Increased susceptibility to fatigue of slow- and fast-twitch muscles from mice lacking the MG29 gene

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Nagaraj, Ramakrishnan Y., Christopher M. Nosek, Marco A. P. Brotto, Miyuki Nishi, Hiroshi Takeshima, Thomas M. Nosek, and Jianjie Ma. Increased susceptibility to fatigue of slow- and fast-twitch muscles from mice lacking the MG29 gene. Physiol Genomics 4: 43–49, 2000.—Mitsugumin 29 (MG29), a major protein component of the triad junction in skeletal muscle, has been identified to play roles in the formation of precise junctional membrane structures important for efficient signal conversion in excitation-contraction (E-C) coupling. We carried out several experiments to not only study the role of MG29 in normal muscle contraction but also to determine its role in muscle fatigue. We compared the in vitro contractile properties of three muscles types, extensor digitorum longus (EDL) (fast-twitch muscle), soleus (SOL) (slow-twitch muscle), and diaphragm (DPH) (mixed-fiber muscle), isolated from mice lacking the MG29 gene and wild-type mice prior to and after fatigue. Our results indicate that the mutant EDL and SOL muscles, but not DPH, are more susceptible to fatigue than the wild-type muscles. The mutant muscles not only fatigued to a greater extent but also recovered significantly less than the wild-type muscles. Following fatigue, the mutant EDL and SOL muscles produced lower twitch forces than the wild-type muscles; in addition, fatiguing produced a downward shift in the force-frequency relationship in the mutant mice compared with the wild-type controls. Our results indicate that fatigue affects the E-C components of the mutant EDL and SOL muscles, and the effect of fatigue in these mutant muscles could be primarily due to an alteration in the intracellular Ca homeostasis.

excitation-contraction coupling; ryanodine receptor; sarcoplasmic reticulum; muscle tension; triad junction

The process of excitation-contraction (E-C) coupling in skeletal muscle involves two major proteins: 1) the ryanodine receptor (RyR)/Ca release channel (28) present in the sarcoplasmic reticulum (SR), and 2) the dihydropyridine receptor (DHPR)/voltage-gated Ca channel present in the transverse-tubule (TT) membrane (30). The DHPRs serve as the voltage sensor across the sarcolemmal membrane (23), and the RyRs act as the pathway for Ca release from the SR (9, 26). A functional unit of E-C coupling involves the triad junction formed by the TT and junctional SR membranes, where the DHPR and RyR proteins presumably interact with each other (10).

The DHPR and RyR genes have been cloned and the proteins expressed in heterologous cell systems (28, 30). The functional aspects of these proteins, such as voltage-gated Ca current in the surface membrane and Ca-induced Ca release from the endoplasmic reticulum, have been reproduced in these cells (22). However, these studies have shown neither close opposition of the DHPR and RyR nor depolarization-induced Ca release (25, 27), suggesting that expression of DHPR and RyR in heterologous cells does not result in formation of the triad junction. On the other hand, intact triad junctions are retained in mutant mice lacking either DHPR (dysgenic mouse) or RyR (dyspedic mouse) (11, 15), indicating that proteins other than DHPR and RyR play an important role in the assembly of the triad and possibly in the signal transduction step of E-C coupling in skeletal muscle.

Recently, Takeshima et al. (29) have identified a new transmembrane protein named mitsugumin 29 (MG29) in the triad junction of skeletal muscle. Sequence alignment revealed that MG29 exhibits a high degree of homology to the synaptophysin family of proteins, a group of membrane proteins with presumed roles in neurotransmitter release (5, 18, 20). Subsequently, Nishi et al. (19) showed that the extensor digitorum longus (EDL) muscles isolated from MG29 knockout mice produced significantly lower twitch force than muscles from wild-type mice. These mice also had morphological abnormalities of membrane structures around the triad junction. The absence of MG29 could alter the proper functioning, or cause conformational changes in, DHPR and RyR that may lead to improper signaling between these molecules, thereby causing the observed alterations in muscle contraction.

An important property of muscle function is fatigability. Skeletal muscle fatigue is defined as a reversible decrease in the isometric contractile force in response to an increase in the frequency or duration of stimula-
Optimal muscle performance revolves around the maintenance of intracellular Ca homeostasis (8), such that inadequate Ca release from the SR could lead to the reduced force output observed in muscle fatigue. The deficient Ca release process could result from improper coupling between the DHPR and RyR, reduction of the SR Ca content, or direct modification of the RyR function (18, 33). The defective ultrastructure of the triad junction and muscle function in the MG29 knockout mice could have an impact on the Ca recycling in the muscle and therefore the susceptibility of the muscle to undergo fatigue.

In this study, we compared the contractility and fatigability of EDL, soleus (SOL), and diaphragm (DPH) muscles isolated from the MG29 knockout mice with those from control mice. We found that the fast-twitch muscle fatigued faster, and both fast- and slow-twitch muscles fatigued to a greater extent in the mutant mice, compared with the wild-type controls. Our data suggest that MG29, an accessory protein component of E-C coupling, could play important roles in the Ca signaling of muscle contraction.

**METHODS**

**Generation of mutant mice.** The procedure for the generation of MG29 knockout mice has been published elsewhere (19). These mice were generated from the C57BL/6J background. For comparative studies, the SV129/J × C57BL6 strain was used as the wild-type control. Care was taken to ensure that all procedures relating to the living animals were in accordance with the “Guiding Principles in the Care and Use of Animals” approved by the American Physiological Society.

**Intact muscle preparation.** We used a modified protocol of Broto and Nosek (4) and Kolbeck and Nosek (16) in our experiments. The mice were euthanized by CO2 inhalation, and the intact EDL, SOL, and DPH muscles were removed and placed in a dissecting dish containing a modified HEPES-Ringer solution with the following composition (in mM): 142 NaCl, 5.0 KCl, 2.5 CaCl2, 1.8 MgCl2, 5 HEPES, and 10 glucose, pH 7.35 (adjusted with NaOH). The muscles were continuously aerated with 100% O2. The experiments with the wild-type muscles (Fig. 1, A and B) were performed at room temperature (22–24°C).

The intact whole muscle or muscle strips were mounted vertically on a Radnoti glass apparatus with platinum stimulating electrodes (Monrovia, CA), and were immersed in a 20-ml bathing chamber containing the aerated HEPES-Ringer solution. The muscles were attached to an isometric force transducer and to the stationary post of the stimulating apparatus. The muscles were stretched to the length, which provided the maximal tetanic force and left at this length for the duration of the experiment. The contractile status of each muscle was monitored on a strip-chart recorder. The output of the force transducer was digitized and stored in a computer and was analyzed with Labview Software (National Instrument, Austin, TX).

**Stimulation protocols.** The muscles were allowed to equilibrate for 20 min, each minute receiving one tetanic (100 Hz) pulse-train, 500-ms duration. The muscles were then subjected to several frequencies of stimulation ranging from 1 to 140 Hz to produce the force-frequency relationship. The frequencies at which maximum isometric tetanic force (Tmax) and 50% Tmax were produced were used in the fatigue-recovery protocol. The muscles were fatigued by being subjected to the Tmax frequency and 50% of Tmax frequency for 5 min at 1-s intervals (50% duty cycle), and the time course of changes in force production was recorded. Following fatigue, recovery in these muscles was measured by subjecting the muscles to the Tmax and 50% of Tmax frequency at 1-min intervals for 20 min. The postfatigue force-frequency relationships of the muscles were then determined.

**Normalization of data.** The twitch force and tetanic force were normalized to force/cross-sectional area by using the following relationship: F/cm² = [force (g) × muscle length × 1.06]/muscle weight. To follow the time course and recovery from fatiguing stimulation, all force data were normalized to the single high-frequency tetanic force measured just prior to the fatiguing protocol. Force vs. frequency data were normalized to the maximum force generated by each muscle. The numbers of muscle preparations used in this study were as follows: 8, 8, and 13 for the mutant EDL, SOL, and DPH muscles; and 10, 9, and 13 for the wild-type EDL, SOL, and DPH, respectively. All the data were analyzed by Student’s t-test, and significant differences were determined when P < 0.05.

**Caffeine stimulation.** Following the 20-min recovery period, the muscles were subjected to the Tmax frequency and 50% of Tmax frequency for 12 min at 1-min intervals. The muscles were exposed to increasing concentrations of caffeine (0.1–35 mM) that occurred once in 2 min.

**RESULTS**

To eliminate possible age- and sex-related variabilities, all muscle tension measurements were performed with 8- to 10-wk-old male mice. We compared the normalized maximum tetanic force between the wild-type and mutant mice in the three different types of muscles, EDL, SOL, and DPH. The muscles were stimulated at various frequencies, and the Tmax was determined from the peak of the force-frequency curve. As shown in Fig. 1, the Tmax in the EDL muscle was similar in the wild-type and MG29 knockout mice (Fig. 1A). This data is similar to those reported in Ref. 19. The mutant SOL and DPH muscles showed a slight but not significant increase in the tetanic force compared with the wild-type muscles (Fig. 1, B and C). In both

![Fig. 1. Measurement of tetanic force in mitsugumin 29 (MG29) knockout and control extensor digitorum longus (EDL, A), soleus (SOL, B), and diaphragm (DPH, C) muscles. The muscles were stimulated at the frequency that produced the maximal force. The tetanic force was calculated by using the following relationship: F/cm² = [force (g) × muscle length × 1.06]/muscle weight. For all three muscles, the open bars represent wild-type muscle, and the solid bars represent the mutant muscles; error bars are SE.](http://physiolgenomics.physiology.org)
the wild-type and mutant muscles, the T\text{max} of EDL muscle was the highest, followed by DPH and then by SOL.

When the muscles were subjected to fatigue, significant differences between muscle types and between the wild-type and MG29 knockout mice were identified. Figure 2, top, shows representative traces of the three muscle types subjected to the fatigue protocol, and the bottom shows the average ± SE of the tetanic force for each time interval (2 s) wherein the T\text{max} frequency was used for the induction of fatigue, plotted as a percentage of T\text{max}. The mutant EDL muscle fatigued faster and to a greater extent than the corresponding wild-type muscles (Fig. 2A). The time-dependent changes in contractile forces following fatigue could be fitted with a single exponential decay function, with $\tau = 20 \pm 0.3$ s for the MG29 knockout mice and $\tau = 29 \pm 0.5$ s for the wild-type mice. The extent of fatigue (percentage change from T\text{max}) was greater in the mutant muscle (3 ± 0.2%) compared with the wild-type mice (6 ± 0.2%).

The difference between the fatigability of knockout and control muscles was the greatest in SOL muscles (Fig. 2B). The rate of fatigue in SOL muscle did not show significant difference between the MG29 knockout and the wild-type mice ($\tau = 38 \pm 0.8$ s, MG29 knockout; 44 ± 0.8 s, wild type), whereas the extent of fatigue was more pronounced in the mutant SOL muscles (31 ± 0.1%) than that in the wild-type muscles (49 ± 0.1%). Interestingly, the mutant DPH muscles did not show any difference in fatigability compared with the wild-type muscles (Fig. 2C). The rate and extent of fatigue in mutant DPH muscles were practically identical to the wild-type muscles.

We also compared the twitch force in the mutant and wild-type muscles prior to and after fatigue. The force produced at a frequency of 1 Hz was taken as the twitch force. The mutant EDL muscles showed a slight, but not significant reduction in the twitch force prior to fatigue (Fig. 3A). However, fatiguing these mutant EDL muscles had a more pronounced effect on the twitch force (Fig. 3A). The mutant EDL muscles showed a 72 ± 5% (mean ± SE, $P < 0.0001$, t-test) reduction in the absolute twitch force compared with just 51 ± 5% ($P < 0.002$) in the wild-type muscles after being fatigued. Fatiguing had a greater negative effect on the twitch force of the mutant SOL muscles (Fig. 3B).
3B). While the wild-type SOL muscles showed a meager 18 ± 4% (P < 0.5) reduction in twitch force, the mutant muscles showed a 49 ± 9% (P < 0.02) reduction after fatigue. The twitch force in the mutant DPH muscles was slightly but not significantly elevated. However, upon fatigue, the mutant DPH muscles showed a greater reduction in the twitch force compared with the wild-type controls (Fig. 3C). The mutant DPH muscles showed a 47 ± 4% (P < 0.002) reduction in the twitch force compared with the 39 ± 2% (P < 0.03) in the wild-type muscles. After being fatigued for 5 min, the muscles were allowed to recover for 20 min. Figure 4 shows the average rate and extent of recovery in all the muscles to the T_{max} stimulation frequency. The rate of recovery was calculated as the time taken for the muscle to recover to 50% of the final tetanic force (in minutes) achieved at the end of the recovery period, and the extent of recovery was calculated as a percentage of T_{max} of this final tetanic force. The extent of recovery for the mutant EDL muscle was significantly less than for the wild-type muscle (30 ± 0.3% and 52 ± 2%, respectively). The wild-type SOL muscles recovered to even higher than the T_{max} value (115 ± 0.1%). This pattern of recovery is a characteristic feature of a slow-twitch muscle, as has been reported earlier (3). In contrast, the mutant SOL muscle recovered to only 78 ± 2%. The mutant DPH muscles did not show any change in the extent of recovery compared with the wild-type muscles (63 ± 5% and 71 ± 4%, respectively). The rate of recovery after fatigue was not altered in any of the muscles compared with their respective controls.

Fatigue also has a significant impact on the force-frequency relationship of the three muscle types (Fig. 5). Prior to fatigue, all the three mutant muscle types exhibited rightward shifts in the force-frequency relationships compared with their respective controls (Fig. 5, solid lines). After fatigue, the force-frequency curves of the EDL muscle shifted downward in the MG29 knockout mice compared with the wild-type controls (Fig. 5A). SOL muscles are more resistant to fatigue (3). Fatiguing the wild-type SOL muscles produced an upward shift in the force-frequency relationship. However, fatiguing the mutant SOL muscles produced a dramatic downward shift in the force-frequency curve (Fig. 5B). The downward shift observed in the mutant EDL and SOL muscles after fatigue could be due to a reduction in cytosolic Ca or a reduction in the sensitivity of the myofilaments to Ca (13, 31, 32). The mutant DPH muscles did not show any change in the force-frequency curve after fatigue compared with the wild-type muscles (Fig. 5C), indicating that the effect of MG29 knockout is probably restricted to purely slow-twitch or purely fast-twitch muscle fiber type.

Treatment of isolated skeletal muscles with caffeine will enhance Ca release from the SR and determine whether the reduction of force with fatiguing stimulation is due to lesser Ca release or due to a reduction in the SR Ca pool/lower sensitivity of contractile proteins to Ca. The muscles were stimulated at the T_{max} frequency and were subjected to a dose-response curve of caffeine in all the muscles after 20 min of recovery from the fatiguing stimulation. Figure 6 shows the effect of caffeine on the three muscles studied. The response of muscles to caffeine was represented as a percentage change from T_{max} that was determined prior to subjecting the muscles to fatigue. In wild-type EDL muscles, increasing doses of caffeine (0.1–35 mM) produced an increase in the force generated by the muscle. At the highest caffeine concentration, the muscles produced the same amount of force obtained prior to fatigue, suggesting that fatigue only affected Ca release from the SR and not the Ca pool or the Ca sensitivity of the myofilaments. However, in the mutant EDL muscles, even the highest concentration (35 mM) produced only about 60% force of T_{max} (Fig. 6A). In the SOL muscles, while low concentrations of caffeine (1 mM) produced more than 100% response in the wild-type muscles, even the highest concentration of caffeine (35 mM) produced only a 9% increase in force compared with the untreated muscles.
produced only about 80% of $T_{\text{max}}$ in the knockout mice (Fig. 6B). The caffeine response was essentially identical between the mutant and wild-type DPH muscles (Fig. 6C). The lower response to caffeine in the mutant EDL and SOL muscles could be due to a reduction in the Ca pool or an effect on the contractile proteins.

**DISCUSSION**

Our data indicate that by knocking out the MG29 gene in mice, we can significantly depress the E-C coupling process of both fast- and slow-twitch muscles. Compared with control muscles, fatiguing the muscles from knockout animals produces significant reductions in contractile force, ability of the muscles to recover from fatigue, and the response to caffeine of EDL and SOL but not DPH. This has helped us to identify two phenotypes of the MG29 knockout mice: the increased susceptibility to fatigue and decreased sensitivity to caffeine of the EDL and SOL muscles.

One of the main effects of muscle fatigue is the alteration in the SR Ca release channel, which can cause a reduction in the force generation of the contractile proteins (1, 4, 7, 12, 17). The hallmark of our experiments has been the increased susceptibility of both the EDL and SOL muscles to fatigue in muscles where MG29 is absent. The alterations in the twitch and tetanic force production in the mutant muscles became apparent only after they were fatigued. The downward shift in the force-frequency curve seen in both the EDL and SOL muscles after fatigue indicates that the mutant mice probably had less Ca available.
for contraction. Despite of several differences in the contractile properties of the EDL and SOL muscles (24), the negative effect of MG29 knockout on the contractile properties is seen equally in both muscle types. This could occur if knocking out the MG29 gene affects intracellular Ca homeostasis. The reduced caffeine sensitivity of the mutant EDL and SOL muscles further supports this hypothesis. It is probable that in the event of fatigue, Ca release from the RyR is suppressed because of conformational changes in the triad junction induced by MG29 deletion.

Earlier studies by Nishi et al. (19) have shown that the MG29 knockout mice exhibited abnormal structures in the triad junction linking the TT and SR membranes. These ultrastructural changes could affect the function of the DHPR and the interaction between DHPR and RyR, leading to improper function of the RyR/Ca release channel.

Events such as buildup of intracellular metabolites, such as inorganic phosphate or lactate, or reduction in intracellular pH can also cause depression of force production during fatigue (6, 10). It is possible that the mutant EDL and SOL muscles are somehow rendered more sensitive to the intracellular milieu of changes or could have a higher buildup of metabolites than the wild-type muscles, which would eventually make them more susceptible to fatigue. It is known that the EDL and SOL muscles have different susceptibilities to fatigue due to the buildup of intracellular metabolites (24). Given that the general characteristics of fatigue in the mutant EDL and SOL muscles were similar, it is unlikely that the mutant muscles developed greater fatigue due to a higher buildup of intracellular metabolites compared with the wild-type muscles. The myofilament sensitivity to Ca may be reduced in the mutant EDL and SOL muscles, since they responded to a lesser extent than the wild-type muscles to caffeine.

In conclusion, our experiments have identified MG29 to play important roles in skeletal muscle E-C coupling process. We have identified two phenotypes of mice that do not express MG29. They are 1) the tendency of the EDL muscle to fatigue faster, and 2) the tendency of the EDL and SOL muscles to fatigue to a greater extent and recover less after fatigue. The detrimental effects of MG29 knockout are seen only in a purely fast-twitch muscle such as the EDL muscle or purely slow-twitch muscle such as the SOL muscle.

For reasons that are not clear at this time, the DPH muscles in the MG29 knockout mice did not show significant changes in their fatigue properties compared with the wild-type controls. It is possible the type 3 RyR, which is only present in the DPH muscles of adult muscles, could play a role in the fatigue process. Further experiments are required to test this hypothesis.

Mussle fatigue is a complicated process that involves the complex interaction of Ca regulatory proteins and several contractile proteins. Although our data indicate the primary involvement of the SR Ca release channel in muscle fatigue, we cannot rule out the possibility that the myofilaments of the mutant mice are less sensitive to Ca and thereby show a decreased generation of force upon fatigue. Other muscle proteins that can affect the intracellular Ca homeostasis could also be affected in the mutant muscles. Therefore, more experiments are needed to understand the exact role of MG29 in muscle fatigue.

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