2), p70 S6 kinase ($p70^{S6k}$), or p38 signaling pathways. The studies detected that consistent with an earlier dystrophic phenotype, phosphorylation of $p70^{S6k}$ is elevated by 40% in the diaphragm with no change in limb muscle. In addition, phosphorylation of p38 kinase was decreased by 33% in the *mdx* diaphragm muscle. Levels of ERK1/2 as well as phosphorylation states were elevated in the diaphragm and limb muscle of *mdx* mice compared with age-matched control muscles. These results indicate that distinct signaling pathways are differentially activated in skeletal muscle of *mdx* mice. The specificity of these responses, particularly in the diaphragm, provides insight for potential targets for blunting the progression of the muscular dystrophy phenotype. Exp Biol Med 229:503–511, 2004.

Key words: intracellular signaling; skeletal muscle; muscular dystrophy; p70s6 kinase; p38; ERK

Duchenne muscular dystrophy (DMD) is a progressive disease characterized by extensive muscular degeneration, pathological hypertrophy, and myofiber necrosis. Patients present symptoms in early childhood, typically require wheelchairs by age 11, and succumb to respiratory or heart failure in their late teens or early twenties. The primary defect in DMD is the absence of a membrane-bound structural protein, dystrophin, which links the myofiber cytoskeleton to the extracellular matrix through the dystrophin-glycoprotein complex (DGC; Ref. 1). It has been hypothesized that dystrophin and the DGC participate in a mechanical link that stabilizes the muscle membrane. Therefore, the absence of dystrophin would cause the plasma membrane to be more fragile, leading to disruptions of the sarcolemma during muscle contractions and resulting in muscle degeneration and muscular dystrophy (2).

Interestingly, not all muscles that lack dystrophin are equally susceptible to muscle degeneration. The *mdx* mouse strain, which lacks the protein dystrophin from all of its skeletal muscles (3), exhibits great variability in myofiber necrosis and contractile properties between different muscles. These mice exhibit a pathology similar to a mild form of muscular dystrophy, as seen by elevated serum levels of creatine kinase and histological changes consistent with myofiber damage (3–5). Their hind limb muscles undergo extensive myofiber degeneration and regeneration from 3 to 5 weeks of age (6), which continues on a more limited scale for the duration of their lifespan. Although hind limb muscles of *mdx* mice undergo successful regeneration, the diaphragm (DIA) muscle in *mdx* mice exhibits a muscle pathology very similar to DMD. Perimysial and endomysial fibrosis, a decrease in fiber size accompanied by functional decreases in active tension, maximal velocity of shortening, and twitch to tetanus ratio are present, as well as a decrease in myosin and total protein content (7–10). Despite the successful regeneration of the hind limb muscles and a lack of *in vivo* muscle dysfunction, the longevity of *mdx* mice is decreased compared with controls (5).

The underlying mechanisms by which dystrophin deficiency mediates the observed pathophysiological changes are unclear. Hack *et al.* (11) implied that mechanisms other than mechanical injury may be involved in muscular dystrophies where the DGC is compromised.

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Lately more evidence has become available indicating a potential role for the DGC in cellular signaling, besides its mechanical role (reviewed in Ref. 12). β-dystroglycan, part of the DGC complex, has been shown to interact with Grb2, an adapter protein involved in linking signaling molecules containing phosphotyrosine residues and proline-rich domains (13). Moreover, β-dystroglycan is also associated with focal adhesion kinase (FAK; Ref. 14). Grb2 and FAK are both involved in the Ras/mitogen-activated protein kinase (MAPK) signaling pathway and could therefore play an important role in cell survival. Recently, Langenbach and Rando (15) found that disruption of the DGC resulted in a decrease in signaling through the protein kinase B (Akt) pathway, which is involved in numerous cellular functions and is upstream of a number of targets including the 70 kD ribosomal S6 kinase ($p70^{S6k}$). Therefore the loss of dystrophin in muscle cells will likely alter cellular signaling pathways, which could lead to changes in growth control and gene expression.

A number of studies have recently investigated changes in gene expression due to alterations in dystrophin using microarray technology (16–18). Chen *et al.* (16) analyzed skeletal muscle samples from patients with DMD or α-sarcoglycan deficiency (16). Increased gene expression was identified for multiple genes, including extracellular matrix proteins such as fibronectin and signaling proteins such as calcyclin. Many genes exhibited decreased expression, such as the p38 protein kinase substrate, MAPK activated protein kinase (MAPKAPK), and metabolic proteins such as glucose transporter 4 (GLUT4). Tkatchenko *et al.* (18) reported that mainly mitochondrial transcripts were upregulated in DMD muscles. These data indicate that the lack of dystrophin in muscle is associated with a large number of changes at the transcription level, which reflect changes in cellular signaling.

Many protein kinases have been shown to be involved in the transduction of extracellular signals leading to changes in gene expression. The extracellular regulated kinase (ERK1/2) and the stress activated protein kinase (SAPK), p38, are downstream targets of multiple growth factors, nitric oxide, and integrin-mediated signaling (19, 20). $p70^{S6k}$ has also been shown to play important roles in the response to hypertrophic stimuli in skeletal muscle (21) and growth factor stimulation (22), as well as anchorage-dependent signaling (23). A recent study has identified the jun N-terminal kinase 1 (JNK1) as being important in contributing to the dystrophic muscle phenotype (24), but also indicated that it may not be the only player in the dystrophic phenotype.

In the current study, we have investigated 3 signaling molecules for their involvement in muscular dystrophy: (i) $p70^{S6k}$, because of its dependence on the Akt signaling pathway, which is disrupted in muscular dystrophy (15); (ii) ERK1/2, because it is a downstream target of stretch-induced signaling (25), and this has been hypothesized to be altered in muscular dystrophy; and (iii) p38, which is also involved in stretch signaling (25), and members of the pathway were shown to be changed in dystrophic muscle (16). Diaphragm and tibialis anterior (TA) muscle were compared at 3 months and 1 year of age. Tibialis anterior is a muscle of mixed fiber type and has been characterized extensively for changes associated with the dystrophic phenotype in *mdx* mice. At 3 months of age, TA muscle has recovered from the initial regenerative phase and is functionally similar to control, although some deficits exist. At this age, the DIA is also functionally similar to control, but shows signs of muscular dystrophy at the histological level, not observed to the same extent in TA muscles. However, at 12 months of age, the DIA muscle exhibits decreased function and is showing a severe morphological phenotype, unlike the TA muscle, which is not as severely compromised at this age. Therefore, the purpose of this study was to determine whether the activation of ERK1/2, $p70^{S6k}$, and p38 was altered in a muscle-specific pattern in control and dystrophic (*mdx*) mice using the DIA and TA muscles. The hypothesis was that different signaling pathways will be distinctly activated depending on the severity of the dystrophic phenotype.

Materials and Methods

Animals. Breeding pairs for C57BL/10SNJ (control) and *mdx* mice were purchased from Jackson Laboratories, Bar Harbor, ME. Mice were bred and housed in accredited facilities at the Veterinary Medical Unit of the Audie L. Murphy VA Hospital, San Antonio, Texas. All animals were cared for according to Institutional Animal Care and Use Committee (IACUC) guidelines. Mice were housed on a 12:12-hr light:dark cycle and received *ad libitum* food and water. Male control (n = 5) and mdx (n = 5) mice were sacrificed at 3 or 12 months of age by cervical dislocation after anesthesia with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL). One half of the costal DIA and TA muscle were rapidly dissected, flash frozen in liquid nitrogen, and stored at –80°C. A sample size of 5 animals was used because power analysis indicated that this was the minimum number of animals needed to observe statistically significant differences.

Western Blot Analysis. DIA and TA muscles were homogenized in a buffer containing 10 mM $MgCl_2$, 10 mM KH_2PO_4, 1 mM EDTA, 5 mM ethylene glycol tetraacetic acid (EGTA), 1% Nonidet NP-40, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM phenylmethanesulfonyl flouride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 nM okadaic acid. All samples were then centrifuged for 5 mins at 5000 *g* at 4°C. The supernatant was collected, and the protein concentration was determined with a DC Protein Assay (Bio-Rad, Hercules, CA). For immunoblots, 10 μg of protein were boiled with an equal volume of 2× Laemmli sample buffer for 5 mins and resolved by SDS-PAGE. Each muscle was used as an individual sample; no pooling of muscles was performed. Electrophoresis was

conducted using 7.5% acrylamide gels for $p70^{S6k}$ blots and 10% acrylamide gels for p38 and ERK blots on a Bio-Rad Protean II system or Mini-Protean III system. Proteins were then transferred to polyvinylidene diflouride (PVDF) membranes and blocked in 5% milk in Tris-buffered saline (pH = 7.5) and 0.1% Tween-20 (TBS-T). Membranes were then reacted with antibodies that recognize only the phosphorylated amino acid residues of activated kinases. Phosphorylated protein kinases were detected through the use of antibodies for phospho-$p70^{S6k}$ (Thr389), phospho-ERK1/2 (Thr202/Tyr204), and phospho-p38 (Thr180/Tyr182) from Cell Signaling Technology (Beverly, MA). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody detects endogenous levels of p42 and p44 MAP kinase (Erk1 and Erk2) only when catalytically activated by phosphorylation at Thr202 and Tyr204 of human Erk, or Thr183 and Tyr185 of rat Erk. All primary antibodies were used at a 1:2000 dilution with 5% bovine serum albumin (BSA) in TBS-T. A horseradish peroxidase conjugated anti-rabbit secondary antibody (Vector Labs, Burlingame, CA) was then used at a 1:5000 dilution. Immunoblots were visualized using Kodak X-OMAT film and enhanced chemiluminescence substrate kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Following visualization, membranes were stripped with 1× Re-Probe (Geno Tech, St. Louis, MO) and blocked for 1 hr at room temperature in 5% milk/TBS-T. Membranes were then incubated with their respective pan antibody (1:2000 dilutions), which recognizes both phosphorylated and unphosphorylated forms of the respective protein kinase. The pan antibodies for p38 and ERK were from Cell Signaling Technology. The $p70^{S6k}$ pan antibody is from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All of the above mentioned primary antibodies are polyclonal and have been characterized extensively for specificity (26–28). Membranes were then incubated with secondary antibody and visualized as described above. Finally, PVDF membranes were stained with India ink to verify equivalent loading conditions. Only blots with equal loading conditions were used for the density calculation. The molecular weights of the immunodetected proteins were verified by using both low range and kaleidoscope molecular weight markers (Bio-Rad).

Densitometric and Statistical Analyses. Protein immunoblots were quantified using scanning densitometry (Alpha Scan, San Leandro, CA). Percent phosphorylation of p38 and ERK1/2 were calculated by dividing the arbitrary density units of the phosphorylated form of the protein by the density units of the total protein as detected by the respective pan antibody. Changes in the $p70^{S6k}$ phosphorylation state were assessed by mobility shift caused by different phosphorylation sites of the protein with a pan antibody, as described previously (21). Percent phosphorylation of $p70^{S6k}$ was calculated by dividing the amount of phosphorylated $p70^{S6k}$ (all of the one to three slower migrating bands) by total $p70^{S6k}$ (all bands). To test for statistically significant differences, a one-way analysis of variance (ANOVA) was used; in the case of significant differences, the Tukey multiple comparisons test was applied. Statistical significance was set at $P < 0.05$.

Results

$p70^{S6k}$ Is Activated in *mdx* DIA Muscle. Diaphragm muscle of *mdx* mice exhibit significant increases in phosphorylation of the $p70^{S6k}$ at both 3 and 12 months compared with the DIA from age-matched control mice (Fig. 1A). Percent phosphorylation of $p70^{S6k}$ in *mdx* DIA is increased relative to control DIA by 40% at 3 months of age and 45% at 12 months. Moreover, percent phosphorylation is increased by 45% in *mdx* DIA of 12-month-old mice compared with 3-month-old mice. The increase in phosphorylation is due, in part, to an increase in phosphorylation at Threonine-389, the site critical for $p70^{S6k}$ kinase activity (Fig. 1B). Additionally, an age-associated increase in $p70^{S6k}$ percent phosphorylation of 37% was seen from 3 to 12 months in control DIA muscle. However, this increase was not associated with increased phosphorylation at Thr389, unlike in *mdx* muscle. No change in $p70^{S6k}$ migration or Thr389 phosphorylation was found in the TA muscle of *mdx* mice (Fig. 1C and D). These results indicate that $p70^{S6k}$ phosphorylation is elevated only in the *mdx* muscle that has the most severe dystrophic phenotype.

Total Levels and Phosphorylation of ERK1/2 Increase in *mdx* Skeletal Muscle. Phospho-specific antibodies reveal that ERK1/2 phosphorylation is elevated at both 3 and 12 months of age in DIA (Fig. 2C) and TA (Fig. 3C) muscles of *mdx* mice, relative to age-matched controls. Additionally, there is an increase in total ERK1/2 in the *mdx* muscle (Figs. 2B and C, and 3B and C), resulting in the percent of phosphorylated ERK1/2 being not different from that found in control DIA and TA muscles (Figs. 2A and 3A). Total ERK1/2 in the DIA muscle shows a 36% and 39% increase at 3 and 12 months, respectively, whereas the TA muscle shows a 36% and 32% increase at 3 and 12 months, respectively. These results indicate that the lack of dystrophin in *mdx* skeletal muscle is associated with an increase in total ERK1/2 activity regardless of the phenotypic expression of the dystrophy.

P38 Phosphorylation Decreases in DIA Muscle of *mdx* Mice. The DIA muscle of *mdx* mice shows a 35% and 33% decrease in p38 phosphorylation at both 3 and 12 months relative to age-matched controls, respectively (Fig. 4A and B). No change is seen in total p38 in this muscle. No significant changes in total or phosphorylated p38 were seen in the TA muscle of *mdx* mice (Fig. 4C and D), although a trend toward decrease was seen in the p38 phosphorylation state at 3 months of age. Due to the high variability in the data, this decrease did not reach significance. These results indicate that p38 activity is decreased in muscles that display the more severe dystrophic phenotype.

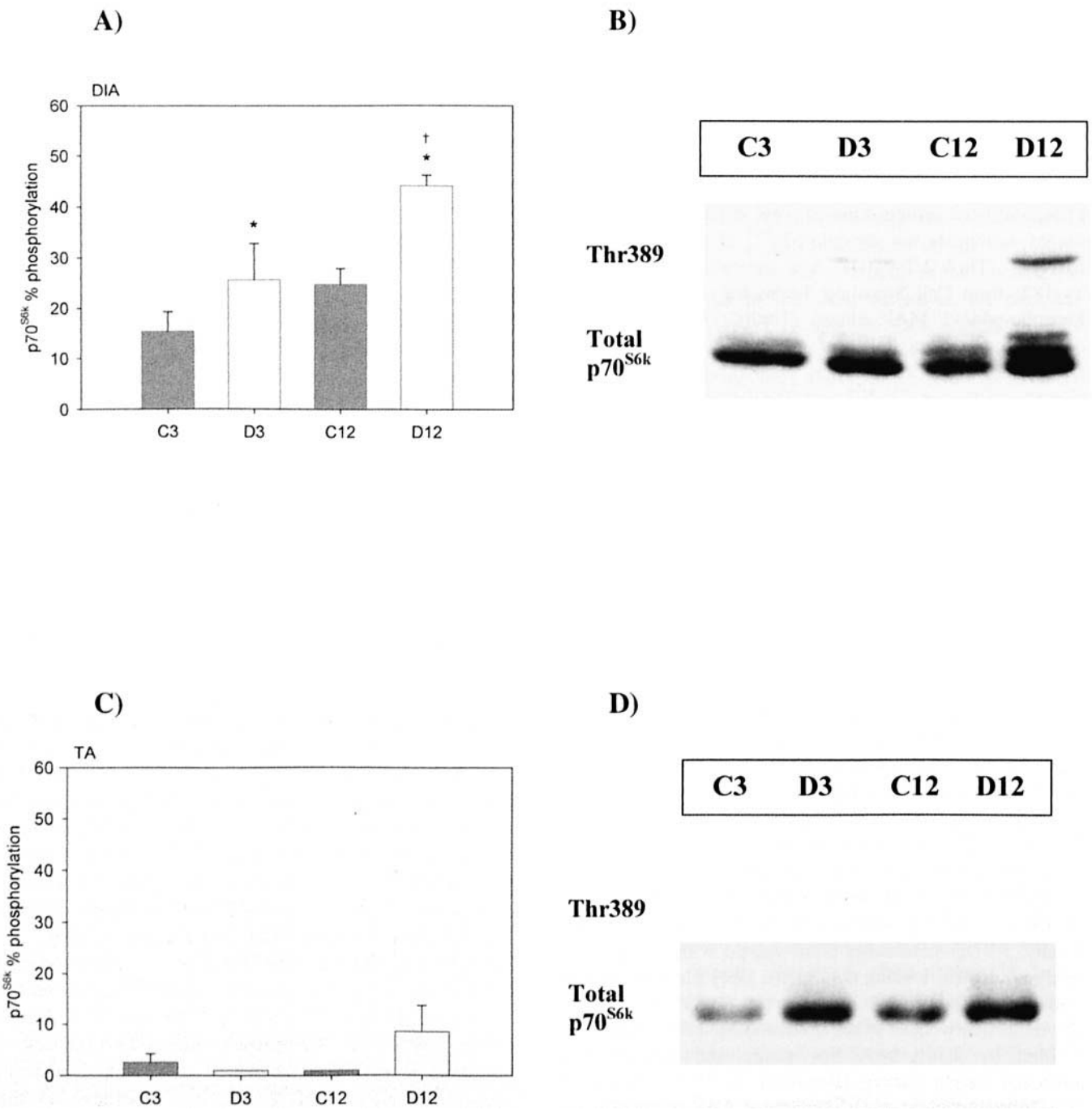

Figure 1. Increased $p70^{S6k}$ phosphorylation in diaphragm (DIA) muscle of *mdx* mice. Percent $P70^{S6k}$ phosphorylation in (A) DIA and (C) tibialis anterior (TA) muscle of 3-month-old control (C3), 3-month-old dystrophic (D3), 12-month-old control (C12), and 12-month-old dystrophic (D12) mice. Bars represent means ± SE. Gray bars represent control and white bars *mdx* muscles. Representative Western blots of total $p70^{S6k}$ (total p70) and $p70^{S6k}$ phosphorylated on residue Threonine 389 (Thr389) of (B) DIA and (D) TA are shown. Asterisk (*), significantly different from age-matched control. Dagger (†), significantly different from respective 3-month muscle group, $P < 0.05$.

Discussion

The activity of multiple signaling proteins in skeletal muscle of mice lacking the dystrophin protein was investigated in this study. We showed that two of these kinases, $p70^{S6k}$ and p38, demonstrated a specificity of response correlated to the dystrophic phenotype. The phosphorylation state of p38 was decreased and that of $p70^{S6k}$ was increased in *mdx* DIA muscle only, but not in *mdx* TA muscle. The increase in total protein of ERK1 and ERK2, and thus total kinase activity, was associated with the lack of dystrophin in skeletal muscle but not disease progression. Taken together, these results suggest that specific kinases were correlated with the transduction of cellular signals that may lead to the dystrophic phenotype,

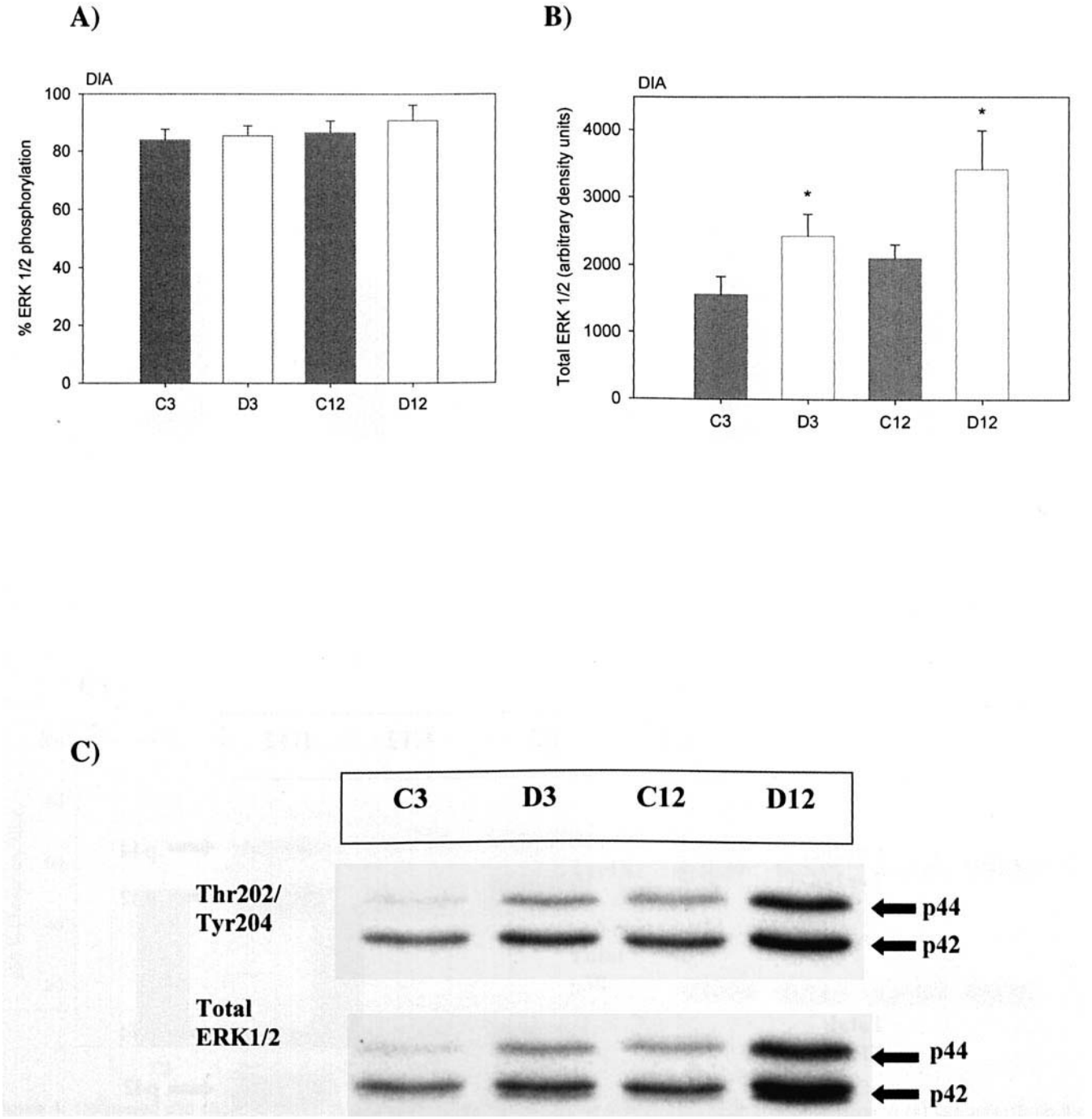

Figure 2. Increased total ERK1/2 protein in diaphragm (DIA) muscle of *mdx* mice. Percent ERK1/2 phosphorylation (A) and total ERK1/2 protein level (B) of DIA muscle. Abbreviations are as in Figure 1. Bars represent mean ± SE. Gray bars represent control and white bars *mdx* muscles. Representative Western blots of total ERK1/2 and ERK1/2 protein phosphorylated on residues Threonine 202 and Tyrosine 204 (C) are shown. Asterisk (*), significantly different from age-matched control, $P < 0.05$.

whereas other kinases were correlated with the genotype but not to the disease progression.

$p70^{S6k}$ is a downstream target of the PI3kinase-Akt-mTOR (target of rapamycin) signaling cascade (29, 30). This pathway plays a significant role in the hypertrophic response of skeletal muscle to high resistance exercise, whereas inactivation of this pathway is associated with muscle atrophy (31). Nader and Esser (28) demonstrated that $p70^{S6k}$ is activated in skeletal muscle following high-resistance exercise, whereas running exercise had no effect on phosphorylation state. Here we report activation of $p70^{S6k}$ in the DIA, but not in the TA, of *mdx* mice at both 3 and 12 months of age. The $p70^{S6k}$ activity was even greater at 12 months of age in the DIA, which correlated with the increase in disease severity. This holds potential significance as the DIA muscle of *mdx* mice shows the most

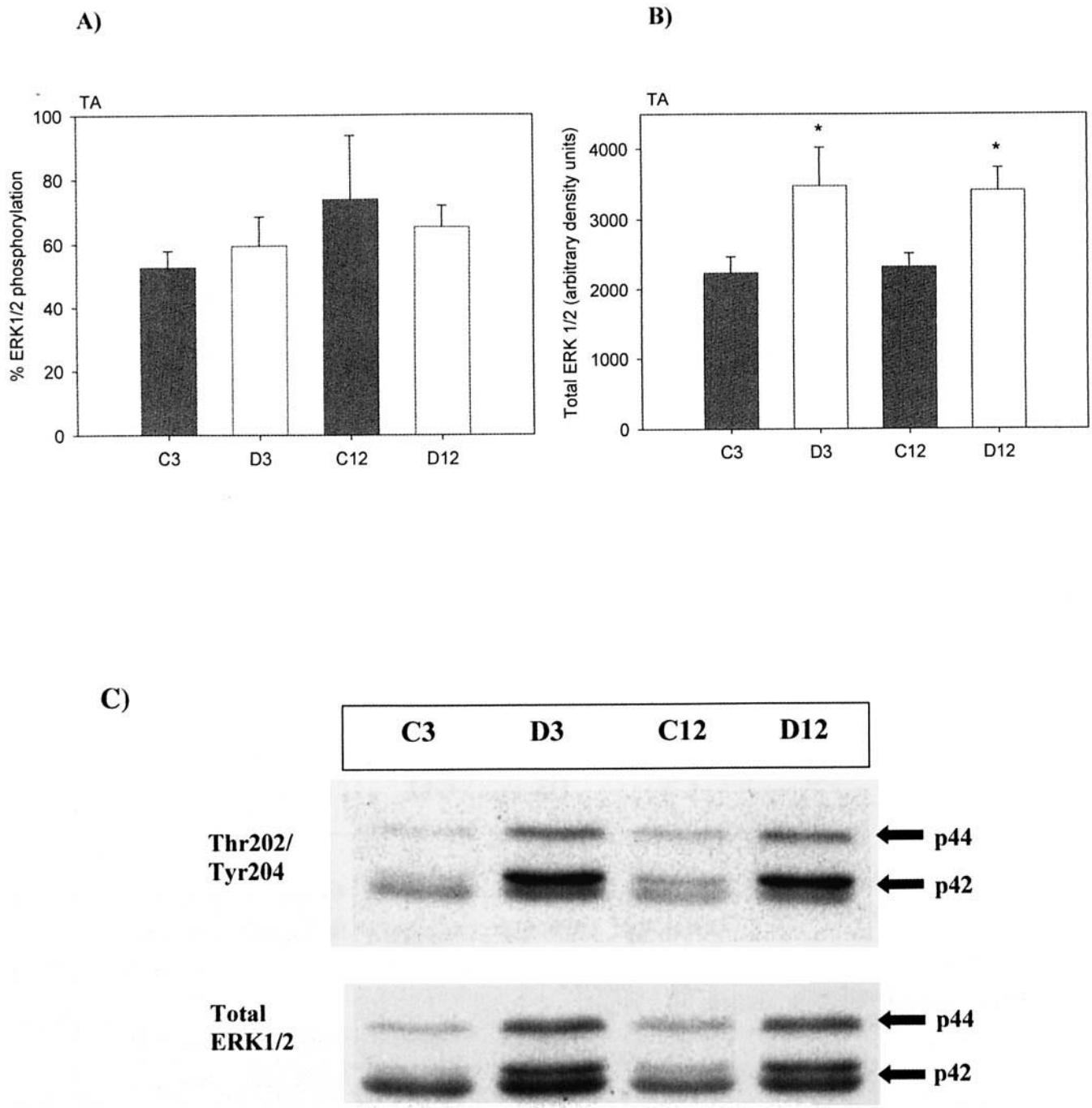

Figure 3. Increased total ERK1/2 protein in tibialis anterior (TA) muscle of *mdx* mice. Percent ERK1/2 phosphorylation (A) and total ERK1/2 protein level (B) of TA muscle. Abbreviations are as in Figure 1. Bars represent mean ± SE. Gray bars represent control and white bars *mdx* muscles. Representative Western blots of total ERK1/2 and ERK1/2 protein phosphorylated on residues Threonine 202 and Tyrosine 204 (C) are shown. Asterisk (*), significantly different from age-matched control, $P < 0.05$.

pathological similarity to DMD muscles and is characterized by cycles of extensive degeneration and regeneration (10). Diaphragm muscles also demonstrated decreased protein and myosin content as well as functional deficits in active tension and fatigue index (7, 32). Limb muscles are not affected to such an extent in *mdx* mice, as seen in the TA muscle, which shows approximately 2%–4% regenerating fibers at 3 and 12 months (33) and moderate decreases in active tension. We suggest that the activation of $p70^{S6k}$ may be a compensatory mechanism of the dystrophic DIA muscle, which contains a high amount of atrophic fibers, to overcome the ongoing muscle degradation and atrophy at both 3 and 12 months of age. Because phosphorylation of $p70^{S6k}$ at Thr389 specifically is downstream of the mTOR signaling cascade, and this particular site showed increased phosphorylation in DIA of *mdx* mice, it would be interesting

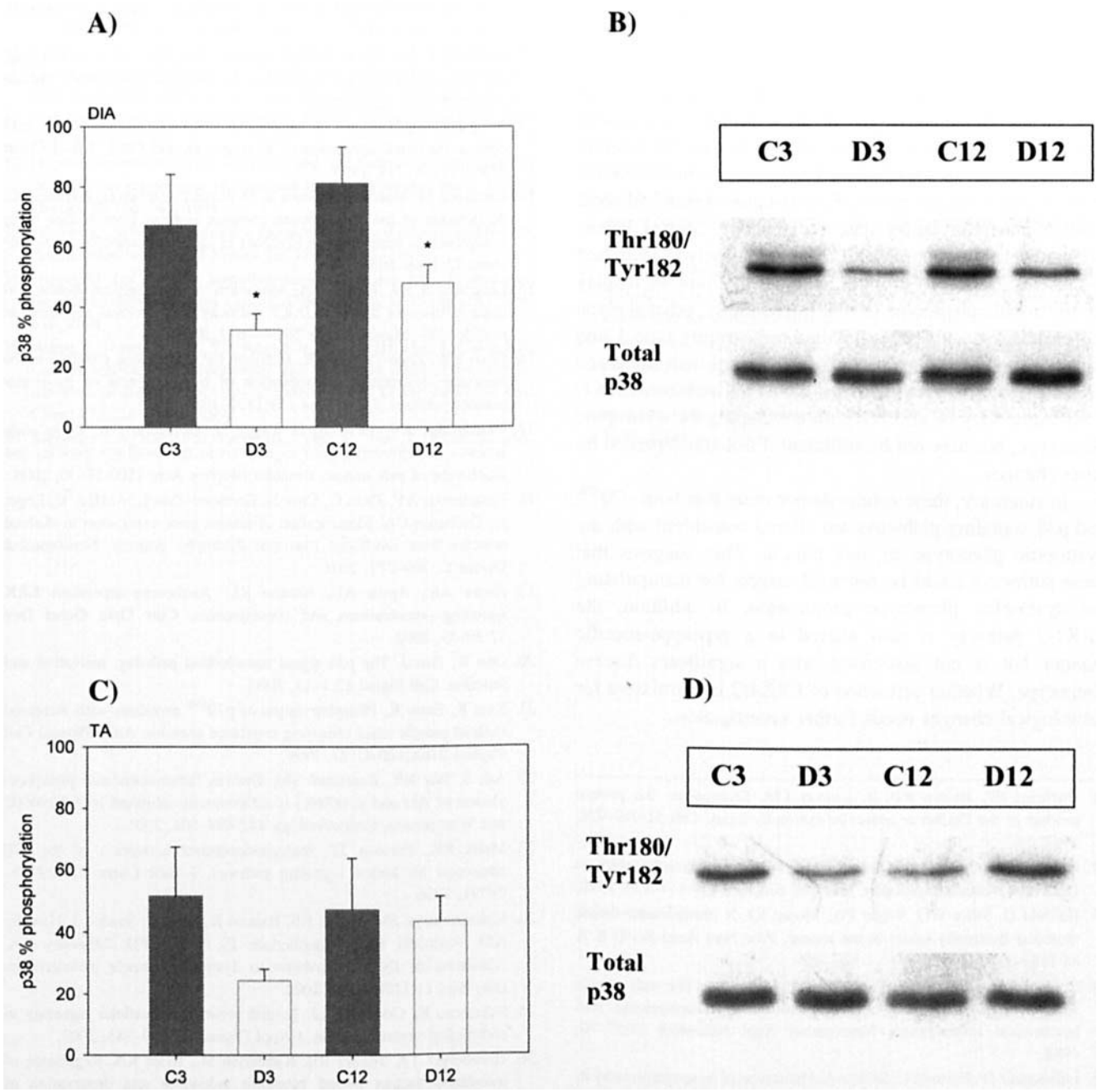

Figure 4. Decreased p38 phosphorylation in diaphragm (DIA) muscle of *mdx* mice. Percent p38 phosphorylation in (A) DIA and (C) tibialis anterior muscle. Abbreviations are as in Figure 1. Bars represent means ± SE. Gray bars represent control and white bars *mdx* muscles. Representative Western blots of total p38 and p38 phosphorylated on residues Threonine 180 and Tyrosine 182 (Thr180/Tyr182) of DIA (B) and TA (D) are shown. Asterisk (*), significantly different from age-matched control, $P < 0.05$.

to see whether treatment with rapamycin would exacerbate or alleviate the dystrophic symptoms.

The stress-activated protein kinase, p38, phosphorylates several transcription factors, including activating transcription factor-2 (ATF-2), p53, and myogenic enhancer factor-2 (MEF-2; Refs. 20, 34). This signaling molecule has also been shown to play an important role in cardiac hypertrophy (35), apoptosis, and cell cycle regulation (34). Surprisingly, we found that p38 showed a decreased phosphorylation state at 12 months of age in the dystrophic DIA muscle and no response in the TA muscle. It has previously been reported that decreased p38 phosphorylation is associated with cardiac fibrosis in the disease state of cardiac tissue from a double knockout mouse strain of dystrophin and MyoD (36). Also, Chen *et al.* (16) found that MAPKAPK, a substrate for p38, was decreased 9-fold in DMD muscles. Therefore, downregulation of the p38 pathway may be an event that correlated with a more severe dystrophic phenotype, but not with dystrophin deficiency, since TA muscles did not show a decrease in p38 phosphorylation.

ERK is a serine/threonine protein kinase that is a member of the MAPK family. There are two isoforms, ERK1 and ERK2, that share approximately 90% homology and are downstream of the Raf1-MEK signaling cascade (19). ERK1/2 kinase activity has been shown to increase in response to endurance exercise, growth factor, and integrin stimulation, among others (37). We found that total ERK1/2 protein levels increased in all dystrophic tissue, whereas relative phosphorylation state remained at control levels. This indicates that total ERK1/2 kinase activity is increased in muscles lacking dystrophin, whether the muscles display the dystrophic phenotype or not. Interestingly, other skeletal muscle diseases, including myotonic dystrophy type 1 and inclusion body myositis, are also associated with increased ERK1/2 expression and activity (38–41). Therefore, ERK1/2 activation may be necessary for developing the dystrophic phenotype, but may not be sufficient if not accompanied by other changes.

In summary, these results demonstrate that both $p70^{S6k}$ and p38 signaling pathways are altered coincident with the dystrophic phenotype in *mdx* muscle. This suggests that these pathways could be potential targets for manipulating the dystrophic phenotype progression. In addition, the ERK1/2 pathway is also altered in a genotype-specific manner but is not associated with a significant disease phenotype. Whether activation of ERK1/2 is permissive for pathological changes needs further investigation.

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