Dominant-negative inhibition of Ca\(^{2+}\) influx via TRPV2 ameliorates muscular dystrophy in animal models

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Received August 19, 2008; Revised and Accepted December 1, 2008

Muscular dystrophy is a severe degenerative disorder of skeletal muscle characterized by progressive muscle weakness. One subgroup of this disease is caused by a defect in the gene encoding one of the components of the dystrophin–glycoprotein complex, resulting in a significant disruption of membrane integrity and/or stability and, consequently, a sustained increase in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). In the present study, we demonstrate that muscular dystrophy is ameliorated in two animal models, dystrophin-deficient mdx mice and \(\delta\)-sarcoglycan-deficient BIO14.6 hamsters by dominant-negative inhibition of the transient receptor potential cation channel, TRPV2, a principal candidate for Ca\(^{2+}\)-entry pathways. When transgenic (Tg) mice expressing a TRPV2 mutant in muscle were crossed with mdx mice, the [Ca\(^{2+}\)]\(_i\) increase in muscle fibers was reduced by dominant-negative inhibition of endogenous TRPV2. Furthermore, histological, biochemical and physiological indices characterizing dystrophic pathology, such as an increased number of central nuclei and fiber size variability/fibrosis/apoptosis, elevated serum creatine kinase levels, and reduced muscle performance, were all ameliorated in the mdx/Tg mice. Similar beneficial effects were also observed in the muscles of BIO14.6 hamsters infected with adenovirus carrying mutant TRPV2. We propose that TRPV2 is a principal Ca\(^{2+}\)-entry route leading to a sustained [Ca\(^{2+}\)]\(_i\) increase and muscle degeneration, and that it is a promising therapeutic target for the treatment of muscular dystrophy.

INTRODUCTION

Muscular dystrophy is a heterogeneous genetic disease that causes severe skeletal muscle degeneration characterized by fiber weakness and muscle fibrosis, severe local inflammation and, at least initially, muscle regeneration. A subset of muscular dystrophy is caused by a mutation in the gene encoding one of the components of the dystrophin–glycoprotein complex (DGC) (1–3), a multi-subunit complex (2,4,5) that spans the sarcolemma to structurally link the extracellular matrix and actin cytoskeleton (6). Therefore, disruption of the DGC could significantly disrupt membrane integrity or stability during muscle contraction and relaxation and prevent myocyte survival. The enhanced susceptibility to exercise-induced muscle fiber damage is observed in dystrophic animals, such as \(\delta\)-sarcoglycan (SG)-deficient BIO14.6 hamsters and dystrophin-deficient mdx mice, which are genetic homologues of human limb-girdle and Duchenne muscular dystrophy (DMD), respectively.

Although many studies have molecularly characterized the genes responsible for and the histological pathology of dystrophic tissues, little is known about the pathways by which the genetic defects lead to muscle degeneration. A number of studies have reported the chronic elevation of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) beneath the sarcolemma or within other cell compartments of skeletal muscle fibers, or in cultured myotubes from dystrophin-deficient DMD patients and mdx mice. Increased [Ca\(^{2+}\)]\(_i\) could activate Ca\(^{2+}\)-dependent proteases and promote protein degradation and cell necrosis (7–9). Several pathways leading to an increase of the [Ca\(^{2+}\)]\(_i\) have been suggested to be involved in the pathology of muscular dystrophy (10–15). We previously identified a stretch-activated channel, the transient receptor potential (TRP) cation channel
TRPV2, that may function in the pathogenesis of myocyte degeneration caused by DGC disruption (16). The TRP channels form a large family of cation channels that likely function as tetramers in various processes, such as sensory signaling (17–19). The cellular function of TRPV2, however, remains to be characterized. We previously found that TRPV2 normally localizes in the intracellular membrane compartments but translocates to the plasma membrane in dystrophic muscle fibers, thus contributing to a sustained [Ca$^{2+}$]$_i$ increase (16).

To determine the relationship between TRPV2 activation and muscle degeneration, and determine whether TRPV2 inhibition potentially prevents muscular dystrophy, we introduced mutant TRPV2 into dystrophic muscles to inhibit endogenous TRPV2 activity via a dominant-negative effect. Here we report that transgenic (Tg) or adenoviral expression of dominant-negative TRPV2 ameliorates muscle pathology in mdx mice and BIO14.6 hamsters by preventing abnormal Ca$^{2+}$ handling.

RESULTS

Characterization of dominant-negative TRPV2 mutants

Conserved acidic residues in the putative pore region of TRP cation channels are known to be crucial for cation permeation (20,21). We generated three TRPV2 mutants by substituting two highly conserved glutamate residues (Glu594 and Glu604) in the putative pore region with lysine, either individually (E594K and E604K) or together (DK). The TRPV2 mutants were stably expressed in Chinese hamster ovary (CHO) cells, as confirmed by immunoblot, which showed two protein bands with different degrees of glycosylation (Fig. 1B). The immunofluorescence of TRPV2 expressed in CHO cells revealed that, in the absence of serum, most TRPV2 was localized in the intracellular membrane compartments (Fig. 1C). In contrast, upon stimulation with serum, a portion of TRPV2 translocated to the surface membranes (Fig. 1C) as previously reported (22). Interestingly, a similar serum-induced translocation occurred with the three TRPV2 mutants (Fig. 1C, data not shown for E594K and DK).

We next assessed TRPV2 activity by measuring the Ca$^{2+}$-induced change of the [Ca$^{2+}$]$_i$, by ratiometric scanning of the fura-2 fluorescence. As shown in Fig. 1D, the perfusion of cells with a 2 mM CaCl$_2$ solution resulted in a rapid and large increase in the [Ca$^{2+}$]$_i$ of cells expressing wild-type TRPV2 compared to non-transfected control cells (Supplementary Material, Fig. S1). In contrast, the increase in [Ca$^{2+}$]$_i$ did not occur in cells expressing E604K (Fig. 1D) or the other two mutants (Fig. 1E), suggesting that these mutations abolished Ca$^{2+}$-permeation via TRPV2. Because the TRP channel family likely functions as tetramers (17,18), we predicted that the TRPV2 mutants would have a dominant-negative effect on wild-type TRPV2 activity. We transiently transfected green fluorescent protein (GFP)-tagged TRPV2 mutants in cells stably expressing wild-type TRPV2 and measured the [Ca$^{2+}$]$_i$ in GFP-positive cells (Supplementary Material, Fig. S1). Transfection with the mutants dramatically diminished the Ca$^{2+}$-induced rise (Fig. 1F), suggesting that the TRPV2 mutants exerted a strong dominant-negative effect. Similar inhibitory effects of the mutants were also observed in HEK293 cells when the [Ca$^{2+}$]$_i$ increase was induced with high Ca$^{2+}$ solution or the TRPV channel agonist 2-aminoethoxydiphenyl borate (2-APB) (Supplementary Material, Fig. S2). Wild-type and mutant TRPV2 were co-localized in the plasma membranes of HEK293 cells (Supplementary Material, Fig. S2). Furthermore, surface biotinylation and co-immunoprecipitation assays revealed that mutant TRPV2 is capable of forming oligomers with wild-type TRPV2 in the cell surface (Supplementary Material, Fig. S3).

Transgenic expression of dominant-negative TRPV2 blocks abnormal Ca$^{2+}$ handling in mdx mice

To assess the relationship between TRPV2 activation and muscle degeneration, we analyzed whether the expression of dominant-negative TRPV2 prevents muscle damage in dystrophin-deficient mdx mice. We first generated transgenic mice (C57/BL6J background) expressing the hemaglutinin (HA)-tagged E604K (E604K-HA) mutant under the control of the α-skeletal actin promoter in skeletal muscle. Two lines of male Tg mice were then crossed with female mdx mice, and because muscular dystrophy is X-linked recessive in mdx mice, the resulting male mice were predicted to express the E604K-HA mutant and be deficient in dystrophin. The expression of the E604K-HA mutant was confirmed by anti-HA immunoblotting only in the Tg and mdx/Tg mice, although only half level of E604K-HA expression was detected in the latter mice as expected from heterozygotes (Fig. 2A). Interestingly, the level of endogenous TRPV2 was elevated by approximately 2-fold in mdx mice compared to controls, but TRPV2 levels were reduced in the mdx/Tg mice (Fig. 2A). While endogenous TRPV2 was broadly distributed in the skeletal muscle of control mice (Fig. 2Ba), it almost exclusively localized in the sarcolemma of mdx muscles (Fig. 2Bb). On the other hand, exogenous E604K-HA was mostly observed in the intracellular membranes, although a small portion was localized in the sarcolemma (Fig. 2Bg and h). In addition, the expression of E604K-HA reduced the sarcolemmal localization of endogenous TRPV2 in mdx muscles (Figs 2Bc and 3Ad, also see Supplementary Material, Fig. S4).

We isolated the Flexor digitorum brevis (FDB) fibers from each mouse and immunostained with the anti-TRPV2 antibody, revealing that, consistent with immunostaining in muscle sections, most TRPV2 localized in the sarcolemma of mdx fibers (Fig. 3Ab), but not in the fibers from wild-type mice (Fig. 3Aa); this sarcolemmal localization was much lower in the mdx/Tg mice (Fig. 3Ad). Consistent with the sarcolemmal localization of TRPV2 in mdx fibers, the perfusion of fibers with 2-APB induced a large and rapid increase in [Ca$^{2+}$]$_i$ in fibers from mdx mice, which was completely inhibited by the TRPV channel antagonist ruthenium red (Fig. 3B). An increase in [Ca$^{2+}$]$_i$ was also observed to occur upon perfusion with high Ca$^{2+}$ solution (2–5 mM, see Fig. 3B and Supplementary Material, Fig. S5). These Ca$^{2+}$ increases were reduced in fibers from mdx/Tg mice (Fig. 3B–D and Supplementary Material, Fig. S5), suggesting that the introduction of E604K-HA inhibited Ca$^{2+}$-entry via endogenous TRPV2. Since TRPV2 is a stretch-activated channel, we analyzed the effect of mechanical stress on membrane deformation of isolated fibers. We observed that hypo-osmotic stress (70% osmolarity) resulted in severe damage in fibers from mdx
mice, but not much in fibers from mdx/Tg mice. Bleb formation in mdx fibers was inhibited by more than 60% upon expression of E604K-HA (data not shown). A sustained increase in [Ca$^{2+}$]$_i$ via surface TRPV2 would result in various phenotypic changes in the skeletal muscles of mdx mice via activation of Ca$^{2+}$/calmodulin-dependent enzymes. Consistent with this idea, the phosphorylation of Ca$^{2+}$/calmodulin-dependent protein kinase (CaMK)II was significantly higher in mdx skeletal muscle compared to controls (Fig. 2A). In contrast, phosphorylated CaMKII was markedly reduced in the mdx/Tg mice (Fig. 2A), suggesting a reduced [Ca$^{2+}$]$_i$ in muscle fibers from these mice.

**Transgenic expression of dominant-negative TRPV2 ameliorates muscular dystrophy in mdx mice**

To determine whether dominant-negative inhibition of TRPV2 could prevent muscular dystrophy, we first analyzed the overall symptoms for muscle degeneration. Expression of E604K-HA in mdx mice (mdx/Tg) markedly (40–60%) reduced the level of serum creatine kinase (CK), a marker for muscle damage (Fig. 4A), and improved muscle performance as evaluated by a grip test (50–70% of wild-type) (Fig. 4B). The improvement of muscle damage was observed in mdx/Tg mice produced from two Tg lines (I and II).

We next analyzed the histological characteristics of skeletal muscles. Skeletal muscle-specific expression of E604K-HA produced no striking morphological changes in the muscles of Tg mice (Fig. 5Ab), and muscle fibers from mdx/Tg mice (Fig. 5Ad and f) presented with a healthier appearance compared to their mdx counterparts (Fig. 5Ac and e). Among several abnormal morphological indices, dystrophic muscle fibers are known to display a greater variation in their cross-sectional area (23). Muscles from mdx/Tg mice exhibited a greater homogeneity compared to mdx mice in respect to fiber size variability, determined by averaging the standard deviation of the cross-sectional areas (Fig. 5B). Furthermore, the expression of E604K-HA reduced (60% reduction) the number of fibers with central nuclei (Fig. 5C) in 10 week mdx/Tg mice, the area of inflammatory infiltrate (more than 90% reduction) and Evans blue dye (EBD) uptake (marker of membrane integrity, 80% reduction) (Fig. 5D) in similar-aged...
mice, suggesting that the degeneration of myofibers markedly decreased and subsequent muscle regeneration was reduced in the mdx/Tg mice. In addition, Masson’s trichrome staining revealed that the foci of fibrosis, reflecting the progressive replacement of myofibers with connective tissue, was frequently detected in mdx mice, but the expression of E604K-HA reduced fibrosis more than 60% (Fig. 5E and F). The high level of apoptosis seen in mdx muscle was also reduced in mdx/Tg muscle (Fig. 5G and H). We observed that the expression of E604K-HA may be somewhat mosaic in mdx/Tg muscle as well as Tg muscle as seen in Fig. 2Bg and h, thus exerts a different dominant-negative effect on individual muscle fibers. In order to directly check the involvement of this expression, we compared the area stained with incorporated EBD, a marker of cell injury, to the area immunostained with anti-HA or anti-TRPV2 using serial sections (Supplementary Material, Fig. S6). Fibers stained with EBD, which were sometimes observed in mdx/Tg mice, corresponded to those showing relatively low expression level of E604K-HA, but the condensed expression of endogenous TRPV2 well correlates with pronounced EBD uptake. Taken together, these data suggest that the inhibition of TRPV2 significantly improves muscular dystrophy in mdx mice, and TRPV2 may play a pivotal role in causing dystrophic muscle damage.

Adenoviral expression of dominant-negative TRPV2 ameliorates muscular dystrophy in BIO14.6 hamsters

We next examined whether the inhibition of TRPV2 also reduces muscle damage in BIO14.6 hamster, another model of dystrophy. Infection of cultured BIO14.6 myotubes with...
Suppressed Ca\(^{2+}\) changed the distribution pattern of endogenous TRPV2 and exogenous TRPV2. However, the expression of DK-HA clearly inhibited the expression of endogenous TRPV2 in myotubes (Fig. 6A), suggesting that the exogenous adenovirus carrying DK-HA resulted in the expression of HA-tagged TRPV2 mutant (upper panel of Fig. 7A) and reduction in plasma membrane localization of TRPV2 (lower panel of Fig. 7A). Fourteen days later, the muscle degeneration was reduced in DK-HA virus-infected BIO14.6 hamsters compared to those infected with adenoviral β-gal (Fig. 7Ba and b). The number of central nuclei and fiber size variability was reduced (40–50%) upon DK-HA infection (Fig. 7C), and TUNEL staining additionally revealed a marked reduction (~80%) in apoptosis in DK-TRPV2-infected muscles (Fig. 7Be and d, and C). Together, these data demonstrate that, similar to the mdx mouse model, the inhibition of TRPV2 is capable of greatly ameliorating muscular dystrophy in the BIO14.6 hamster model.

DISCUSSION

Abnormal Ca\(^{2+}\)-handling is a hallmark of muscle dysfunction in muscular dystrophy. In the present study, we demonstrated that the 50% decrease in plasma membrane permeation and reduced plasma membrane expression. In addition to the decreased Ca\(^{2+}\) influx in isolated FDB fibers, we observed that the enhanced phosphorylation of CaMKII in mdx mice was reduced after crossing them with Tg mice, strongly suggesting that the increase in intracellular Ca\(^{2+}\) level was reduced in the skeletal muscle of mdx/Tg mice. This inhibition likely occurs from the oligomerization of the mutant subunit with endogenous TRPV2 subunits upon assembly of TRPV2 channels because TRPV2 is a homo-oligomer (17,18,24). In fact, this is supported by the finding that the subcellular localization of endogenous TRPV2 is markedly affected by the exogenous expression of mutant TRPV2 in mdx/Tg mice. Although a recent study reported that TRPV2 may also be capable of forming hetero-oligomers with TRPV1 and/or TRPV3 (25), the expression of TRPV1 and TRPV3 was not detected by immunoblot analysis in mouse skeletal muscle (Supplementary Material, Fig. S7). On the other hand, the TRPC family has been reported to be involved in abnormal Ca\(^{2+}\) handling in the skeletal muscle of mdx mice (14). However, we observed that the [Ca\(^{2+}\)] was increased by 2-APB, a TRPC channel antagonist (26). Furthermore, a significant difference was not observed in the expression of TRPC family members (TRPC1, 3, 4 and 6) among four kinds of mice (Supplementary Material, Fig. S7). Therefore, the involvement of TRPC channels would be minor. Thus, our present data, together with previous data (16), strongly suggest that TRPV2 plays a crucial pathological role in the Ca\(^{2+}\)-induced muscle degeneration of dystrophic muscles.

Our findings also provide convincing validation of the therapeutic potential of TRPV2 for muscular dystrophy. This protein has several advantages as a therapeutic target. First, most TRPV2 localizes to the intracellular membranes in normal, healthy skeletal muscle, but it translocates to the surface membrane upon muscle degeneration (16); hence, specific inhibitors against TRPV2 are predicted to only act...
on degenerative muscles. Second, surface translocation and the subsequent activation of TRPV2 may occur in a wide range of genetic or non-genetic muscle diseases (16). For example, we recently observed that the surface translocation of TRPV2 also occurs in the hearts of idiopathic cardiomyopathy patients (unpublished observation). Thus, specific inhibitors against TRPV2 could be potentially useful for the treatment of various degenerative muscle diseases.

In this study, we presented evidence that the inhibition of TRPV2 is able to ameliorate several indices characterizing the dystrophic pathology. However, it is important to consider that the pathology of dystrophin-deficient muscles covers a broader area that includes susceptibility to exercise-induced injury, oxidative stress and an impaired regenerative capacity. We observed that the inhibition of TRPV2 resulted in a different degree of amelioration in dystrophic indices examined in this study, reflecting the complexity of this disease. For example, good amelioration (60–90%) was seen in all dystrophic parameters in young mdx/Tg mice (4–10 weeks), but in old mice (more than 26 weeks) only 30–40% amelioration was seen in fiber size variability and in number of centrally nucleated fibers, although fibrosis was markedly reduced even in old mice. In young mdx/Tg mice, it is likely that dominant-negative TRPV2 decreases the susceptibility of dystrophic muscles to the on-going cycles of degeneration and regeneration induced by contraction, while in older mice such a cycle may be saturated and no more changed. Thus, the inhibition of TRPV2 appears to primarily promote the retardation of muscle degeneration onset. Furthermore, we observed a partial amelioration (40–60%) of serum CK levels, when compared with apparently better improvement of tissue injury in limbs muscles such as quadriceps examined. This would be due to the fact that serum CK levels reflect the overall state of all muscles including diaphragm and back muscle not examined in this study. Although dominant-negative TRPV2 does not completely prevent dystrophic pathology, our present data suggest that TRPV2 would be a key molecule to link the disruption of DGC with muscle degeneration.

An important issue is the molecular mechanism by which TRPV2 translocates to the plasma membrane and becomes activated in dystrophic muscles. In both mdx and mdx/Tg mice, it is likely that the muscles are subjected to continuous mechanical stress caused by the dystrophin deficiency. Such mechanical stretch may enhance the secretion of various hormones or bioactive substances, which potentially promote TRPV2 translocation via receptor stimulation because translocation is known to occur in response to various signals,

Figure 5. Dominant-negative TRPV2 attenuates muscle degeneration in mdx mice. (A) Representative images for H&E staining of quadriceps sections from wild type (a), Tg (b), mdx (c and e) and mdx/Tg (d and f) mice. (a–d) Twenty-six weeks old, (e and f) 10 weeks old. Scale, 100 μm. (B–D) Histological parameters were measured using H&E stained sections. Sections covering more than 1000 fibers from three to four mice were used for an analysis of each group. Cross-sectional areas of each individual fiber were measured and the variability of muscle fiber size was determined by averaging the standard deviations of data (B). The number of centrally nucleated fibers (C) was also measured using the same histological sections. (D) Percentage of inflammatory infiltration area measured using the histological sections from 7 to 10 week old mdx and mdx/Tg (line I) mice. Data are means ± SD of four mice. The percentage of EBD-positive area (cf. Supplementary Material, Fig. S6). Data are means ± SD of four mice. (E and F) Masson’s trichrome staining of 26 week old mdx (a) and mdx/Tg (b) mice, and measurements of the area of fibrosis. (G and H) TUNEL labeling of sections of gastrocnemius muscle samples from 10 week old mdx (a) or mdx/Tg (b) mice, and the number of fibers exhibiting apoptosis. For histological analysis, sections covering more than 1000 fibers from three to four mice were used for measurements. Scale bar, 100 μm.
such as growth factors and chemotactic peptides (16,22,27). We recently reported that stretch-induced ATP release contributes to abnormal Na\(^{+}\) and Ca\(^{2+}\) handlings in dystrophic muscles via the P2 receptor (28). In addition, the release of hormones such as IGF-1 and TGF\(\beta\) has been well documented in dystrophic pathogenesis (28–30). The stretch signal is expected to be generated in both \(mdx\) and \(mdx/Tg\) mice. However, unexpectedly, dominant-negative TRPV2 markedly inhibited the plasma membrane retention of TRPV2 in \(mdx/Tg\) muscle fibers (see Figs 2B and 3A and Supplementary Material, Fig. S4 and S6), suggesting that TRPV2 activity may be required for surface localization in \(mdx\) muscles. In fact, we have previously shown that the removal of extracellular Ca\(^{2+}\) or stretch-activated channel inhibitor Gd\(^{3+}\) and general TRPV inhibitor ruthenium red markedly promote the internalization of TRPV2 in dystrophic myotubes (16). It is likely that the increase in local Ca\(^{2+}\) levels mediated by TRPV2 contributes to the persistent plasma membrane retention of TRPV2 in dystrophic muscles by blocking their internalization. In this regard, a recent study reported that TRPV2 may function as an endosomal Ca\(^{2+}\) release channel, which would control endosome fusion and/or endocytosis (31).

Understanding the molecular mechanism of TRPV2 recycling will require further investigation and may accelerate the development of novel therapeutic strategies towards TRPV2.

We observed that the expression of dominant-negative TRPV2 itself exerts no apparent detrimental effect on the function and histological characteristics of muscles in Tg mice, suggesting a minor physiological role of TRPV2 in skeletal muscle. On the other hand, we presented evidence that TRPV2 can provide a Ca\(^{2+}\) pool, leading to the activation of CaMKII in \(mdx\) mice. Since the CaMKII-induced phosphorylation of histone deacetylase, together with the calcineurin/NFAT pathway, is known to
be critical for inducing skeletal muscle remodeling (32), TRPV2 may also play an important physiological role in Ca\(^{2+}\)-dependent remodeling processes during muscle injury and/or exercise. Although we do not exclude such a possible physiological role, the present findings suggest that activated TRPV2 becomes a major risk factor in dystrophic muscles.

In conclusion, the specific inhibition of TRPV2 led to a significant amelioration of muscle pathology in dystrophic animal models, and this channel is a promising therapeutic target for muscular dystrophy.

**MATERIALS AND METHODS**

**Antibodies**

Affinity-purified rabbit polyclonal anti-TRPV2 antibody was described previously (16). Other antibodies were obtained from the following sources: mouse monoclonal anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), rabbit polyclonal anti-TRPV1, Chemicon; mouse monoclonal anti-dystrophin, Sigma; rat anti-HA antibody (3F10), Roche; rabbit anti-GFP, Medical & Biological Laboratories co., LTD; mouse monoclonal anti-β-dystroglycan, Novocastra Laboratories, Newcastle, UK; rabbit polyclonal anti-CalMKII and anti-TRPV3, Santa Cruz Biotechnology, INC; mouse monoclonal anti-phosphorylated CaMKII, ABR Golden Co.; rabbit polyclonal anti-TRPCs (TRPC1, 3, 4 and 6) and anti-TRPV4, Alomons lab Ltd.

**DNA manipulation and adenovirus production**

All plasmid constructions of TRPV2 were essentially carried out by PCR-based strategy using the full-length mouse TRPV2 cDNA cloned into the pIRES expression vector (Invitrogen, Carlsbad, CA, USA). In some constructs, a HA-tag or GFP was cloned to the C-terminus of TRPV2 in pIRES or pEGFP-N1 (Clontech, Palo Alto, CA, USA) vector, respectively. GFP was inserted at the C-terminus just after the HA-tag. Three mutants were produced by substituting the conserved Glu residues (Glu594 and Glu604) of TRPV2 with Lys, either singly (E594K or E604K) or together (DK). For adenoviral gene transfer, we inserted the TRPV2 mutant cDNAs or β-gal as a control into the Adeno-X™ viral vector (Clontech). Adenovirus was produced according to the manufacturer’s protocol. One-day-old myotubes in differentiated medium were infected with adenoviruses at an MOI of 5–10 viral particles per cell for 24 h and cultured for an additional 36–48 h.

**Transgenic mice**

The transgene was constructed by inserting a cDNA encoding the full-length mouse TRPV2 mutant tagged with HA (E604K-HA) into the cloning site between the human α-skeletal actin promoter and the SV40 polyadenylation sequence of the plasmid [kindly provided by Dr Jeffrey S. Chamberlain (33)]. The transgene was used to generate Tg mice from C57BL/6j mice according to standard procedures. Two lines (I and II) of male F2 transgenic homozygotes were mated with female mdx mice. Male pups that expressed transgene were identified through PCR screening of genomic DNA extracted from tail tissue. Only the male mdx/Tg offspring (mdx/Tg I or II) were analyzed. The absence of dystrophin from these mice was confirmed by immunological detection with anti-dystrophin antibody. Genotyping was carried out by PCR with specific primers. PCR of the tail DNA was used to identify Tg mice using primers in the 3’ end of the human α-skeletal actin promoter (5’-AACGCAATTCCTAATGTCGA-3’) and a sequence within TRPV2 (5’-AATGAATCCGAGTTAGGTACCTTTTAA-3’). Normal and abnormal dystrophin genes were detected by PCR using the following primers: sense primer, 5’-AATCTCATCAATATGGCTGTTAGTG-3’; antisense primer for normal dystrophin, GTCACTCA-GATAGTTGAAGCCTATTATTTGA; antisense primer for abnormal dystrophin present in mdx mice, GTCACTCATATAAGGCAATTTAG.

**Animal experiments**

All animal experiments were performed according to the Guidelines for Animal Experimentation at the National Cardiovascular Center. Muscle strength was evaluated by a grip-test as previously described (28). Briefly, mice were placed to hold onto a fine wire net with their forelimbs and the length of the time they could support their body weight was recorded. CK activity was measured as previously described (28).

**Histology**

Skeletal muscle was fixed in phosphate buffer saline (PBS) containing 10% formalin and embedded in paraffin. Serial sections (5 μm) were stained with hematoxylin and eosin (H&E) or Masson’s trichrome for morphological analysis. The extent of muscle regeneration was determined by counting the number of fibers with central nuclei. The variability in fiber size was determined by averaging the standard deviations of the area from the myofiber cross-sectional views (>1000 fibers) of three to four animals per group (28). To detect apoptosis, muscle fibers were TUNEL stained using an apoptosis detection kit (Takara Biomedical). For the measurement of EBD uptake, EBD in PBS (10 mg/ml) was injected intraperitoneally into each kind of mice (0.1 ml/10 g body weight). The mice were sacrificed 24 h after injection. Muscles were excised and embedded in optimal cutting temperature compound (Tissue-Tek, Torrence, CA, USA) and snap-frozen using liquid nitrogen. Blocks were then sectioned into 6-μm thick slices, dried for 10 min and washed briefly in PBS. The EBD was detected as red auto-fluorescence. All histochemical analysis was done by investigators blinded to genotype.

**Quantification of histological data**

Stained serial sections were viewed under a light microscope (OLYMPUS BX41) and images were analyzed using a computer-assisted imaging system (FLOVEL Filing System) by investigators blinded to the genotypes. Images were acquired using a digital camera (Olympus FX380) equipped with image filing software (Fловел FLVFS-LS, Tokyo, Japan). The extent of damage occurring in muscles was determined by comparing the
number of centrally located nuclei (CLN) between samples. The variability of fiber size was obtained by averaging the standard deviations of cross-sectional myofiber views. Fibrosis was assessed by measuring the Masson’s trichrome-positive area. Briefly, color images were converted to binary images by setting a threshold so only blue-stained fibrotic areas were detected. These areas were summed and reported as a percentage of the total area. The infiltrate area was defined as the region infiltrating non-muscle cells, such as lymphocytes and macrophages, and showing the accumulation of small nuclei and weak staining with eosin. Such areas were selected by eye and represented as the percentage of the total area.

Immunoblot and immunohistochemistry

Immunoblotting and immunohistochemistry were carried out as previously described (16,34). Briefly, the immunoblot was visualized using an enhanced chemiluminescence detection system (Amersham Biosciences) after blotting, blocking with PBS containing 5% non-fat milk and incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies. For immunohistochemistry, several different procedures were used. Frozen muscle sections (5–6 μm thick) or methanol fixed myotubes were double immunostained by incubating for 1 h with fluorescein isothiocyanate (FITC)-conjugated rat anti-HA antibody and rabbit anti-TRPV2 antibody, followed by further incubation with rhodamine-conjugated secondary antibody. Frozen muscle sections were also immunostained with anti-dystrophin antibody. For the immunostaining of isolated fibers, fibers immobilized on glass slides were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% TritonX-100 and then stained with anti-TRPV2 antibody followed by FITC-conjugated secondary antibody. Stained samples were observed by a confocal laser scanning microscopy (FLUOVIEW FV1000, Olympus) mounted on an objective lens (Olympus). Serial sections of BIO14.6 skeletal muscles were immunostained with an enzymatic color detection system. Sections were incubated with anti-HA or anti-TRPV2 overnight, then treated with Simple Stain MAX-PO (NICHIREI CO.) for rat antibody or DAKO EnVision™ +System ( Peroxidase) for rabbit antibody, followed by color development with diaminobenziden (DAB). Samples were observed by a light microscope (OLYMPUS BX41).

Isolation of fibers

FDB muscles were removed and incubated for 40 min at 37°C in Krebs solution containing 124 mM NaCl, 1.2 mM MgCl2, 5.9 mM KCl, 11.5 mM glucose, 11.5 mM Hepes-Na, 1.5 mM CaCl2 and 0.2% collagenase type IV (Sigma-Aldrich). Muscles were then removed, washed twice in Krebs buffer and suspended in Ham’s F12/DME (Sigma-Aldrich) supplemented with 2% FCS. Single fibers were mechanically dissociated by repeatedly passing the muscle through fire-polished Pasteur pipettes. Dissociated fibers were plated onto glass-bottom dishes coated with BDcell-Tak™ (BD Biosciences) and allowed to adhere to the bottom of the dish for 2 h.

Cell culture, plasmid transfection and myotube preparation

Chinese hamster ovary (CHO-K1) cells and corresponding transfectants were maintained in DMEM containing 25 mM NaHCO3 and supplemented with 7.5% (v/v) fetal calf serum. cDNAs were transfected into CHO cells with Lipofectamine 2000 (Invitrogen Corp., CA, USA) and stable clones were isolated after selection with puromycin or G418. Myotube culture was performed using muscles from normal or BIO14.6 hamsters by enzymatic dissociation essentially as described previously (35). Briefly, we prepared satellite cells from the gastrocnemius muscle of hamsters using an enzyme cocktail containing 0.5 mM CaCl2 for cell dissociation. After enrichment of the myoblasts by several preplatings, cells were placed on the culture dishes; 2 days later, culture medium was switched to DMEM containing 2% horse serum to induce myotube formation. Two to 4 days after start of fusion, the generated myotubes were analyzed.

Application of stretch

Myotubes were subjected to a uniaxial sinusoidal stretch of up to 120% at 1 Hz and 25°C for 1 h using a temperature-controlled stretching apparatus (NS-300: SCHOLAR-TEC Co., Osaka, Japan) as described previously (36). Cells were cultured in a silicon-rubber chamber with 400 μm thick side walls and a 200 μm thick transparent bottom coated with collagen I. In this way, uniform stretch was applied to most of cells cultured on the bottom (36). Osmotic stress-induced cell damage was observed in myofibers preloaded with 5 μM calcine-AM as previously described (15).

Ca2+ measurement

CHO cells and HEK293 cells were loaded with 4 μM fura-2-acetoxymethyl ester (fura-2/AM) for 30 min at 37°C, and maintained in balanced salt solution (BSS) (146 mM NaCl, 4 mM KCl, 2 mM MgCl2, 0.5 mM CaCl2, 10 mM glucose, 0.1% bovine serum albumin and 10 mM HEPES/Tris, pH7.4); fura-2 fluorescence was measured by a ratiometric fluorescence method using a fluorescence image processor (Aquacosmos, Hamamatsu Photronics). The excitation wavelength was alternated at 340 and 380 nm (1 Hz), and the emitted fluorescence light was detected at 510 nm. The fluorescence ratio at 340/380 nm was calculated and [Ca2+]i was determined using a Kd of 135 nM for the dissociation of fura-2/Ca2+ complex (37). Muscle fibers were loaded for 1 h at room temperature with 4 μM fura-2/AM in BSS. For the experiments, the working medium contained 50 μM N-Benzyl-p-toluene sulphonamide (BTS), an inhibitor of the myosin II ATPase. Stimulation with 2APB was performed in BSS containing 5 mM CaCl2 (pH6.8). Myotubes were loaded with 4 μM fluo-4-acetoxymethyl ester for 30 min at 37°C, and maintained in BSS. Fluorescence signal was detected with a confocal microscope (MRC-1024; Bio-Rad, Richmond, CA, USA) mounted on an Olympus BX50WI microscope. Images were acquired at a rate of one image every 1 s and single-frames or the single cell-integrated signal density were analyzed by Laser-Sharp software (Bio-Rad). All Ca2+ measurements were carried out at room temperature.
Statistical analysis

Unless otherwise stated, data are represented as means ± SD of at least three determinations. We used an unpaired t-test, one-way analysis of variance followed by Dunnett’s test for statistical analyses. Values of P < 0.05 (indicated as asterisks in figures) were considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Ms H. Otake for her technical assistance.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by Grant-in-Aid for Priority Areas 18077015 (to S.W.), Grants-in-Aid 19390800, 17659241 (to S.W.), 18590796 (Y.I.) and a Grant for the Cooperative Link for Unique Science and Technology for Economy Revitalization (S.W.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant for the Promotion of Fundamental Studies in Health Sciences of National Institute of Biomedical Innovation (NIBIO), research grants for Cardiovascular Diseases (17A-1) (S.W.) and for Nervous and Mental Disorders (16B-2 and 19A-7) from the Ministry of Health, Labor, and Welfare (to Y.I.), and grants from Takeda Science Foundation (to Y.I.) and the Salt Science Research Foundation, No. 0737 (to S.W.).

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