PTEN Contributes to Profound PI3K/Akt Signaling Pathway Deregulation in Dystrophin-Deficient Dog Muscle

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Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder that affects 1 newborn boy in 3500. This recessive disease is caused by mutations in the dystrophin gene, resulting in total lack of the protein,1–3 and is characterized by severe degeneration of muscle fibers, progressive paralysis, and death. Dystrophin is located under the sarcolemma of muscle fibers, and is associated with a complex comprising several integral, peripheral membrane and cytoplasmic proteins: the dystrophin-glycoprotein complex (DGC).4–7 By providing a strong physical link between the cytoskeleton network and the extracellular matrix, the DGC ensures the integrity of skeletal muscle fibers. In the absence of dystrophin, the complex is destabilized and this integrity is lost.5,8 However, the impaired structural role of the DGC alone may not be sufficient to account for the massive degenerative process observed in DMD muscles. Numerous observations suggest that signaling pathway alterations may also participate in DMD pathogenesis.

Dystrophin and various DGC proteins have been demonstrated to interact with a number of signaling proteins, including growth factor receptor-bound protein 2,9 neuronal nitric oxide synthase,10 calmodulin,11 focal adhe-

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sion kinase,\cite{12} and caveolin-3.\cite{13,14,15} Moreover, studies of the X chromosome-linked muscular dystrophy (mdx) mouse\cite{16} revealed modulations in mitogen-activated protein kinase (MAPK) signaling cascades, as dystrophic animals exhibited increased phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2)\cite{17,18} and c-jun N-terminal kinases 1 and 2 (JNK1/2),\cite{19–21} and decreased phosphorylation of p38.\cite{18} Also, the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway has been shown to be affected in the mdx mouse, with increased synthesis and phosphorylation of Akt.\cite{22,23}

In addition to the limited information related to the origin of signal perturbations in dystrophic muscle, almost no information is available regarding signaling pathways in clinically relevant animal models or human tissue samples.\cite{23} It is noteworthy that the mdx mouse model of DMD is characterized by successive degeneration/regeneration processes, but does not exhibit the progressive muscle wasting and accumulation of connective tissue observed during the development of the human disease.\cite{24,25,26} The Golden Retriever muscular dystrophy (GRMD) dog, characterized by rapidly progressive clinical dysfunction, severe muscle weakness, and abundant fiber necrosis, displays a disease progression that is far more similar to human DMD.\cite{27,28}

In this study, we used antibody arrays to assess the global phosphorylation status of key proteins of the PI3K/Akt and MAPK signaling pathways in skeletal muscles of 4-month-old healthy and GRMD dogs. Our data indicated that Akt1, glycogen synthase kinase-3β (GSK3β) and p70S6K, as well as ERK1/2 and p38α and γ kinases all displayed a decreased phosphorylation level in GRMD muscle. Western immunoblot, immunohistochemistry analysis, and enzymatic assays allowed us to confirm these results and demonstrated that they were associated with a reduction in Akt activity and with enhanced GSK3β expression and activity. Analysis of key enzymes involved in Akt regulation revealed that phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) was present at a much higher level and was more active in GRMD muscle. Moreover, immunohistochemistry analysis showed that all of the GSK3β-positive fibers observed in GRMD muscle sections exhibited a strong cytosolic labeling of PTEN, suggesting that the accumulation of the phosphatase could play a central role in PI3K/Akt signaling pathway deregulation. The observation of PTEN/GSK3β-positive fibers in muscle sections from 3- and 36-month-old GRMD dogs further demonstrated that both the early and late stages of the disease share deregulation of the pathway. Collectively, our findings highly suggest that alterations in PTEN exist in GRMD muscle, which leads to long-term and deep modulation of the PI3K/Akt signaling pathway.

Materials and Methods

Antibodies

Mouse monoclonal antibodies (mAbs) anti-Akt1 (dilution used for Western immunoblot analysis: 1:2000) and anti-PTEN (1:500) were from BD PharMingen (San Diego, CA). Mouse mAbs, including anti-caveolin-3 (1:25,000), anti-GSK3β1 (1:1000), anti-p70S6K (1:1000), and anti-protein phosphatase 2A (PP2A) catalytic α (1:5000), were obtained from BD Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies anti-phospho-Akt (Ser473) (1:250), anti-phospho-GSK3β (Ser9: 1:50), anti-phospho-p70S6K (Thr421/Ser424: 1:100), anti-phospho-3-phosphoinositide-dependent protein kinase-1 (PDK1) (Ser241: 1:250), and anti-lactate dehydrogenase (1:750) were all obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody anti-histone H2AX (1:750) was from Bethyl Laboratories Inc. (Montgomery, TX). A mouse mAb anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1: 5000) from Santa Cruz Biotechnology (Santa Cruz, CA) was used as an internal reference. The anti-Akt1, anti-GSK3β, a rabbit monoclonal anti-phospho-Akt (Ser473), a rabbit polyclonal anti-phospho-p70S6K (Thr389), and a rabbit monoclonal anti-PTEN antibodies from Cell Signaling Technology were also used for immunohistochemistry analysis at dilutions of 1:50; 1:50; 1:10; 1:10, and 1:10, respectively. Goat polyclonal anti-mouse and anti-rabbit secondary antibodies (IgG) conjugated to Alexa Fluor 680 (1:10,000), as well as anti-rabbit and antimouse IgGs conjugated to Alexa Fluor 488 (1:300) were from Molecular Probes (Eugene, OR). Mouse monoclonal anti-Akt1 and anti-PP2A antibodies from Upstate Biotechnology (Lake Placid, NY) were also used for immunoprecipitation tests.

Animals

The animals came from a GRMD dog-breeding colony established in France. Affected dogs were identified during the first days of life by PCR genotyping using appropriate oligonucleotide primers and immunohistochemical localization of dystrophin.\cite{29} The dogs were housed and cared for at the Boisbonne Center for Gene Therapy of the National Veterinary School of Nantes. Protocols complied with the principles outlined in the French National Institute for Agronomic Research (INRA) Guide for the care and use of laboratory animals in biological experimentation.

Muscles

GRMD dogs and healthy littermates were sacrificed by intravenous sodium pentobarbital administration, and the muscles were removed immediately after euthanasia. The vastus lateralis and biceps brachii muscles were removed from two healthy and two GRMD dogs at 4 months of age. Each muscle biopsy was equally divided, with one sample used for protein extraction (samples were immediately frozen in liquid nitrogen and stored at −80°C until processing), and the other one for immunohistochemical observation. The vastus lateralis muscle was also removed from 3- and 36-month-old GRMD dogs and used for immunohistochemistry analysis.
For protein analysis, the frozen muscles were thawed in lysis buffer containing phosphatase inhibitor cocktails 1 and 2, and protease inhibitor cocktail (purchased from Sigma, St. Louis, MO) at 1:100 and 1:200, respectively. Lysis buffer 6 (R&D Systems, Minneapolis, MN) and Nonidet-P40 lysis buffer (1% Nonidet-P40, 20 mmol/L Tris-HCl pH 8.0, 137 mmol/L NaCl, 10% glycerol, and 2 mmol/L EDTA) were used for the antibody microarray assays and the Western immunoblot analysis, respectively. Fibroic and calcified portions of the biopsies were carefully removed manually, under a binocular microscope. The proteins were extracted from muscle samples using a Tissue-Lyser (Qiagen, Courtaboeuf, France) under the optimum conditions established during preliminary experiments. Typically, 200 mg of muscle samples were suspended in 1 ml lysis buffer, and treated using the Tissue-Lyser at 30 Hz, with a 3-mm stainless steel bead, for 15 minutes at 4°C.

For the immunohistochemistry and histopathological analysis, the sectioned blocks were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and frozen in isopentane cooled with liquid nitrogen. Transverse vastus lateralis muscle cryosections (8 μm) were prepared using a Leica CM 3050S cryostat. For immunofluorescent labeling, muscle sections were permeabilized by incubating in PBS (pH 7.4) containing 0.2% Tween-20 (except for PTEN: 0.3% Triton X-100) at room temperature for 5 minutes, blocked with 10% goat serum in PBS containing 0.2% Tween-20 (except for PTEN: 0.1% Triton X-100) for 30 minutes, and then incubated in blocking solution at 4°C overnight with primary antibodies anti-GSK3β, anti-Akt1, anti-phospho-Akt (Ser473), anti-phospho-p70S6K (Thr389), or anti-PTEN. After washing with PBS, the samples were incubated with goat anti-rabbit or anti-mouse antibodies labeled with Alexa Fluor 680 (1:10,000) in PBS containing 0.1% Tween-20 and 2.5% bovine serum albumin at room temperature for 1 hour, and incubated with the corresponding secondary goat anti-mouse or anti-rabbit IgG labeled with Alexa Fluor 488 (1:100) and protease inhibitor cocktail (1:200) were added to the buffers used to obtain subcellular cytosolic, membrane, and nuclear protein fractions. The protein concentration was determined using a detergent-compatible colorimetric assay (DC protein Assay kit, Biorad, Hercules, CA). The purity of the fractions was verified using anti-lactate dehydrogenase (cytosolic marker), anti-caveolin-3 (plasma membrane marker), and anti-histone H2AX (nuclear marker) antibodies.

**Western Immunoblot Analysis**

Total proteins or subcellular fraction proteins extracted from healthy and GRMD muscles were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a Protran BA83 nitrocellulose membrane (Whatman, Maidstone, UK) by electro-blotting with a Mini Trans-Blot cell (BioRad). A Ponceau-red staining was used to verify that the lanes were equally loaded (data not shown). The membranes were blocked with PBS containing 0.1% Tween-20 and 2.5% bovine serum albumin at room temperature for 1 hour, and incubated with the corresponding antibody in PBS containing 0.1% Tween-20 and 1% bovine serum albumin for 2 hours. After three washes in PBS, the membranes were incubated with secondary goat anti-mouse or anti-rabbit IgG labeled with Alexa Fluor 680 (1:10,000) in PBS containing 0.1% Tween-20 and 1% bovine serum albumin for 1 hour. The membranes were washed, and the fluorescence emitted by the protein bands was monitored using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE). The signal intensity (pixel.mm⁻²) was quantified using the Odyssey Application Software Version 1.1 (LI-COR Biosciences). For total extracts, preliminary immunoblot analysis was performed on separate biopsies (four samples dissected from distinct parts of the muscle). No difference was observed in the protein yield of the different biopsies and protein extracts were usually pooled, and analyzed in a single sample. The values were normalized with respect to the reference GAPDH and the average value was calculated from at least three experiments. Quantitative evaluation of the fluorescent intensity of the protein bands was performed using statEL software (Excel, Microsoft).

**Kinase Activity Assays**

Healthy and GRMD vastus lateralis muscles were lysed in modified RIPA lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.4, 1% Nonidet-P40, 1% glycerol, and 1 mmol/L EDTA) containing phosphatase and protease inhibitors. Protein concentration was determined using a detergent-compatible colorimetric assay (DC protein Assay kit, Biorad, Hercules, CA). The purity of the fractions was verified using anti-lactate dehydrogenase (cytosolic marker), anti-caveolin-3 (plasma membrane marker), and anti-histone H2AX (nuclear marker) antibodies.
using the bicinchoninic acid method and 500 μg of total proteins were precleared with 30 μl of Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Buckinghamshire, UK) for 2 hours at 4°C. Lysates were incubated with 5 μg of anti-Akt1 or 2.5 μg of anti-GSK3β antibody overnight at 4°C under gentle agitation and immune complexes were immunoprecipitated with 30 μl of Protein G Sepharose beads for 4 hours at 4°C. Immobilized complexes were washed twice with lysis buffer and twice with a specific buffer: kinase assay dilution buffer (100 mM/L 3-[N-morpholino]propanesulfonic acid pH 7.2, 125 mM/L β-glycerol phosphate, 25 mM/L ethylenebis(oxyethylenenitrilo)tetraacetic acid, 5 mM/L sodium orthovanadate, and 5 mM/L dithiothreitol) for the Akt assay and cold kinase assay buffer (25 mM/L HEPES pH 7.4, 10 mM/L MgCl₂, and 1 mM/L dithiothreitol) for the GSK3β assay. Kinase reactions were performed for 30 minutes at 30°C in the presence of 1 μCi [γ-32P]ATP. For the Akt kinase activity assay, 10 μl of kinase assay dilution buffer, 10 μmol/L of protein kinase A inhibitor peptide, 100 μmol/L of Akt/SGK peptide [RPRAATF] (immunoprecipitation phosphatase assay from Upstate Biotechnology), 150 μmol/L of ATP, and 20 mM/L of MgCl₂ were added, for a total volume of 40 μl. For the GSK3β assay, 10 μmol/L of ATP, and a GSK3β-specific peptide substrate (YRRAAVPPSPSLSRHLDI) were used to evaluate the phosphorylation of a preferred PP2A substrate phosphopeptide (KRpTIRR; Upstate Biotechnology).

PTEN Activity Assay

Healthy and GRMD vastus lateralis muscles were lysed in modified RIPA lysis buffer containing protease inhibitors. Total extracts were clarified by centrifugation at 100,000 g for 40 minutes and supernatants were desalted on a PD-10 column after immunoprecipitation. Phospholipid vesicles were prepared using 100 μmol/L of di(C8)-Pi(3,4,5)P3 (Echelon Biosciences, Inc., Salt Lake City, UT) and 0.5 mM/L of phosphatidyl-serine (Sigma) in 20 mM/L HEPES, pH 7.4, and 1 mM/L ethylenebis(oxyethylenenitrilo)tetraacetic acid by a sonication of 1 hour at 4°C. The lipid phosphatase assay was performed by incubating 10 μl of supernatant with 10 μl of phospholipid vesicles and 10 μl of buffer (100 mM/L Tris-HCl pH 8.0 and 10 mM/L dithiothreitol) for 15 minutes at 37°C and stopped by the addition of 100 μl of Malachite Green Reagent (Echelon Biosciences). Optical density at 650 nm was read after 15 minutes of color development at room temperature.

PP2A Activity Assay

PP2A activity was evaluated in healthy and GRMD vastus lateralis muscles by using an immunoprecipitation phosphatase assay from Upstate Biotechnology. In brief, immunoprecipitates of PP2A were obtained by incubating the muscle extracts with 4 μg of anti-PP2A C subunit antibody and the activity was assessed by the dephosphorylation of a preferred PP2A substrate phosphopeptide (KRpTIRR; Upstate Biotechnology).

Antibody Arrays

A human phosho-MAPK array kit (R&D Systems) was used to evaluate the phosphorylation of 19 protein kinases involved in major signaling pathways in skeletal muscle from healthy and GRMD dogs. Protein sequence homology between human and dog was preliminary controlled by determining the identity score of the different kinases and the antibody cross-reactivity was validated by Western immunoblot (data not shown). Capture antibodies directed against pan-epitopes of the corresponding proteins are immobilized on a nitrocellulose membrane (two spots of each antibody) and, after binding of both unphosphorylated and phosphorylated kinases, the latter are detected thanks to a cocktail of phosphosite specific antibodies (Proteome Profiler Array, R&D Systems).

Binding assays were performed following the Supplier’s instructions with minor modifications. The membranes were blocked for 1 hour at 20°C, and then incubated overnight at 4°C with 300 μg of total proteins. The membranes were washed three times for 10 minutes and then incubated with the detection antibody cocktail for 2 hours at 20°C. After washing, the membranes were incubated with streptavidin-horseradish peroxidase for 30 minutes and the chemiluminescent signal was recorded on Kodak Biomax MR film (Eastman Kodak, Rochester, NY). Films were scanned, and the spot images were analyzed using GenePix Pro 4.0 software (Axon Instruments, Union City, CA).

The assays were conducted in duplicate, using two biopsies removed from two different dogs, and similar results were obtained. A background value was determined for each array, using negative controls and empty spots positioned in clear areas. This average background signal was subtracted from each spot to normalize the values obtained. A ratio of signal intensity (GRMD: healthy) was calculated for each of the four replicates (two duplicates per assay), and transformed into a log value (base 2). A cut-off value was determined by analysis of variance analysis at the 95% confidence level (P < 0.05, n = 4). Statistically significant variations in the fluorescence signals determined from the spots are shown on the corresponding histograms.

Results

Profiling Protein Kinase Phosphorylation in Dog Skeletal Muscle

To obtain a global overview of the modulations that could impact the PI3K/Akt and MAPK (ERK, JNK, and p38) signaling pathways in GRMD muscle, standard antibody array technology was used. A phosho-MAPK antibody
array was applied to compare the phosphorylation profile of 19 different protein kinases in skeletal muscle from 4-month-old healthy and GRMD dogs (Figure 1A). Among the 15 spots that could be analyzed, seven corresponded to kinases exhibiting a phosphorylation level statistically reduced in GRMD muscle (Figure 1B). Akt1 and the direct and indirect Akt targets GSK3β and p70S6K all exhibited a reduced phosphorylation level in GRMD muscle, revealing that the PI3K/Akt pathway was severely affected. Also, two of the three MAPK pathways appeared to be modulated in GRMD muscle, as a reduced phosphorylation level was demonstrated for both ERK1 and ERK2 (confirmed by Western immunoblot experiment, data not shown), and p38 and γ kinases. Collectively, these results showed that GRMD skeletal muscle displayed a deep remodeling of various major cell signaling pathways.

Figure 1. Antibody array analysis revealed PI3K/Akt and MAPK signaling pathway modulation in GRMD skeletal muscle. A: Healthy and GRMD muscle extracts from 4-month-old dogs were incubated with two antibody arrays. Phosphorylated kinases were detected thanks to a cocktail of phospho-site specific biotinylated antibodies, via streptavidin-HRP and chemiluminescence. The position of the antibodies (double spots for each antibody) relative to the relevant protein kinases is shown. 1) Positive control, 2) ERK1 (right) and ERK2 (left), 3) JNK pan (bottom, right), JNK1 (top, right), JNK2 (top, middle), and JNK3 (top, left), 4) p38α (top, left), p38β (bottom, right), and p38γ (top, right), 5) GSK3α/β (right) and GSK3β (left), 6) p70S6K, and 7) Akt pan (bottom, left), Akt1 (top, right), and Akt2 (bottom, right). B: Quantification of the antibody array data. The chemiluminescence signal intensity of individual spots was analyzed using Genepix Pro 4.0 software. The normalized spot intensity was determined by subtracting the averaged background signal from the spots corresponding to each kinase. A ratio of signal intensity (GRMD:healthy) was calculated, and log transformed (base2). The graph was constructed from two independent assays and error bars show the SEM. The numbers under the graph refer to Figure 1A. A cutoff value was determined by analysis of variance analysis at a P-value of 0.05.

Figure 2. Akt exhibited increased expression, decreased phosphorylation, perturbed localization, and reduced activity in GRMD skeletal muscle. A: Western immunoblot analysis of Akt expression in skeletal muscle from 4-month-old healthy (H) and GRMD dogs. Total proteins (75 µg) were migrated with SDS-PAGE and the blotted proteins were incubated with anti-Akt1 antibody. The anti-GAPDH mAb was used to detect the corresponding control protein. Signals were analyzed by densitometry and quantified using the Odyssey Application Software Version 1.1. Immunohistochemical analysis for Akt1 was performed on transverse cryosections from 4-month-old healthy and GRMD dogs. Nuclear Akt1 immunolabeling was observed in healthy muscle fibers whereas strong Akt1 immunolabeling was observed in the cytosol of small GRMD muscle fibers (green fluorescent staining). Nuclear counterstaining is shown in blue. Scale bar = 50 µm. B: Western immunoblot analysis of Akt phosphorylation level. Subcellular (cytosol, membranes and nucleus) protein fractions (10 µg) were migrated with SDS-PAGE and the blotted proteins were incubated with anti-phospho-Akt (Ser473), anti-lactate dehydrogenase (cytosolic marker), anti-caveolin-3 (plasma membrane marker), and anti-histone H2AX (nucleus marker) antibodies. C: Immunohistochemical analysis for phosphorylated Akt (Ser473) showed strong fluorescent labeling of the nucleus and membrane in the healthy muscle, and a fall in the intensity of the phosphorylated Akt immunolabeling in the GRMD one (green fluorescent staining). Scale bar = 50 µm. D: Akt activity assay revealed that the kinase was almost half as active in the GRMD muscle as in the healthy one. The histogram represents the relative level of Akt kinase activity in healthy and GRMD total extracts. The mean variation between the healthy and the GRMD muscle was calculated and the GRMD value was represented as a percentage of the healthy level taken as 100% (*P < 0.05).

Decreased Phosphorylation and Perturbed Localization of Akt in GRMD Muscle

Antibody array analysis revealed an important deregulation of the PI3K/Akt signaling pathway in GRMD skeletal muscle, with notably a reduction in Akt1 phosphorylation level. Western immunoblot experiments showed a marked increase in total Akt1 proteins in GRMD muscle (Figure 2A). Immunofluorescence analysis revealed that the Akt1 labeling was slight in healthy skeletal muscle, whereas GRMD muscle was characterized by the presence of many Akt1-expressing fibers exhibiting a cytosolic labeling and dispersed throughout the muscle tissue section. Some of
these Akt1-positive fibers, regularly observed in cluster (Figure 2A, asterisk), were defined by a very intense labeling indicative of a massive accumulation of the kinase. To determine how Akt phosphorylation was altered at the subcellular level, compartment-specific fractions were prepared from healthy and GRMD skeletal muscles and analyzed by Western immunoblot. In healthy dog muscle, phosphorylated Akt was not visible in the cytosolic fraction but was found to be concentrated in the membrane and nuclear compartments (Figure 2B). In GRMD muscle, phosphorylated Akt was neither detected in the cytosolic fraction and a dramatic reduction in the amount of the protein was showed within both the membrane and nuclear fractions. Immunofluorescence analysis revealed the presence of phosphorylated Akt in the nucleus and membrane of healthy muscle fibers (Figure 2C) whereas in GRMD muscle sections, phosphorylated Akt-positive nuclei appeared less numerous and were characterized by a less intense labeling. Using a kinase activity assay, we also demonstrated that the reduced presence of phosphorylated Akt in GRMD skeletal muscle was associated with a large decrease in its activity, as the kinase was found to be almost half as active as in healthy counterparts (Figure 2D). To explore whether the alteration of the phosphorylated form of Akt was main-

Figure 3. Decreased phosphorylation of GSK3β in GRMD skeletal muscle was associated with an increase in expression and enzymatic activity. A and B: Total extracts (75 µg) obtained from healthy and GRMD skeletal muscles (4-month-old dogs) were separated by SDS-PAGE. The blotted proteins were incubated with anti-phospho-GSK3β (Ser9) (A) or anti-GSK3β (B) antibody. The anti-GAPDH mAb was used as a loading control. C: Subcellular (cytosol, membranes and nucleus) protein fractions (10 µg) obtained from healthy and GRMD skeletal muscle were separated by SDS-PAGE and the blotted proteins were incubated with anti-GSK3β antibody. D: Immunohistochemical analysis revealed weak cytosolic labeling of GSK3β in healthy muscle fibers and strong cytosolic immunolabeling in GRMD muscle fibers (green fluorescent staining). Nuclear counterstaining is shown in blue. Scale bar = 50 µm. E: The calcium-sensitive dye alizarin red failed to stain any GSK3β-positive fibers (arrow and arrowheads). An alizarin red-positive fiber is shown (asterisk). H&E staining revealed classical pathological changes of DMD, including fiber size variation, fiber splitting, and central nucleation in skeletal limb muscle. Some of the GSK3β-positive fibers were centro-nucleated (arrowheads). F: GSK3β activity assay revealed that the kinase was more than twice as active in GRMD muscle. For further details, see Figure 2D, (**P < 0.002).
GSK3β and p70S6K Phosphorylation Is Reduced in GRMD Muscle

We next wanted to determine the consequences of the reduced Akt activity detected in GRMD muscle on the expression and phosphorylation pattern of GSK3β and p70S6K. First, Western immunoblot experiments confirmed the previous antibody array results revealing that the phosphorylation of GSK3β was lower in GRMD muscle than in the healthy counterpart (Figure 3A). Subsequently, we observed that GSK3β synthesis was enhanced in GRMD muscle, suggesting a greater elevation of the non-phosphorylated and active form of the kinase (Figure 3B). Subcellular fractionation experiments revealed that GSK3β was present in the healthy cytosolic, membrane, and nuclear fractions (Figure 3C). In GRMD muscle, GSK3β was also observed in these three compartments but with an increased amount. To determine the muscle tissue location of GSK3β, immunolabeling directed against the kinase was performed. It revealed that, while any healthy fibers were found to express GSK3β-positive fibers displayed centrally located nuclei characteristic of regenerated fibers (Figure 3E, arrowhead). This indicates that GSK3β overexpression concerns mature fibers as well as young myotubes. Above all, the enhanced expression of GSK3β and its reduced phosphorylation in GRMD muscle were associated with a twofold increase in its kinase activity (Figure 3F).
Western immunoblot experiments also confirmed that p70S6K phosphorylation level was notably lower in the GRMD muscle extract than in the healthy muscle, yet the level of total proteins remained similar between the two conditions (Figure 4A). Confocal analysis of the phosphorylated p70S6K immunolabeling revealed that the protein was present in nearly all of the nuclei of the healthy muscle fibers whereas its presence was limited to a few nuclei in the GRMD fibers (Figure 4B).

Collectively, these results demonstrated that both the PI3K/Akt/GSK3β and the PI3K/Akt/mammalian target of rapamycin (mTOR)/p70S6K pathways were significantly impacted by the reduced activity of Akt detected in GRMD muscle.

**Increased PTEN Expression and Activity in GRMD Muscle**

To understand the mechanisms that affect Akt phosphorylation in GRMD muscle, three enzymes known to modulate Akt signaling were analyzed: PDK1, which phosphorylates Akt, and protein phosphatase type 2A (PP2A), and PTEN, which dephosphorylates Akt and the lipid product phosphatidylinositol 3,4,5-triphosphate (PiP3), respectively. Western immunoblot experiments revealed that the active phosphorylated form of PDK1 was elevated in GRMD muscle (Figure 5A), which could not account for the reduction detected in Akt phosphorylation level. The expression level of PP2A was also slightly increased but the enzyme assay did not reveal any difference in its activity (Figure 5, A and B). Interestingly, the amount of the PTEN phosphatase was highly elevated in GRMD muscle, with a 4.5-fold increase when compared with healthy muscle (Figure 5C). Moreover, a notable increase in PTEN activity was observed in the GRMD muscle extract (Figure 5D). Immunolabeling directed against PTEN showed that healthy muscle fibers displayed a weak expression of the phosphatase whereas nearly all of the GRMD muscle fibers were PTEN-positive (Figure 5E). These fibers exhibited either a strong labeling dispersed in whole cytosol, and evoking an important phosphatase accumulation (Figure 5E, arrow), or only an intense subsarcolemmal labeling (Figure 5E, arrowhead). To determine whether this PTEN altered expression was specific to the age of 4 months or if it could be observed during the GRMD disease progression, H&E staining,
and PTEN-immunolabeling were performed on serial muscle tissue sections from 3-, 4-, and 36-month-old GRMD dogs. Immunohistochemistry was also performed in parallel to the downstream-located kinase GSK3β. Three-month-old GRMD dog muscles, in which the morphological features of muscular-located kinase GSK3β were yet very few, with only heterogeneity of fiber diameter, occasional fibers containing central nuclei and focal inflammatory cells (Figure 6A), already displayed a large number of PTEN-positive fibers randomly dispersed in the section (Figure 6B, arrowhead). Of note, it appeared that these PTEN-positive fibers were all GSK3β-positive (Figure 6C, arrow), demonstrating that PTEN alteration contributes to deregulate the PI3K/Akt signaling pathway. Moreover, these observations highly suggest that the signaling pathway deregulation is present precociously in the dystrophic context, when the primitive histological muscle abnormalities are identified. In 4-month-old GRMD dog muscles that were characterized by high fiber-size variation, individual necrosis fibers, centrally nucleated fibers and fibrosis (Figure 6D), PTEN/GSK3β-positive fibers were also found (Figure 6, E and F, arrow). They mainly had a small diameter (Figure 6, D–F, empty arrowhead) or displayed central nuclei indicative of a stage of muscle regeneration (Figure 6, D–F, thick arrow). Also, some PTEN/GSK3β-positive fibers corresponded to fibers undergoing phagocytosis (Figure 6, D–F, round). Finally, 36-month-old GRMD dog muscles that displayed areas of extensive endomysial fibrosis typically associated with marked fiber atrophy and massive infiltration of inflammatory cells (Figure 6G) were characterized by a large proportion of fibers expressing both PTEN and GSK3β (Figure 6, H and I, arrow). As this age point defined by exhausted myogenic regeneration potential, several PTEN/GSK3β-positive fibers were identified as necrotic fibers characterized by pale homogeneous cytoplasm and variable invading macrophages (Figure 6, G–I, round), which could indicate that PTEN/GSK3β up-regulation defines muscle fibers that have accumulated damages and that are intended to degenerate. Of interest, the few hypertrophic fibers that were still present in the section were PTEN and GSK3β-negative (Figure 6, H and I, square). The immunolabeling directed against the phosphorylated form of Akt performed in parallel on serial muscle sections co-labeled with PTEN and GSK3β at 3, 4, and 36 months revealed that PTEN/GSK3β-positive fibers were defined by a faint phospho-Akt signal, whatever the stage of the disease (see supplemental Figure 1 at http://ajp.amjpathol.org/). All together, these results showed that an increased PTEN expression exists in muscle fibers during the progression of the GRMD disease and demonstrated that PTEN/Akt/GSK3β alteration could not be attributed to a feature of regeneration or a consequence of inflammatory changes.

**Discussion**

Recent studies have indicated that DMD is associated with alterations in signal transduction pathways that may influence the muscle phenotype. \(^{17–19,21–23}\) Nevertheless, these studies have generally focused on one protein rather than assessing the global changes that may precipitate or exacerbate the pathology. Moreover, they have been mainly performed in the mdx mouse, a model that most likely will exhibit species specific changes that may not be reflected in the course of the human disease. Here, we applied antibody array technology to compare the phosphorylation profile of proteins from the PI3K/Akt and MAPK signaling pathways between skeletal muscles from healthy and GRMD dogs, that correspond to the only clinically relevant animal model for DMD. For the first time, we showed that Akt1, GSK3β, and p70S6K, along with ERK1/2, and p38β and γ kinases, exhibited reduced phosphorylation in GRMD muscle, revealing that cell signaling function is largely altered in the DMD context. Given the importance of the PI3K/Akt signaling pathway for regulating muscle mass, \(^{30–32}\) we chose to focus our analysis on the cascade and on the proteins involved in its regulation. Subcellular fractionation and immunohistochemistry experiments revealed that the reduced phosphorylation level of Akt in GRMD muscle, as initially detected by the antibody array method, was the result of a dramatic decrease in the amount of the phosphorylated isoform in the membranes and nucleus. Using a kinase activity assay, we showed that this was associated with a severe decrease in its activity. Akt directly phosphorylates GSK3β at Ser9, thereby repressing its activity, \(^{34}\) and catalyzes, via mTOR, p70S6K phosphorylation and activation\(^{31,35–38}\) (see Figure 7 for a schematic representation of the signaling pathway). The enhanced GSK3β activity and reduced p70S6K phosphorylation detected in GRMD muscle are thus coherent with the reduction in
its activity. Three enzymes involved in its regulation were then analyzed. We detected changes in phosphorylated PDK1 and total PP2A levels, but those could not account for the perturbations observed. Indeed, subsequently to the generation of PIP$_3$ by the PI3K kinase, Akt is recruited to the plasma membrane and phosphorylated by PDK1. Thereby, the increased level of the phosphorylated (and presumably active) form of PDK1 detected in GRMD muscle would presumably lead to a decreased phosphorylation and activity. PP2A dephosphorylates Akt and p70S6K downstream in the cascade but, even if its expression was slightly increased in GRMD muscle, no change was detected in its activity. In contrast, we revealed enhanced expression and activity of the PTEN phosphatase. PTEN opposes PI3K action by dephosphorylating PIP$_3$, and the increased activity detected in GRMD muscle would presumably lead to a decreased level of the phosphoinositide, which should limit the recruitment and activation of Akt (see Figure 7). This hypothesis is strongly supported by the observation that the same fibers that had greatly elevated PTEN level, induced hypertrophy, activation of the Akt/mTOR pathway and p70S6K downstream in the cascade but, even if its expression was slightly increased in GRMD muscle, no change was detected in its activity. In contrast, we revealed enhanced expression and activity of the PTEN phosphatase. PTEN opposes PI3K action by dephosphorylating PIP$_3$, and the increased activity detected in GRMD muscle would presumably lead to a decreased level of the phosphoinositide, which should limit the recruitment and activation of Akt (see Figure 7). This hypothesis is strongly supported by the observation that the fibers that accumulated GSK3$\beta$ in GRMD muscle were the same fibers that had greatly elevated PTEN level. The PI3K/Akt/GSK3$\beta$ and PI3K/Akt/mTOR/p70S6K pathways have been implicated in the regulation of skeletal muscle mass. Akt/mTOR signals were found to be up-regulated during hypertrophy and down-regulated during atrophy and the activation of Akt or p70S6K (or inactivation of GSK3$\beta$) appeared to be sufficient to induce hypertrophy. Moreover, in addition to acting as an inductive cue for hypertrophy, activation of the Akt/mTOR pathway could also prevent muscle atrophy in vivo. Furthermore, it has been shown in vitro that the overexpression of Src homology 2 domain-containing inositol-5’-phosphatase 2, which like PTEN decreases PIP$_3$ level, led to atrophy, whereas the overexpression of a dominant negative mutant, which increases PIP$_3$ level, induced hypertrophy. The overexpression of inositol-5’-phosphatase 2 in healthy mice muscle had no effect on fiber size but the overexpression of the phosphatase in a model of compensatory hypertrophy completely blocked the hypertrophy response. It is thereby likely that the overexpression and increased activity of PTEN detected in GRMD muscle (by decreasing Akt activity and p70S6K phosphorylation, and by activating GSK3$\beta$) could prevent compensatory muscle hypertrophy.

In dystrophin-deficient mdx mice, in which an hypertrophic response is seen, an activation of Akt has been reported in hindlimb skeletal muscles at the pre-necrotic, peak necrotic, and hypertrophic stages of the disease, even if this was surprisingly associated with a decrease in phosphorylated GSK3$\beta$ level. Intriguingly, Akt has also been shown to be activated in the diaphragm muscle at both the pre-necrotic and necrotic stages, in association with increased GSK3$\beta$ and mTOR phosphorylation, suggesting that the activation of the kinase in mdx mice would not depend on the muscle phenotype (i.e., necrotic for the diaphragm muscle or hypertrophic for hindlimb skeletal muscles). The mdx mouse and the GRMD dog develop completely different answers to the absence of dystrophin and the discrepancies in the observations concerning Akt phosphorylation status underlie the necessity of studying signaling pathway modulation in an animal model that closely mimics the human disease. An activation of Akt has been reported in the muscle of two patients (aged of 8 months and 3 years), suggesting that a compensatory mechanism could exist in young DMD boys. However, as a single study exists, with only two biopsies studied, these results need further confirmation. Moreover, the activity of Akt, as well as the status of the downstream GSK3$\beta$ and p70S6K kinases, has not already been assessed.

The question that arises next is what, at the structural level, could explain the enhanced expression and activity of PTEN detected in GRMD muscle. PTEN activity is largely restricted to the plasma membrane. Under normal conditions, the phosphatase is in a constrained conformation that would permit to maintain PIP$_3$ at a basal level but would not prevent stimulus-induced activation of the PI3K/Akt pathway. Moreover, a fraction of the molecules seems to be localized in caveolin-enriched membrane fractions. The absence of dystrophin is associated with multiple plasma membrane rehandling, along with structural and conformational changes, among which we can mention the large variability of caveolae size, shape, or number. It therefore appears possible that the alteration of PTEN detected in GRMD muscle could be a consequence of the membrane instability generated by the absence of dystrophin. Considering the impact of this deregulation on the PI3K/Akt pathway, and the putative consequences on the compensatory hypertrophic response that muscles could eventually develop, the question of its precise origin and the demonstration of its link with the lack of dystrophin appear of great interest. PTEN is the second most frequently deleted gene in human cancer and mutations have been associated with Cowden disease (a cancer predisposition syndrome). Mutations of PTEN have also been associated with type 2 diabetes but this is the first time that a deregulation is reported in a neuromuscular disease. The status of the phosphatase should now be carefully examined in mdx mice and DMD patients. It would also be interesting to see whether the observations made in the GRMD dog can be seen in other muscular dystrophies (due to mutations in genes coding for caveolins, like limb-girdle muscular dystrophy LGMD-1sC or in genes coding for some component of the DGC complex) or if they are restricted to DMD.

Taken together, our present results establish that increased PTEN activity is a signature of muscular dystrophy pathogenesis in the GRMD dog. This elevation leads to long-term and deep PI3K/Akt signaling pathway alteration, which could limit compensatory hypertrophy and exacerbate muscle degeneration.

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