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Pathways Implicated in Tadalafil Amelioration of Duchenne Muscular Dystrophy

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Numerous therapeutic approaches for Duchenne and Becker Muscular Dystrophy (DMD and BMD), the most common X-linked muscle degenerative disease, have been proposed. So far, the only one showing a clear beneficial effect is the use of corticosteroids. Recent evidence indicates an improvement of dystrophic cardiac and skeletal muscles in the presence of sustained cGMP levels secondary to a blocking of their degradation by phosphodiesterase five (PDE5). Due to these data, we performed a study to investigate the effect of the specific PDE5 inhibitor, tadalafil, on dystrophic skeletal muscle function. Chronic pharmacological treatment with tadalafil has been carried out in mdx mice. Behavioral and physiological tests, as well as histological and biochemical analyses, confirmed the efficacy of the therapy. We then performed a microarray-based genomic analysis to assess the pattern of gene expression in muscle samples obtained from the different cohorts of animals treated with tadalafil. This scrutiny allowed us to identify several classes of modulated genes. Our results show that PDE5 inhibition can ameliorate dystrophy by acting at different levels. Tadalafil can lead to (1) increased lipid metabolism; (2) a switch towards slow oxidative fibers driven by the up-regulation of PGC-1α; (3) an increased protein synthesis efficiency; (4) a better actin network organization at Z-disk.

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The numerous studies performed in the field of muscle degenerative diseases such as Duchenne and Becker muscular dystrophies (DMD and BMD, respectively) have proposed different approaches with therapeutic potentialities (Di Certo et al., 2010; Tedesco and Cossu, 2012; Fairclough et al., 2013; Konieczy et al., 2013; Kornegay et al., 2014; Berardi et al., 2014; Jarmin et al., 2014; Strimpakos et al., 2014). Unfortunately, all of these genetic and cellular approaches are currently far from having an important impact on the treatment of human patients affected by dystrophy, and the only treatment showing a clear beneficial effect for this disease is the use of corticosteroids (Baltgalvis et al., 2009). Corticosteroids can delay the loss of motility and improve the ventilatory function of patients. Unfortunately, corticosteroid therapy has many adverse side effects and is not feasible for long-term use. For this reason, we recently investigated the effect of different types of non-steroidal anti-inflammatory drugs (NSAIDs) on mdx mice. The results revealed that these compounds have a beneficial effect on dystrophic muscle morphology that is comparable to glucocorticoid treatment (Serra et al., 2012). Other molecules that have shown efficacy in slowing down or arresting the progression of the disease include nitric oxide (NO) donors and histone deacetylases inhibitors (HDACi) (Colussi et al., 2008; Rovere-Rovere-Querini et al., 2013). In dystrophic patients, a pilot study has been performed to evaluate the possibility of a combinatorial therapy based on NSAIDs and NO donor drugs (D'Angelo et al., 2012). In addition, NO is implicated in the regulation of HDAC signaling, which is another pathway that is impaired in dystrophic muscles (Colussi et al., 2008). The main biological target of NO is the enzyme guanylate cyclase, which generates the second

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messenger cGMP (Stamler and Meissner, 2001). cGMP signaling is under the negative control of a family of enzymes called phosphodiesterases (PDEs) (Conti and Beavo, 2007). cGMP-specific PDEs include PDE5, PDE6, and PDE9; several classes of drugs inhibiting these enzymes are already extensively used in human therapy, and much is known regarding their toxicology, potential side effects, drug regimens, and pharmacoepidemiology, as well as regarding tolerance to their long-term use. PDE5 plays a critical role in heart physiology (Movsesian et al., 2011). The over-expression of guanylyl cyclase in mdx mice or treatment with sildenafil significantly improved heart physiology in dystrophic mice (Percival et al., 2011). Following PDE5 inhibition, an improvement of vascular activity in dystrophic mice and a reduction of skeletal myofibril damage were observed (Asai et al., 2007; Kobaiashi et al., 2008). Furthermore, Percival et al. (2012) demonstrated that chronic sildenafil treatment improves respiratory function in mdx mice by reducing diaphragm fibrosis. In both murine and fish models of muscular dystrophy, a beneficial effect was observed following the sildenafil-mediated up-regulation of the heme oxygenase signaling pathway (Kawahara et al., 2014).

In patients with BMD, tadalafil treatment ameliorated muscle perfusion by restoring the sympathetic vasoconstriction response (Martin et al., 2012), while Witting et al. (2014) did not observe significant amelioration of muscle function and blood flow in BDM patients following a 4 weeks treatment with sildenafil. Here we investigated the molecular alterations that could possibly contribute to the therapeutic effects of drugs blocking PDE5 on mdx mice, an animal model of DMD.

Materials And Methods

Animals

All procedures involving mice were carried out in accordance with the ethical guidelines for animal care of the European Community Council (directive 2010/63EU). Dystrophin-deficient C57BL/10ScSn-DMDmdx/J mice (mdx) and wt mice from the same mouse strain (Charles River Laboratories Italia s.r.l.-Calco, Lecco, Italy) were housed under a 12 h light-dark schedule at a constant temperature, with food and water provided ad libitum. The tadalafil (Cialis, Ely Lilly and Company, IN) treatments were started in pregnant mice at 10 days postcoitum (p.c.) by oral administration through the drinking water (30 mg/kg/day) and were continued in the pups after birth until 16 weeks of age. At birth, the treated female mice showed no significant differences in number of pups born per litter compared with the untreated mice. No growth differences were observed between the pups given the treatment or not. Only male mice were used in the different experiments performed. The animals were sacrificed by carbon dioxide asphyxiation. After the animals were sacrificed, skeletal muscle tissues were collected and frozen in liquid nitrogen for RNA and protein extractions or were mounted in OCT embedding compound and frozen in liquid nitrogen-cooled isopentane for histological and immunological studies. All samples were stored at −80°C.

Treadmill exercise

The running performance assay was carried out using a five-lane motorized treadmill equipped with an electronic control unit (Treadmill Model LE8710, PanLab, Barcelona, Spain). For the running task, the mice were first acclimated with the treadmill for 2 min, after which the treadmill was run at a 12 m/min fixed speed for 5 min. After 5 min, the speed was increased by 1 m/min every 2 min. The test was stopped when the mouse remained on the shocker plate for more than 5 sec without attempting to reengage the treadmill. The time to exhaustion was automatically recorded from the beginning of the test (Di Certo et al., 2010; Strimpakos et al., 2014). Groups of 10 mice were used in each test. After the treadmill performance test, five mice were intraperitoneally injected with 1% Evans blue dye solution in saline buffer. Twenty-four hours later, the muscle specimens were collected and processed for histological analysis by fluorescence microscopy. Color images were captured using a digital camera.

Contractile activity

The muscle force was measured in isolated extensor digitorum longus (EDL) and diaphragm muscles (Di Certo et al., 2010; Strimpakos et al., 2014). Dissected muscles were mounted in a chamber filled with oxygenated Krebs solution maintained at 37°C, were stretched to a tension of 1.0 g and directly stimulated by single stimulations carried out with rectilinear pulses of 0.5 ms duration at 0.05–0.2 Hz (Electric Stimulatore Digit 3T, Lace Elettronica, Pisa, Italy). Muscle excitability was examined by increasing the voltage until the supramaximal voltage was reached. The mechanical activity of the muscle was isotonically recorded by a strain-gauge transducer (7006 isotonic transducer, Basile, Milano, Italy) and was displayed on a recording micro-dynamometer (Unirecord 7050, Basile, Milano, Italy). Groups of muscles from five mice were used in each test. At the end of the tension recordings, muscles were subjected to a period of repetitive stimulation. Contractions of the muscles were elicited by trains of stimuli at a frequency of 40 Hz for 250 ms every second for 3 min. After this repetitive stimulation, the muscles were incubated in 0.2% Procion Orange dye in phosphate buffer solution (PBS) for 45 min at 30°C. The muscles were washed three times with PBS for 5 min, embedded in Tissue-Tek OCT medium (Sakura Finetechnical, NL), and rapidly frozen in liquid nitrogen-cooled isopentane. Cross sections were prepared and viewed under a fluorescent microscope. Color images were captured using a digital camera.

Morphological analysis

Skeletal muscles frozen in OCT compound were sectioned by a cryostat. Transversal or longitudinal sections were fixed with paraformaldehyde, permeabilized with 0.1% Triton-X-100, blocked with 5% BSA–PBS and incubated with primary antibodies. The specific proteins were visualized using secondary antibodies coupled to a fluorescent marker (fluorescein isothiocyanate, FITC or Texas red; Pierce, Thermoscientific, Rockford, IL). The immunostained samples were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Life Technologies[Q2]) and examined by conventional fluorescence microscopy (Olimpus BX51; Tokyo, Japan). The images were captured using a digital camera. The following antibodies were used: embryonic myosin (eMHC) and sarcomeric myosin MF20 (Developmental Hybridoma Bank, Iowa City, IA); laminin (Sigma–Aldrich, St. Louis, MO); α-actinin (Sigma–Aldrich, St. Louis, MO). The actin filaments were detected with fluorescent phalloidin (Sigma–Aldrich, St. Louis, MO). Evans blue and Procion orange uptake analyses were performed as already reported (Di Certo et al., 2010; Strimpakos et al., 2014).

RNA preparation and analysis

RNA was isolated from the quadriceps muscles. Total RNA was extracted using TRIzol reagent (Life Technologies[Q3]) according to the manufacturer's instructions. Gene expression profiling was conducted using microarrays. Aliquots of 500 ng

of total RNA were labeled with fluorescent probes and utilized as probes to hybridize to Agilent oligonucleotide microarrays. TIFF images were analyzed using Genepix Pro 4.0 software, the signal intensities were log-transformed and normalization was performed by the intensity-dependent Lowess method. Analysis of the microarrays was performed using the GeneSpring GX 11 software with the following conditions: normalization to the 75th percentile, baseline transformation to the median of all samples, grouping of replicates and filtering by expression, and a fold change cut-off of 2.0. Bioinformatics analysis was performed for the differentially expressed genes using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Hang et al., 2009; Huang et al., 2009a).

Quantitative reverse transcription PCR (RT-qPCR) was performed using the Applied Biosystems set of products. The cDNA was prepared using the High Capacity cDNA Reverse Transcription kit; the quantitative real-time RT-PCR was performed using the SYBR Green PCR Master mix or the TaqMan Gene Expression Master mix, according to the protocol for the Applied Biosystems 7500 Real-Time PCR System. For quantification analysis, the comparative threshold cycle (Ct) method was used. The Ct values of each gene were normalized to the Ct value of GAPDH and/or Cyclophilin in the same RNA sample. The gene expression levels were evaluated by the fold change using the equation 2^{-ddCt}. The primers used are reported in Supplementary Table 3. The microarray data are available in the GEO database (GSE62639). (n = 3 mice for microarray; n = 5 mice for real time PCR).

G/F Actin measurement

The tissues were homogenized in F-actin stabilization buffer (50 mM PIPES, 50 mM NaCl, 1 mM ATP, 5 mM MgCl2, 5 mM EGTA, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercaptoethanol, and protease inhibitors). The lysates were centrifuged for 1 h at 100,000 g at 37°C to separate the F-actin from the G-actin pool. After centrifugation, the supernatant, representing the G-actin pool, was collected and transferred to a new tube. The pellet, corresponding to the F-actin pool, was resuspended in solubilization buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 2% SDS and protease inhibitors), sonicated, and boiled. Equal amounts of G-actin and F-actin were separated on a 10% SDS PAGE gel and processed for the immunoblotting analysis with an antibody against actin (Clone AC-40, Sigma–Aldrich, St. Louis, MO) and detected by chemiluminescence (ECL, GE Healthcare Life Sciences, Buckinghamshire, UK). A densitometric analysis was performed using the AIDA 2.2 image software, and the optical density of the G- and F-actin signals were quantified from the scanned blots.

Statistical analysis

The data are presented as the mean ± standard deviation (s.d.) or standard error of the mean (s.e.m.). Statistical significance between groups was determined using GraphPad Prism software. The unpaired *t*-test was performed to assess the significance; *P*-values of less than 0.05 were considered statistically significant.

Results

Muscle recovery

We performed in vitro analyses to document the direct effect of PDE5 inhibitors on skeletal muscle cells. When the cells were treated with the specific inhibitor tadalafil at the onset of differentiation, we detected an increased myotube fusion index in the myogenic cell lines and primary culture from dystrophic cells (Supplementary Fig. S1).

For the in vivo experiments, wild type (wt) mice and dystrophic mdx mice were treated with tadalafil at a dosage very close to that commonly used in clinical settings. The pharmacological treatment was started before birth in pregnant females and was then continued in the pups for 16 weeks. To assess the pharmacological treatment efficacy, standardized performance behavioral tests for DMD animal models were performed (Di Certo et al., 2010; Grange, 2013; Strimpakos et al., 2014). Male mice were treated or not with tadalafil until the age of four months and were then subjected to forced physical exercise on an accelerating treadmill. The exercise was repeated weekly for one month, and the running time to exhaustion was recorded in each session. The wt mice showed the same performance in terms of running time for the four consecutive trials whether treated or not. The mdx mice had a drastically reduced running time; however, following the pharmacological treatment, an improved treadmill performance was observed in these dystrophic mice (Fig. 1a, 1b). To monitor muscle injury, the mdx and mdx-treated mice were injected with Evans blue dye at the end of the running tests, and the dye uptake was visualized by fluorescence microscopy of histological muscle sections. The number of Evans blue-positive muscle fibers was clearly decreased in the mdx mice following tadalafil treatment (Fig. 1c).

Furthermore, we evaluated the effect of tadalafil on mechanical activity, under resting conditions. Isolated extensor digitorum longus (EDL) and diaphragm preparations from both hind limbs of the animals were subjected to in vitro physiological assessment of muscle force, using variable voltages until the supramaximal value was reached. Muscle strength increased in EDL dystrophic muscles in tadalafil-treated mice compared with untreated mice (Fig. 2a), while diaphragm muscle contractile function was not considerably affected by the tadalafil treatment (data not shown). At the end of the force test, the sarcolemmal integrity of EDL post-contraction was investigated by evaluating the uptake of procion orange dye into damaged muscle fibers. The level of sarcolemmal damage was higher in the mdx muscle compared with that of the muscle from the mdx-treated mice (Fig. 2b).

A significant recovery from the dystrophic phenotype in tadalafil-treated mdx mice compared with untreated littermates was also assessed by histological analysis of muscle biopsies. H and E staining was performed on cross-sections of tibialis anterior (TA) muscles. The morphological analysis showed a reduction in inflammatory cell infiltration, fiber necrosis and regenerating myofibers; an increased cross-sectional area in muscle sections from the mdx mice treated with tadalafil was also observed (Supplementary Fig. S2). The effects of the tadalafil treatment were also evaluated in mdx mice, at the age of 30 days, immediately after the onset of the first and massive wave of muscle wasting. Morphological analyses revealed comparable results to those observed in adult mdx mice (data not shown).

Global gene expression analysis

We next investigated whether genome-wide modifications of gene expression occurred in skeletal muscle tissues following tadalafil treatment. For this purpose, RNA samples of muscle tissue from wt and mdx mice treated or not with tadalafil were prepared and utilized in microarray analyses. The obtained results indicated that several genes were differently modulated at a two-fold minimum by the pharmacological treatment (GEO database, GSE62639). Under resting conditions, approximately 400 genes were up-regulated and 200 were down-regulated in the mdx mice compared with the wt mice. By comparing muscles from the mdx mice treated or not with tadalafil, 289 genes were up-regulated and 143 were down-regulated. Bioinformatics analysis of the altered genes under

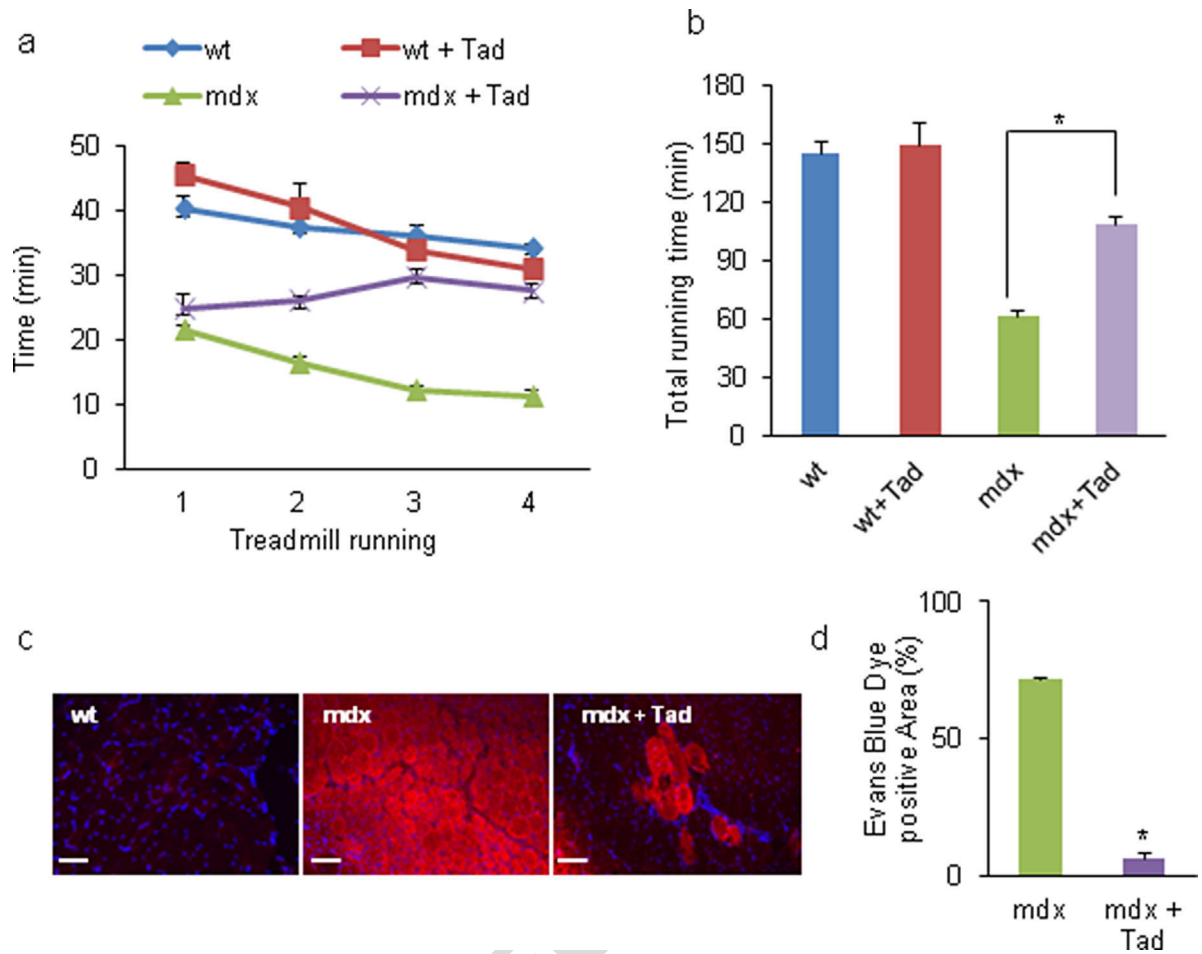

Fig. 1. Treadmill trials (a) Single session performance and (b) total running time relative to 4 weekly treadmill trials with the exhaustive exercise protocol. The values represent the mean +/− s.e.m. (n = 10). *$P < 0.05$ in an unpaired t-test. (c) Uptake of Evans blue dye. The blue signal indicates nuclei, and the red staining detects the uptake of Evans blue dye into the myocytes. Magnification 20x, scale bar represents 100 μm. (d) Graph shows the percentage of Evans blue-positive myofibers. At least 10 muscle sections from five treated and not-treated mdx mice were analyzed. Data represent the mean ± s.e.m. *$P < 0.05$ indicates statistical significance by the t-test.

resting conditions revealed that these genes are implicated in several pathways: PPAR signaling, ECM-receptor interactions, focal adhesion, muscle contraction, and metabolism (Supplementary Table S1). Concerning NOS signaling, the major pathway linked to the cyclic GMP system, we observed a small increase in nNOS mRNA expression following tadalafil treatment (data not shown).

In both muscles from the mdx versus the wt mice and in samples from the tadalafil-treated mdx mice versus the untreated mdx mice, a significant modulation of genes belonging to the peroxisome proliferator-activated receptor (PPAR) family was observed (Supplementary Table S2). The expression of several of these genes was validated by RT-qPCR (Fig. 3). We found that PPARγ, CD36, SCD1, and SCD2 were down regulated in dystrophic muscles compared with wt muscles; additionally, tadalafil treatment counteracted this decreased expression in dystrophic muscles. In vitro experiments revealed that PPARγ levels were also increased in cultured primary myogenic cells following tadalafil treatment (Supplementary Fig. S3a).

The PPARγ coactivator-1 (PGC-1α) mRNA was increased in dystrophic tissues following PDE5 inhibition (Fig. 3). This effect of tadalafil on PGC-1α expression was also evident in primary myoblast cultures from mdx muscles and was mediated by cGMP (Supplementary Fig. S3b). PGC-1α has been demonstrated to improve muscle dystrophy by acting through multiple molecular pathways (Handschin et al., 2007; Selsby et al., 2012; Hollinger et al., 2013). PGC-1α acts as a regulator of mitochondrial biogenesis. Indeed, we observed increased expression of the mitochondrial DNA-encoded gene COX-1 (Supplementary Fig. S3c) as well as of the NADH dehydrogenases ND1, NDL4, and NDUFA4L2 (GEO database, GSE62639).

PGC-1α can also trigger a fiber type switch towards the slow oxidative type. In cross sections of tibialis anterior muscle from mdx mice, we observed a 20% increase in the number of slow-twitch fibers following PDE5 inhibition compared with that in muscle from untreated dystrophic mice (data not shown). In agreement with the implicated effects of tadalafil on the slow-twitch muscle phenotype, we observed an up-regulation of the muscular gene isoforms characteristic of slow-twitch fibers. High levels of the slow myosin isoform MYH7 were observed in tadalafil-treated mdx mice (Fig. 3). Troponin isoforms, specifically expressed in slow-twitch muscles, were also

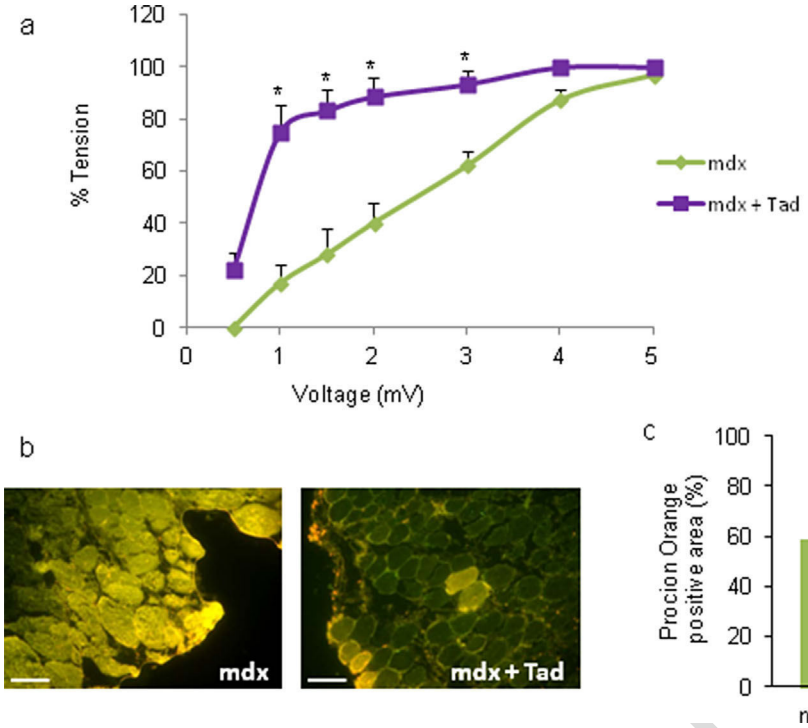

Fig. 2. Mechanical response of isolated muscles (a) EDL isolated from 4-month-old treated (n = 5) and untreated mdx mice (n = 5) under resting conditions; the muscles were stimulated using variable voltage pulses, and the contractile activity was recorded. The values are reported as the % of the maximal force. Each point represents the mean +/− s.e.m. (n = 10 muscles); *$P < 0.05$ indicates statistical significance by the t-test. (b) Procion orange dye uptake in sections of EDL muscles after the force test. Magnification 20x, scale bar represents 100 μm. (c) Graph shows the mean +/− s.e.m. area of dye-positive fibers, expressed as the percentage of the total CSA of muscle sections. At least 10 muscle sections from five treated and not-treated mdx mice were analyzed. *$P < 0.05$ indicates statistical significance by the t-test.

upregulated by tadalafil (Fig. 3). In addition, tadalafil treatment also increased the mRNA levels of utrophin in muscle samples from dystrophic mice compared to the control mice (Fig. 3).

The myosin isoform MHY7, which was induced by more than 10 fold by tadalafil treatment, is also implicated in cardiac hypertrophy. Other hypertrophic markers such as the transcript variant four of PGC-1α (PGC-1α-4; Ruas et al., 2012), FHL-1 D'Arcy et al., 2014) and IGF-1 were found to be overexpressed in dystrophic muscles following tadalafil treatment (Fig. 3).

The muscle hypertrophy induced by PDE5 inhibitor involved protein synthesis, as documented by evaluating the activity of the p70 S6 kinase; in cultured myotubes an increased activity of p70 and its target, the ribosomal protein S6, was observed in the presence of tadalafil (Supplementary Fig. S4).

PDE5 localization and Z-disc organization

Based on previous data indicating restricted localization of PDE5 at the Z-disc (Nagayama et al., 2008; Mokni et al., 2010), we focused our studies on Z-disc organization in muscle sections from mdx and tadalafil-treated mdx mice. The tissue sections were fixed and labeled with α-actinin to identify the Z-disc and were then stained with phalloidin to detect actin filaments. In the wt muscle, a striated pattern of actin filaments intercalated with α-actinin was observed, whereas the mdx muscles displayed diffuse actin distribution (Fig. 4a). In dystrophic muscle treated with tadalafil, we detected better actin distribution in thicker bands compared with that of untreated mdx muscles (Fig. 4a). Similar results were obtained when analyzing heart sections from mdx and tadalafil-treated mdx (data not shown).

To evaluate whether tadalafil could directly affect actin dynamics, the G/F actin ratio was assessed by Western blotting of the purified G and F actin fractions. In tadalafil-treated mdx mice, there seemed to be a slight decrease in actin polymerization (Fig. 4b). Interestingly, we observed reduced acta-1 gene expression (GEO database, GSE62639). However, the pool of G actin is under the control of β-actin (Bunnell et al., 2011). In adult feline cardiomyocytes, cytoskeletal rearrangement is governed by the specific localization of β-actin on the Z-disc following hypertrophic stimulation (Balasubramanian et al., 2010). In terms of β-actin content, we observed increased β-actin expression in samples from tadalafil-treated mice compared with mdx mice (Fig. 4c).

Discussion

In this study, we investigated the effect of drugs blocking PDE5 on dystrophic skeletal muscle function. PDE5 inhibitors improve dystrophic skeletal muscle performance; this effect is likely due to a direct effect on skeletal muscle as documented by the modulation of the genomic response of cells. The microarray studies we performed indicated that more than 200 genes are up-regulated and approximately 140 genes are down-regulated in dystrophic muscles following tadalafil treatment. The beneficial effect observed in mdx mice treated with PDE5 inhibitors could be explained by the modulation of different signaling pathways. The modulated genes fall into the following categories: PPAR signaling, focal adhesion, and ECM

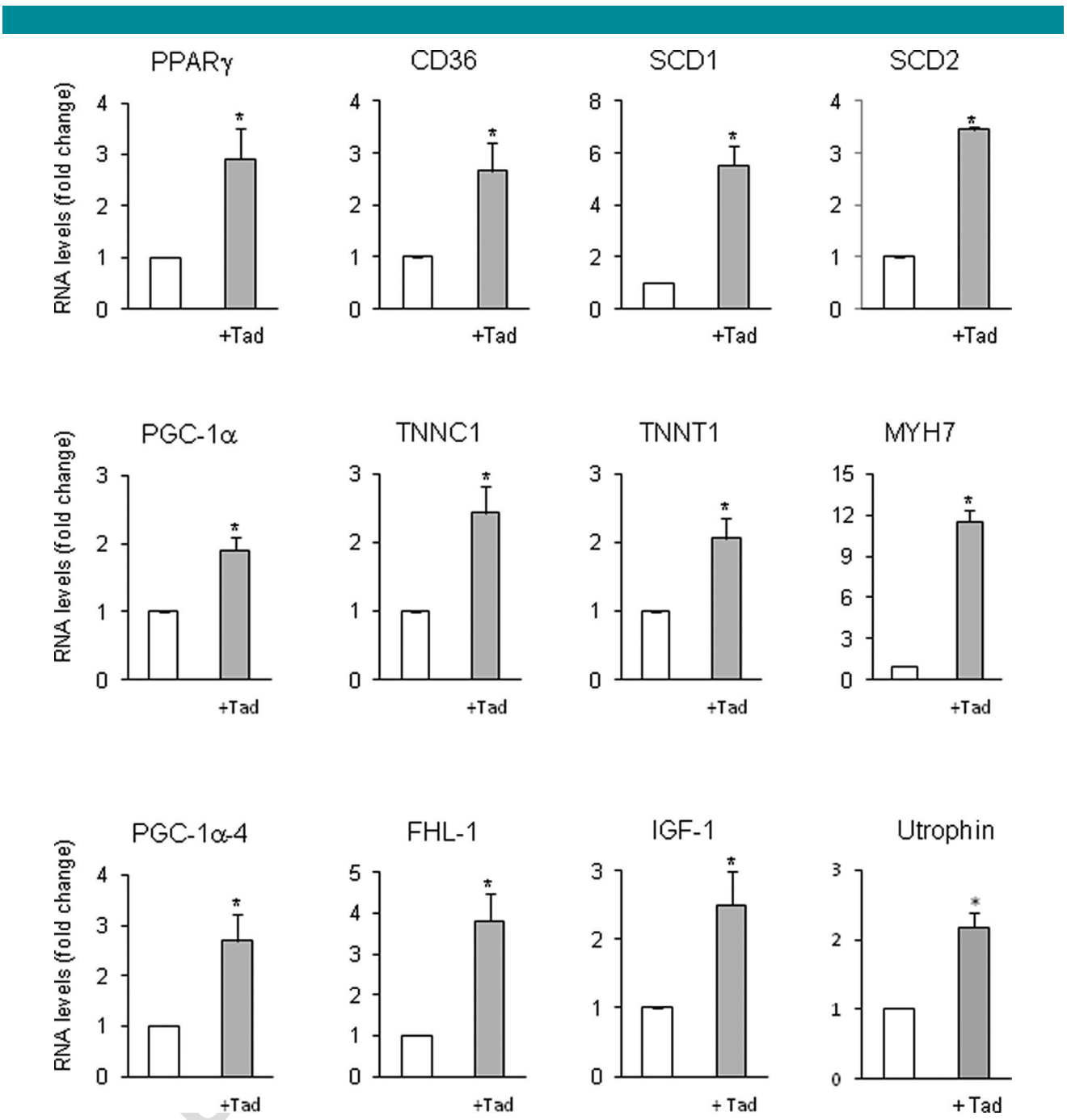

Fig. 3. Changes in gene expression levels RNA was prepared from the quadriceps muscle tissues of the mdx mice treated or not with tadalafil. The mRNA levels of the indicated genes were measured by RT-qPCR and calculated using the comparative Ct method (2^{-ddCt}). The mRNA levels in the samples from tadalafil-treated mdx mice are expressed as the fold change compared with the samples from untreated mdx mice. All values represent the mean +/− s.e.m. of five different RNA preparations. $^{*}P < 0.05$ indicates statistical significance by the t-test.

organization. We found that the treatment of dystrophic mice with tadalafil led to an up-regulation, in terms of mRNA, of ECM and focal adhesion components. This is in contrast with other studies (Percival et al., 2012), in which decreased collagen I and fibronectin expression were detected following sildenafil administration to mdx mice starting at 3 weeks of age, at which point the disease had already developed. A stronger extracellular scaffold could better stabilize the sarcolemma during repeated cycles of contraction and relaxation. A low level of NOS pathway modulation was observed following tadalafil treatment. In our experimental conditions, a modest increase in nNOS transcription and protein expression was detected in tadalafil-treated mdx mice (personal observation). Instead, the most significant changes were observed in PPAR signaling, both between mdx and wt mice and between mdx and tadalafil-treated mdx mice. In the muscle of the tadalafil-treated mdx mice, we detected increased expression of PPARγ, CD36 and other markers of fatty acid metabolism. Although PPARγ is expressed at relatively low levels in skeletal muscle compared with adipose tissue, is required for skeletal muscle cell

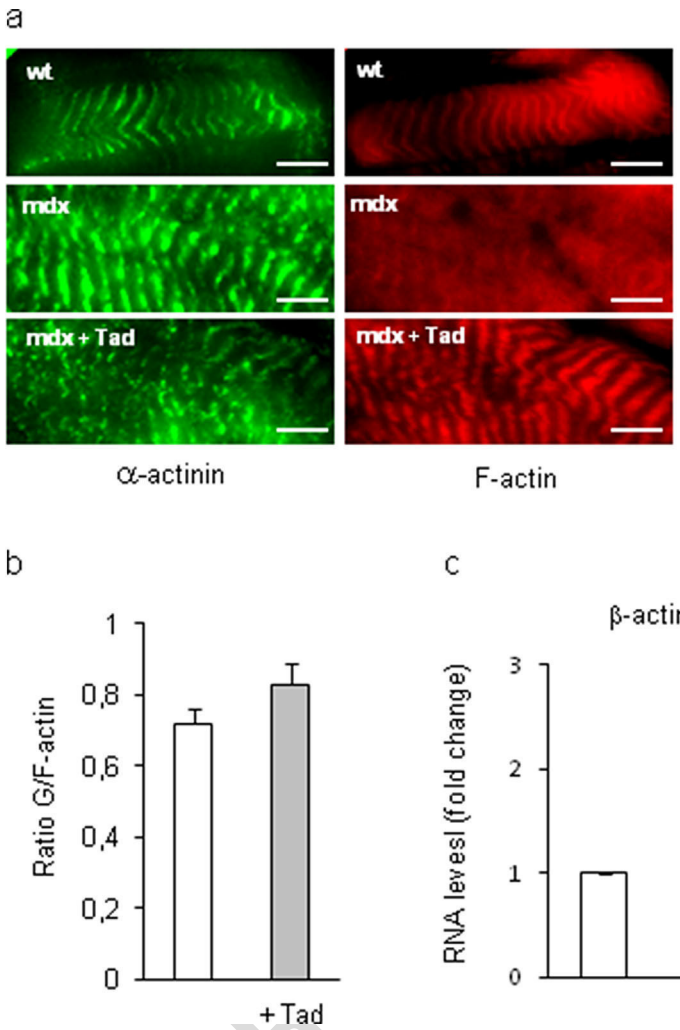

Fig. 4. Sarcomeric actin organization (a) Longitudinal cryostat sections from the TA muscles of wt and mdx mice treated or not with tadalafil were probed with an α-actinin antibody (green). The actin filaments (red) are visualized with phalloidin. A representative analysis out of the four performed on different animals is shown. Magnification 100x, scale bar represents 5 μm. (b) Extracts from the TA muscle of mdx mice treated or not with tadalafil were separated by ultracentrifugation, followed by Western blotting of the supernatants (corresponding to G-actin) and pellets (corresponding to F-actin). All values represent the mean +/− s.d. of four different preparations. (c) β-actin mRNA levels, as determined by RT-qPCR (n = 5). $^{*}P < 0.05$ indicates statistical significance by the *t*-test.

differentiation and improves insulin sensitivity (Ahmadian et al., 2013). CD36 acts as a plasma membrane fatty acid transporter and, as such, can regulate skeletal muscle fatty acid oxidation at rest and during exercise (McFarlan et al., 2012). Reduced CD36 expression has been observed in the muscles of muscular dystrophy patients (Forst et al., 1998). SCD1 and SCD2 are integral membrane proteins of the endoplasmic reticulum and are implicated in the defective regulation of energy metabolism present in dystrophic muscle (Stamatikos et al., 2013). The increased expression of PPARγ following tadalafil treatment seems to be specific to muscle tissue; it was also observed in an in vitro analysis on cultured myogenic cells. However, we cannot exclude the effect of contaminating adipose cells present in the muscle samples. Furthermore, it is important to mention the recently identified fibro-adipogenic precursors (FAPS; Boppart et al., 2013), which are resident muscle interstitial cells that are able to sustain satellite cell differentiation during an efficient myogenic regeneration by providing trophic factors. In dystrophic muscles, FAPs follow the adipogenic lineage that contributes to the advancement of the disease. This adipogenic potential can be overcome by HDAC inhibitor treatment (Mozzetta et al., 2013). It is possible that tadalafil treatment may improve these cells if started before disease onset; these cells, in turn, can exert beneficial effects on muscle cells. In any case, the overall effect of tadalafil treatment is a change in myofiber metabolism. Following tadalafil treatment, we also observed the up-regulation of PGC-1α, a coactivator of PPARγ. The over-expression of PGC-1α in mdx mice, as well as the administration of drugs leading to increased PGC-1α expression such as resveratrol and AICAR is beneficial for the dystrophic phenotype (Handschin et al., 2007; Selsby et al., 2012; Ljubicic et al., 2012; Hollinger et al., 2013; Gordon et al., 2013; Liubicic et al., 2014) being correlated with a shift towards a greater proportion of slow-twitch fibers. Therefore, one way by which tadalafil could exert its beneficial effects on muscle dystrophy is through

increased PGC-1α expression, thereby driving an enhancement of oxidative myofibers, which could compensate for the loss of functional dystrophin by being more resistant to contraction-induced damage. Indeed, an increased expression of slow isoforms of muscle-specific myosins was also detected.

We also observed an increase in the size of myofibers and the up-regulation of several hypertrophy markers such as the specific isoform PGC-1α-4 and the LIM protein FHL-1. This type of tadalafil-induced hypertrophy involves protein synthesis, as suggested by the increased level of phospho S6 kinase. These results are in accordance with Sheffield-Moore et al. (2013) that found increased protein synthesis in human skeletal muscle following sildenafil treatment. In addition, studies from Crescioli et al. (2013) showed that PDE5 inhibitors behaved similarly to insulin on skeletal muscle cells.

PGC-1α, Sirtuin-l, and AMPK activation, as well as FHL-1, also led to the up-regulation of utrophin, the autosomal paralogue of dystrophin (Ljubicic et al., 2012; Gordon et al., 2013; Liubicic et al., 2014). Tadalafil behaved similarly to these drugs in mdx mice by increasing utrophin mRNA expression. A 20% increase in utrophin protein expression was detected in tadalafil-treated samples (data not shown); thus, in a context of better translation conditions, the increased activity of a certain transcript that is normally repressed such as utrophin together with enriched ECM could be beneficial for a stronger connection between the intracellular cytoskeleton and the ECM.

A further effect of the treatment with tadalafil could be observed at the Z-disc. One role of PDE5 at the Z-disc in the heart has already been reported by Bishu et al. (2011). These authors observed the phosphorylation of the sarcomeric protein titin in canine dog hearts following sildenafil treatment and an improved diastolic myofiber stiffness. In addition to its structural-mechanical function, the Z-disc also performs an important cell signaling role by acting as a platform for several signaling molecules. Tridimensional imaging of the sarcomere microarchitecture revealed irregularities in dystrophic fibers without impairments in the actin-myosin interaction (Friedrich et al., 2010). Interestingly, we found that PDE5 is abundantly expressed on the Z-disc in dystrophic muscles, and its expression is reduced following tadalafil treatment. We also found that the expression of other genes localized at the Z-disc and implicated in microfilament organization such as myozenin, tropomyosin four, wasp, was modulated by tadalafil administration (GEO database, GSE62639). Our studies revealed that the pharmacological treatment provoked an improved organization of sarcomeric actin filaments at the Z-disc, a process analogous to that observed following IGF-1 treatment by Takano et al. (2010). Thus, PDE5 inhibition again induces some effects resembling those driven by the insulin signaling pathway.

Currently, a phase 3 study with tadalafil in boys affected by DMD is underway and it will be completed in 2016 (http://clinicaltrials.gov/show/NCT01865084). In this scenario, in two recent clinical trials, sildenafil, and tadalafil treatment had contradictory effects in adult BMD patients; the obtained results probably are due to the different protocols used (Martin et al., 2012; Witting et al., 2014). Our results reveal for the first time the critical pathways implicated in tadalafil-based therapy in muscular dystrophy. Tadalafil can compensate the loss of functional dystrophin in muscle cells by metabolic reprogramming of myofibers towards a profile more resistant to contraction-induced damage. In addition, the beneficial effect of tadalafil correlates with a better actin filament organization at the Z-disc. This latter finding suggests that tadalalafil could provide positive effects also in other pathologies, such as Spinal muscular atrophy (SMA), where a perturbation of the actin network occurs during disease pathogenesis (Coque et al., 2014).

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