Junctophilin damage contributes to early strength deficits and EC coupling failure after eccentric contractions

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Corona BT, Balog EM, Doyle JA, Rupp JC, Luke RC, Ingalls CP. Junctophilin damage contributes to early strength deficits and EC coupling failure after eccentric contractions. Am J Physiol Cell Physiol 298: C365–C376, 2010. First published November 25, 2009; doi:10.1152/ajpcell.00365.2009.—Junctophilins (JP1 and JP2) are expressed in skeletal muscle and are the primary proteins involved in transverse (T)-tubule and sarcoplasmic reticulum (SR) membrane apposition. During the performance of eccentric contractions, the apposition of T-tubule and SR membranes may be disrupted, resulting in excitation-contraction (EC) coupling failure and thus reduced force-producing capacity. In this study, we made three primary observations: 1) through the first 3 days after the performance of 50 eccentric contractions in vivo by the left hindlimb anterior crural muscles of female mice, both JP1 and JP2 were significantly reduced by ~50% and 35%, respectively, while no reductions were observed after the performance of nonfatiguing concentric contractions; 2) following the performance of a repeated bout of 50 eccentric contractions in vivo, only JP1 was immediately reduced (~30%) but recovered by 3-day postinjury in tandem with the recovery of strength and EC coupling; and 3) following the performance of 10 eccentric contractions at either 15° or 35°C by isolated mouse extensor digitorum longus (EDL) muscle, isometric force, EC coupling, and JP1 and JP2 were only reduced after the eccentric contractions performed at 35°C. Regression analysis of JP1 and JP2 content in tibialis anterior and EDL muscles from each set of experiments indicated that JP damage is significantly associated with early (0–3 days) strength deficits after performance of eccentric contractions (R = 0.49; P < 0.001). As a whole, the results of this study indicate that JP damage plays a role in early force deficits due to EC coupling failure following the performance of eccentric contractions.

mitsugumin; muscle injury or damage; mice; excitation-contraction

FORCE-PRODUCING CAPACITY of skeletal muscle is reduced both immediately and for a prolonged period of time after the performance of lengthening or eccentric contractions (11, 20, 30, 38). It is generally accepted that prolonged force deficits (>5 days) are primarily due to a loss of force-generating and force-transmitting proteins mediated by a well-characterized inflammatory response (20, 33, 42, 52). On the other hand, early force deficits (0 to 5 days) can stem from an inability to stimulate sarcoplasmic reticulum (SR) Ca2+ release [i.e., excitation-contraction (EC) coupling failure], although damage to force-generating or force-transmitting proteins also plays a role in these early strength deficits (21, 30). To date, it is not completely understood how EC coupling is impeded in injured skeletal muscle.

In skeletal muscle, EC coupling involves the propagation of an action potential along the plasmalemma and transverse (T)-tubule membranes to triads. At the triad, L-type Ca2+ channels [dihydropyridine receptors or caveolin (Cav).1.1] located in the T-tubule membrane physically couple with approximately every other SR Ca2+ release channel (ryanodine receptor, RyR1) (6, 8, 15). T-tubule membrane potential depolarization results in a conformational change in Cav.1.1 that physically opens RyR1 and promotes release of Ca2+ from the SR (6, 15), which then incites force production.

Immediately after the performance of eccentric contractions, the primary components of the EC coupling process are still present and appear functional when tested in isolation. Specifically, immediately after performance of short-duration (i.e., ~200 ms) eccentric contractions, the ability of the T-tubule to propagate an action potential does not appear to be impeded (21, 53, 54). Cav.1.1 and RyR1 protein content are not diminished (22), and SR Ca2+ release and reuptake rates are at most only modestly reduced (21). However, it does appear that the cellular organization of the T-tubule network is disrupted after the performance of eccentric contractions (45, 53). Thus, the EC coupling failure observed after eccentric contractions may result from a disruption in the communication between Cav.1.1 and RyR1.

Although the physical coupling of Cav.1.1 and RyR1 is essential for voltage-induced Ca2+ release (VICR), the expression of these ion channels in cell culture is not sufficient for channel colocalization or VICR, despite both channels exhibiting signs of independent functionality (e.g., Ca2+-induced Ca2+ release was observed) (44, 46). Therefore, expression of accessory triadic proteins in skeletal muscle is crucial for VICR. There are a number of triadic proteins that have been identified (55); however, in skeletal muscle, two junctophilin isoforms, JP1 and JP2, are thought to be the primary proteins involved in embryonic development and postnatal apposition of T-tubule-SR membranes (24, 26, 47). Junctophilins possess a SR transmembrane domain at the COOH terminus and a membrane occupation and recognition nexus (MORN) motif at the NH2 terminus that binds to the plasmalemma or T-tubule membrane (47). These characteristics allow JP1 and JP2 to tether the T-tubule membrane to the SR membrane, allowing direct interaction between Cav.1.1 and RyR1.

Previous studies have indicated that reductions in junctophilin protein expression carry structural and functional consequences. For example, reduced expression of JP1 and JP2 in adult skeletal muscle results in loss of triad formation, reduced VICR, and diminished store-operated Ca2+ entry (SOCE) (18). Moreover, ablation of JP1, which is lethal, impedes SR-T-tubule membrane junction development and reduces force-producing capacity of neonatal skeletal muscle (24, 26). Similarly, in cardiomyocytes, reductions in junctophilin protein
expression have been associated with a failure to release Ca\(^{2+}\) from the SR (35, 39, 56).

On the basis of the cellular location of junctophilins in skeletal muscle and the functional implications of junctophilin loss, we hypothesized that eccentric contractions would damage junctophilins, and that damage of junctophilins contributes to EC coupling failure and early strength deficits after injury. Therefore, our first objective was to characterize JP1 and JP2 content in mouse anterior crural muscles after the performance of a single bout of either eccentric or concentric contractions in vivo. Upon finding immediate reductions in JP1 and JP2 with only eccentric contractions, our next objective was to determine the content of JP1 and JP2 in mouse anterior crural muscle under conditions known to alter the magnitude and/or timing of EC uncoupling and strength deficits.

**METHODS**

**Animals**

Female CD1 mice 2 to 5 mo old were used in this study. The mice were housed in groups of 10 animals per cage, supplied with food and water ad libitum, and maintained in a room at 20–22°C with a 12-h photoperiod. Mice were euthanized with an overdose of pentobarbital sodium (200 mg/kg body wt) followed by cervical dislocation. All animal care and use procedures were approved by the institutional animal care and use committee and met the guidelines set by the American Physiological Society.

**Experimental Design**

Three studies were conducted to determine whether junctophilins are damaged by eccentric exercise and to determine whether junctophilin damage is associated with early strength deficits and EC coupling failure. A fourth study was performed to determine whether blocking ion entry through store-operated Ca\(^{2+}\) channels or stretch-activated channels would influence force deficits associated with eccentric contraction-induced injury. In study one, mice performed a single bout of 50 concentric or eccentric contractions with the left anterior crural muscles [tibialis anterior (TA) and extensor digitorum longus (EDL) muscles]. Isometric torque produced by this muscle group as a function of stimulation frequency (20–400 Hz) was measured in anesthetized mice before and immediately after the concentric or eccentric contraction bout. The recovery of anterior crural muscle strength was also evaluated in vivo by measuring isometric torque as a function of stimulation frequency (20–400 Hz) in different groups of mice 1 day after the concentric contractions and 1 and 3 days after performance of eccentric contractions.

For each respective group of mice, junctophilin protein content was assessed via Western blot, and the extent of myocellular damage was assessed in uninjured and injured anterior crural muscles at 0, 1, and 3 days postinjury. Additionally, isometric force production of isolated EDL muscle as a function of stimulation frequency (10–300 Hz) and caffeine contracture force was assessed in vitro for uninjured muscles and muscles that had previously performed concentric [0 or 1 day (1d) post] or eccentric (0, 1, or 3d post) exercise. Because caffeine elicits SR Ca\(^{2+}\) release by acting directly on RyR1 and therefore bypasses the upstream components of EC coupling (27), relative reductions in caffeine-induced and electrically stimulated force were compared to serve as an indirect marker of EC coupling failure (21, 54).

Upon finding that JP1 and JP2 protein content was reduced during the first 3 days after eccentric exercise, we sought to perform experiments that would manipulate the magnitude and time course of eccentric contraction-induced strength deficits and EC coupling failure. In study two, mice performed two bouts of 50 eccentric contractions in vivo separated by 2 wk. It has been shown previously that the performance of repeated bouts of eccentric contractions results in at most a small attenuation in immediate force deficits but a robust enhancement in the rate of recovery of early force deficits (10, 11, 23). Therefore, in this study we hypothesized that JP1 and JP2 protein content would be reduced immediately after performance of a second bout of eccentric contractions, but would recover at a faster rate in conjunction with recovery of force and EC coupling. In this study, there were three groups of mice that performed a single bout of 50 eccentric contractions, with isometric torque-frequency testing occurring immediately before and after the contraction bout. Then, one group of mice performed an isometric torque-frequency test 14 days later, while the other two groups performed a second bout of 50 eccentric contractions with immediate pre- and postinjury isometric torque frequency testing. At this point, one of the repeated eccentric contraction groups ceased testing (i.e., 0d post eccentric bout 2) and the other group performed a follow-up isometric torque frequency test 3 days later. As in study one, on the final day of testing, EDL muscles were isolated and underwent an isometric force-frequency and caffeine contracture test. JP1 and JP2 protein in the TA muscle was determined by Western blot.

In study three, the temperature that EDL muscles were injured in vitro was manipulated, because it has been shown previously that force deficits and EC coupling failure after performance of eccentric contractions in vitro are temperature dependent (51). Specifically, force deficits and EC coupling have been observed when eccentric contractions are performed at ~35°C, while significant force deficits and EC coupling failure are not observed when the same intensity eccentric contractions were performed at ~15°C. Thus, we hypothesized that JP1 and JP2 protein content would only be reduced after the performance of eccentric contractions at 35°C, further supporting that the loss of junctophilins after performance of eccentric contractions is contributing to immediate force deficits and EC coupling failure. In this study, isolated EDL muscles performed 10 eccentric contractions at either 15° or 35°C, or 10 isometric contractions at 35°C. JP1 and JP2 protein content was then determined via Western blot.

In study four, possible contributions of disrupted SOCE to force loss after eccentric contractions were studied in isolated EDL muscles (35°C) exposed to 20 μM gadolinium, a drug reported to block Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels (16). In addition, possible contributions of stretch-activated channel activation of calpains to force loss associated with eccentric contraction-induced injury were studied in isolated EDL muscles (35°C) exposed to 200 μM streptomycin, a drug known to block Ca\(^{2+}\) and Na\(^{+}\) entry through stretch-activated channels (16, 59).

**Experimental Methodology**

In vivo muscle strength analysis and injury induction. Contractile function (i.e., torque-frequency relationship) of the left anterior crural muscles was measured in vivo as previously described (10, 21). After mice were anesthetized (0.3 mg/kg fentanyl citrate, 16.7 mg/kg droperidol, 5.0 mg/kg diazepam), the left hindlimb was aseptically prepared. The mouse was then placed on a heated platform and halogen lamps were directed at the torso of the mouse to maintain core body temperature between 35° and 37°C. The left knee was clamped and the left foot was secured to an aluminum “shoe” that is attached to the shaft of an Aurora Scientific 300B servomotor. Sterilized needles were inserted through the skin for stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with 5 to 15 isometric contractions (200-ms train of 0.1-ms pulses at 300 Hz). Contractile function of the anterior crural muscles was assessed by measuring peak isometric torque as a function of stimulation frequency (20–400 Hz). The anterior crural muscles performed either 50 concentric or eccentric contractions (38° angular movement at 2,000° s\(^{-1}\) starting from a 19° plantarflexed or dorsiflexed position, respectively). It is important to note that we chose to have mice perform 50 eccentric contractions in vivo, as opposed to 150 eccentric contractions, because we wanted to reduce the damage to
force-bearing proteins normally observed in this injury model and therefore isolate strength deficits due to EC coupling failure (10, 11, 21, 23). Core body temperature was monitored continuously during all functional testing with a mouse rectal thermocouple.

In vitro analysis of EDL muscle. EDL muscles were dissected free and studied using an in vitro preparation as previously described (10, 21). EDL muscles were mounted in a chamber containing a Krebs-Ringer bicarbonate buffer (pH 7.4) with (in mM) 144 Na\(^+\), 126.5 Cl\(^-\), 6 K\(^+\), 1 Mg\(^2+\), 1 SO\(_4\)\(^{2-}\), 1 PO\(_4\)\(^{3-}\), 25 HCO\(_3\), 1.25 Ca\(^{2+}\), 0.17 leucine, 0.10 isoleucine, 0.20 valine, 10 glucose, and 10 μg/ml gentamicin sulfate and 0.10 U/ml insulin (the buffer was equilibrated with 95% O\(_2\)-5% CO\(_2\) gas). The distal tendon was attached by silk suture and cyanoacrylate adhesive to a fixed support, and the proximal tendon was attached to the lever arm of a servomotor system (Aurora Scientific 300B). Optimal physiological muscle length (L\(_{po}\)) in the chamber was set with a series of twitch contractions (0.2-ms pulse at 150 V).

In studies one and two, peak isometric force as a function of stimulation frequency (10–300 Hz) was measured at 37°C during isometric contractions (200-ms trains of 0.2-ms pulses), with 3 min between contractions. Caffeine sensitivity was assessed by measuring changes in baseline force during exposure to caffeine (50 mM) and between contractions. Caffeine sensitivity was assessed by measuring isometric contractions (200-ms trains of 0.2-ms pulses), with 3 min stimulation frequency (10 –300 Hz) was measured at 37°C during isometric contractions (200-ms trains of 0.2-ms pulses), with 3 min between contractions. Caffeine contracture force was assessed for all treatment groups as described above for studies one and two.

In study four, once L\(_{po}\) had been set to peak twitch force, EDL muscles were exposed to Krebs-Ringer with either 20 μM gadolinium or 200 μM streptomycin. Five minutes after drug exposure, peak isometric force as a function of stimulation frequency (10–300 Hz) was measured at 35°C during isometric contractions (200-ms trains of 0.2-ms pulses), with 3 min between contractions. Four minutes later, EDL muscles performed 10 eccentric contractions with 3 min between contractions. During the eccentric contractions, the muscle was shortened to 87.5% and then lengthened to 112.5% of L\(_{po}\) over 133 ms at 1.9 muscle lengths per second. Because peak mechanical stress during eccentric contractions has been shown to be a primary determinant of loss of force producing capacity (50), the stimulation frequency during the 35°C eccentric contractions was reduced to 160 Hz to match peak eccentric force at 15°C (62 Hz). The isometric control group performed 10 submaximal tetanic contractions at 160 Hz at 35°C. Caffeine contracture force was assessed for all treatment groups as described above for studies one and two.

In study four, once L\(_{po}\) had been set to peak twitch force, muscles were exposed to Krebs-Ringer with either 20 μM gadolinium or 200 μM streptomycin. Five minutes after drug exposure, peak isometric force as a function of stimulation frequency (10–300 Hz) was measured at 35°C during isometric contractions (200-ms trains of 0.2-ms pulses), with 3 min between contractions. Four minutes later, EDL muscles performed 10 eccentric contractions with 3 min between contractions. During the eccentric contractions, the muscle was shortened to 87.5% and then lengthened to 112.5% of L\(_{po}\) over 133 ms at 1.9 muscle lengths per second; stimulation frequency was 300 Hz. Five minutes after the last eccentric contraction, peak isometric force as a function of stimulation frequency (10–300 Hz) was measured again. Peak isometric force as a function of stimulation frequency (10–300 Hz) was measured again. Three minutes after the last isometric contraction, caffeine contracture force was assessed as described for the other studies.

**Histology**

Muscles intended for histological analysis were embedded in a cryomold with OCT compound, frozen in 2-methylbutane chilled by liquid nitrogen, and stored at −80°C until further use. As described previously, cryosections (10 μm) were obtained from the middle of the

Table 1. **Study one in vivo torque-frequency parameters**

<table>
<thead>
<tr>
<th></th>
<th>Concentric</th>
<th>Eccentric</th>
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<tr>
<td></td>
<td>Pre</td>
<td>0d</td>
</tr>
<tr>
<td>Sample size n</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Body weight g</td>
<td>30.8 ± 1.6</td>
<td>30.5 ± 0.9</td>
</tr>
<tr>
<td>M(_{\text{basem}}) N-mm(^{-1})kg(^{-1})</td>
<td>21.8 ± 1.0</td>
<td>19.4 ± 0.9</td>
</tr>
<tr>
<td>M(_{\text{maxm}}) N-mm(^{-1})kg(^{-1})</td>
<td>111.2 ± 3.1</td>
<td>107.7 ± 2.6</td>
</tr>
<tr>
<td>M(_{\text{basem}}) N-mm(^{-1})kg(^{-1})</td>
<td>20.9 ± 1.1</td>
<td>19.0 ± 0.9</td>
</tr>
<tr>
<td>M(_{\text{maxm}}) N-mm(^{-1})kg(^{-1})</td>
<td>108.9 ± 3.0</td>
<td>106.0 ± 2.7</td>
</tr>
<tr>
<td>EC(_{50}), Hz</td>
<td>91.2 ± 1.9</td>
<td>97.9 ± 2.3</td>
</tr>
<tr>
<td>n Coefficient</td>
<td>−4.8 ± 0.2</td>
<td>−5.1 ± 0.2</td>
</tr>
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</table>

Values are means ± SE. The minimum (Min) and maximum (Max) torques measured and estimated represent twitch and peak tetanic torques, respectively.

EC\(_{50}\) is the stimulation frequency at which half of the rise in amplitude of torque occurred. The n coefficient describes the slope of the steep portion of the torque-frequency curves depicted in Fig. 1. Pre, 0d, 1d, and 3d: time course of contractions, i.e., before and immediately, 1 day, or 3 days after, respectively.

*Different from Pre Eccentric (Ecc); †different from Ecc 0d; ‡different from Ecc 1d (P < 0.05).
Fig. 2. Peak torque produced during either 50 concentric or eccentric contractions performed in vivo. Values are means ± SE.

Fig. 3. In vitro extensor digitorum longus (EDL) muscle isometric force as a function of stimulation frequency. Muscles were dissected and tested immediately (0d) and 1 day (1d) after a single bout of concentric (A) or eccentric (B) contractions, as well as 3 days (3d) after a single eccentric bout. Additionally, measurements were made with EDL muscles that had performed no prior exercise (control). Each data set was modeled with Eq. 1, listed in METHODS. Values are means ± SE.

METHODS. Values are means after a single bout of concentric (and tested immediately (0d) and 1 day (1d) (EDL) muscle isometric force as a function of Fig. 3. In vitro extensor digitorum longus (EDL) muscle isometric force as a function of

Western Blot Analysis

Following dissection, muscles intended for Western blot analysis were weighed, frozen in liquid nitrogen, and stored at −80°C until further use. Muscles were homogenized in a buffer of the following constituents (in mM): 0.1 sucrose, 46 KCl, 10 NaEDTA, and 10 TrisCl (pH 7.4). Total protein content was determined spectrophotometrically using the Bradford assay. A portion of the whole muscle homogenate was diluted in SDS reducing buffer. Twenty-five micrograms protein from the whole muscle homogenate was loaded into a 7% polyacrylamide gel (Acrylamide to Bis-; 37:5:1) and separated according to molecular weight (100 V for 30 min; 150 V for 45 min). The protein was then transferred to a nitrocellulose membrane (100 V for 60 min). Following transfer, the membrane was blocked overnight at 4°C in 5% nonfat dried milk (wt/vol) in Tris-buffered saline with 0.1% Tween-20 (TBS-T). The following morning, the membranes were then probed with rabbit anti-mouse primary antibodies for 2 h at room temperature on an orbital shaker. Junctophilin 1 and 2 (catalog nos. 40-5100 and 40-5300, respectively, Invitrogen) primary antibodies were then probed with rabbit anti-mouse primary antibodies for 1 h at room temperature with shaking. The membranes were then washed as described above and treated with enhanced chemiluminescent solution (Thermo Scientific) before detection with autoradiography film. Optical density of the blot was determined using SigmaGel (Jandel Scientific). JP1 and JP2 optical density was normalized to the tubulin content of each muscle.

Data Modeling and Statistical Analysis

Torque- and force-frequency relationships were modeled with the following equation:

\[
f(x) = \min + \frac{(\max - \min)}{[1 + (x/\text{EC}_{50})^n]}
\]

where \(x\) is the stimulation frequency, \(\min\) and \(\max\) are the smallest (i.e., twitch) and largest (i.e., peak tetanic) respective forces estimated, \(\text{EC}_{50}\) is the stimulation frequency at which half the amplitude of force (max − min) is reached, and \(n\) is the coefficient describing the slope of the steep portion of the curve.

Torque- and force-frequency parameters, anthropometric measurements, histological degeneration, and protein content values were analyzed separately using a one-way ANOVA. Eccentric and concentric contractions during each type of exercise bout in all three studies, as well as pre- and postexercise protocol force values in study three, were analyzed using a group by contraction or time split-plot ANOVA. Post hoc means comparisons testing was performed when a significant ANOVA was observed. Statistical significance was achieved with \(P < 0.05\). All statistical testing was performed with SPSS 12.0.

RESULTS

Study One: A Single Bout of Concentric or Eccentric Contractions

In vivo torque and injury induction. There were no significant differences in preexercise anterior crural muscle isometric torque values between concentric and eccentric groups (Fig. 1 and Table 1). Peak eccentric torque was significantly greater than peak concentric torque and, over the 50 contractions, exhibited a significant decline (~40%), while concentric torque was not significantly reduced (Fig. 2). Following the performance of concentric contractions, there were no significant alterations in isometric torque-frequency parameters compared with preexercise values (Fig. 1 and Table 1). In

\[
\text{EC}_{50} = \frac{\text{stimulus frequency}}{\text{max} \pm \frac{\min - \text{max}}{2}}
\]

where \(\text{EC}_{50}\) is the stimulation frequency at which half the amplitude of force (max − min) is reached, and \(n\) is the coefficient describing the slope of the steep portion of the curve.

\[
\text{EC}_{50} = \frac{\text{stimulus frequency}}{\text{max} \pm \frac{\min - \text{max}}{2}}
\]

where \(\text{EC}_{50}\) is the stimulation frequency at which half the amplitude of force (max − min) is reached, and \(n\) is the coefficient describing the slope of the steep portion of the curve.
contrast, throughout the 3 days following the performance of eccentric contractions, both peak twitch and tetanic isometric torque values were significantly reduced and the stimulation frequency required to elicit half-maximal force (EC_{50}) was shifted to higher frequencies, compared with preinjury values (Fig. 1 and Table 1). By 3 days after eccentric exercise, all altered isometric torque parameters exhibited a significant but incomplete recovery.

In vitro EDL muscle force. EDL muscle isometric specific force was measured as a function of stimulation frequency for mice that had either previously performed in vivo concentric or eccentric exercise or performed no prior exercise (control). Compared with control values, the prior performance of concentric exercise did not significantly alter isometric specific force parameters (Fig. 3 and Table 2). However, within an hour after performance of eccentric exercise, peak twitch and tetanic isometric forces were significantly reduced and EC_{50} was significantly greater compared with control values. By 3 days after eccentric exercise, isometric peak twitch and tetanic specific force exhibited a significant but incomplete recovery, while EDL muscle EC_{50} recovered to control values 1 day after performance of eccentric contractions (Fig. 3 and Table 2).

EDL muscle caffeine (50 mM) contracture force was measured after the force-frequency test (Table 2). Compared with control values, there were no significant reductions of caffeine-contracture force ~1 to 24 h after performance of concentric contractions. In contrast, ~1 to 24 h after performance of eccentric contractions, caffeine contracture force was significantly reduced but was not statistically different from control values 3 days after performance of eccentric contractions. When comparing the caffeine contracture and peak tetanic force deficits to estimate EC coupling failure, ~40%, 58%, and 45% of the force deficit observed 0, 1, and 3 days after the injury bout, respectively, is explained by EC coupling.

Histological indications of TA muscle degeneration. Despite the reduction in caffeine contracture force immediately and 1 day after the performance of 50 eccentric contractions, the degree of muscle degeneration assessed via H&E staining through the first 3 days after injury induction was not significantly different among groups (Fig. 4). The percentage of total fibers counted exhibiting signs of degeneration for unexercised and 0, 1, 1d; *different from Bout 1 Ecc 0d; †different from Bout 1 Ecc 1d; ‡different from Bout 1 Ecc 3d; §different from Bout 1 Ecc 1d; ‡different from Bout 2 Ecc 0d; †different from Bout 2 Ecc 1d (P < 0.05).

Table 2. TA and EDL muscle characteristics and in vitro EDL muscle force parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Concentric</th>
<th>Eccentric Bout 1</th>
<th>Eccentric Bout 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size n =</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>TA muscle wt, mg</td>
<td>55.0 ± 1.7</td>
<td>52.8 ± 3.3</td>
<td>59.0 ± 2.6</td>
<td>64.1 ± 3.5</td>
</tr>
<tr>
<td>TA muscle protein, mg</td>
<td>24.1 ± 1.3</td>
<td>21.0 ± 1.3</td>
<td>21.2 ± 0.7</td>
<td>21.4 ± 2.4</td>
</tr>
<tr>
<td>EDL muscle wt, mg</td>
<td>9.0 ± 0.6</td>
<td>10.9 ± 0.6</td>
<td>11.4 ± 0.5</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td>L_{c}, cm</td>
<td>1.47 ± 0.02</td>
<td>1.52 ± 0.04</td>
<td>1.56 ± 0.02</td>
<td>1.55 ± 0.34</td>
</tr>
<tr>
<td>Min_{isoc}, N/cm²</td>
<td>2.8 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Max_{isoc}, N/cm²</td>
<td>22.2 ± 0.5</td>
<td>20.7 ± 0.4</td>
<td>20.8 ± 0.7</td>
<td>20.5 ± 0.6</td>
</tr>
<tr>
<td>Min_{tet}, N/cm²</td>
<td>2.8 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Max_{tet}, N/cm²</td>
<td>22.8 ± 0.3</td>
<td>21.3 ± 0.5</td>
<td>21.2 ± 0.6</td>
<td>20.8 ± 0.7</td>
</tr>
<tr>
<td>EC_{50}, Hz</td>
<td>116.5 ± 5.5</td>
<td>108.8 ± 5.1</td>
<td>109.9 ± 4.1</td>
<td>109.5 ± 3.1</td>
</tr>
<tr>
<td>N Coefficient</td>
<td>−3.4 ± 0.2</td>
<td>−3.8 ± 0.2</td>
<td>−4.0 ± 0.2</td>
<td>−4.3 ± 0.1</td>
</tr>
<tr>
<td>Caffeine, N/cm²</td>
<td>7.7 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>7.5 ± 0.2</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Tibialis anterior (TA) muscle protein content was determined in whole muscle homogenate. L_{c} is optimal muscle length coinciding with peak twitch force; Minimum and maximum forces measured (meas) and estimated (estim) represent twitch and peak tetanic forces, respectively. EC_{50} is the stimulation frequency at which half of the rise in amplitude of force occurred. The n coefficient describes the slope of the steep portion of the force-frequency curves depicted in Figs. 3 and 8. EDL, extensor digitorum longus muscle. "Different from control; "different from Bout 1 Ecc 0d; "different from Bout 1 Ecc 1d; "different from Bout 1 Ecc 3d; "different from Bout 1 Ecc 1d; "different from Bout 2 Ecc 0d; "different from Bout 2 Ecc 1d (P < 0.05).
in wild-type TA muscle 3 days after the performance of 150 eccentric contractions in vivo (10).

Junctophilin protein content alterations following exercise. JP 1 and JP 2 protein content normalized to tubulin content was not different from unexercised control values immediately or 1 day after performance of 50 concentric contractions in vivo (Fig. 5). In contrast, 0d, 1d, and 3d after performance of 50 eccentric contractions, JP 1 and JP 2 protein content was reduced by \(-50\%\) and \(35\%\), respectively. There were no significant differences among groups for tubulin protein content (\(P = 0.833\)).

Study Two: Repeated Bouts of Eccentric Contractions

In vivo adaptation of torque deficits. In this study, mice performed two bouts of 50 eccentric contractions in vivo separated by 14 days (Fig. 6). Peak torque values during the first eccentric contraction of both bouts were similar, although through the course of the 50 eccentric contractions, eccentric torque was reduced to a greater extent during the first than the second eccentric bout (\(-36\%\) vs. \(-25\%\)).

Isometric torque was measured as a function of stimulation frequency immediately before and after each eccentric bout, as well as 3 days after the second eccentric bout (Fig. 7 and Table 3). In response to the first eccentric bout, isometric torque parameters were immediately altered in a similar fashion as reported above for study one (Table 3). Fourteen days after the initial eccentric bout, peak tetanic torque was still reduced by \(-10\%\), while twitch torque and all other isometric torque parameters had returned to preinjury levels. In comparison to these 14-day postinjury values, immediately after the second eccentric injury bout, all isometric torque parameters were altered similarly to that observed after the initial eccentric contraction bout (Table 3). Twitch and peak tetanic isometric torque values measured immediately after the first and second eccentric contraction bouts were similar, indicating that immediate
strength deficits exhibited little adaptation. Three days after the second injury bout, twitch and peak tetric isometric torque was recovered to values observed before the initial eccentric contraction bout. This is in stark contrast to twitch and peak tetric isometric torque values that were still reduced by 47% and 20%, respectively, 3 days after a single eccentric contraction bout in study one.

*In vitro adaptation of force deficits.* Fourteen days after the initial eccentric contraction bout, EDL muscle twitch and peak tetric isometric force and caffeine contracture force were similar to unexercised control values (Fig. 8 and Table 2). Immediately after the second eccentric contraction bout, twitch and peak tetric isometric force and caffeine contracture force were reduced in comparison to control and 14-day postinjury values. The immediate reductions in peak tetric isometric force were similar after the initial and second bout of eccentric contractions (bout 1 vs. 2; ~45% vs. ~37%), although there was less of a reduction in twitch (bout 1 vs. 2; ~60% vs. ~40%) and caffeine contracture force after the second than the initial eccentric bout (Table 2). Approximately 56% of the strength deficit immediately after the second injury bout is explained by EC uncoupling, as estimated by the comparison of caffeine contracture and peak tetric force deficits. Three days after the second eccentric contraction bout, peak twitch and tetric isometric forces and caffeine contracture force were recovered to unexercised control values.

Junctophilin protein content alterations following two bouts of exercise. Fourteen days after the initial injury bout, JP1 and JP2 protein contents were recovered to control levels (Fig. 5). Immediately after the second bout of 50 eccentric contractions, JP1, but not JP2, protein content was reduced compared with control values. Three days after the second injury bout, JP1 protein content was recovered to control levels.

**Study Three: Temperature Dependence of Muscle Injury**

*In vitro force.* There were no significant differences among isometric or eccentric (15°C and 35°C) groups for average EDL muscle wet weight (10.6 ± 0.2, 10.5 ± 0.2, and 11.1 ± 0.3 mg, respectively), EDL muscle protein content (3.6 ± 0.2, 3.6 ± 0.1, and 3.6 ± 0.1 mg, respectively), and Lo (1.53 ± 0.02, 1.52 ± 0.01, and 1.55 ± 0.02 cm, respectively). As such, initial twitch and peak tetric specific forces (i.e., “Pre” values) were similar among groups (Fig. 9). Peak tetric force produced during the first eccentric contraction was similar between 15°C and 35°C groups (Fig. 10). From the first to the tenth eccentric contraction, there was a similar significant decrease in eccentric force at 15°C and 35°C. In contrast, submaximal isometric force was not significantly reduced from the beginning to the end of the isometric contraction protocol (Fig. 10). Following the 10 isometric or eccentric contractions, twitch and peak tetric forces produced at 35°C were similar between the 35°C isometric and 15°C eccentric groups and, in comparison, were significantly reduced for the 35°C eccentric group (Fig. 9). Despite the 35°C eccentric contraction group exhibiting significant reductions in electrically stimulated force values, caffeine contracture force was similar among 35°C isometric, 15°C eccentric, and 35°C eccentric groups (6.3 ± 0.1, 6.1 ± 0.1, and 6.1 ± 0.1 N/cm², respectively).

**Junctophilin protein content.** The performance of 10 isometric contractions at 35°C or eccentric contractions at 15°C did not result in a significant reduction in JP1 and JP2 protein content in comparison to unexercised control values (Fig. 11). However, within 15 min after performance of eccentric contractions at 35°C, both JP1 and JP2 protein content was

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**Table 3.** Study two in vivo torque-frequency parameters

<table>
<thead>
<tr>
<th></th>
<th>Eccentric Bout 1</th>
<th>Eccentric Bout 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>0d</td>
</tr>
<tr>
<td></td>
<td>Pre/14d</td>
<td>0d</td>
</tr>
<tr>
<td></td>
<td>3d</td>
<td>3d</td>
</tr>
<tr>
<td>Sample size n</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>32.0 ± 0.5</td>
<td>32.8 ± 0.6</td>
</tr>
<tr>
<td>Min measured, N·mm⁻¹kg⁻¹</td>
<td>22.1 ± 1.1</td>
<td>20.6 ± 0.9</td>
</tr>
<tr>
<td>Max measured, N·mm⁻¹kg⁻¹</td>
<td>114.4 ± 2.5</td>
<td>103.3 ± 3.2*</td>
</tr>
<tr>
<td>Min estimated, N·mm⁻¹kg⁻¹</td>
<td>21.5 ± 1.0</td>
<td>20.0 ± 0.9</td>
</tr>
<tr>
<td>Max estimated, N·mm⁻¹kg⁻¹</td>
<td>112.6 ± 2.5</td>
<td>101.6 ± 3.2*</td>
</tr>
<tr>
<td>EC₅₀, Hz</td>
<td>91.7 ± 1.8</td>
<td>88.7 ± 1.4</td>
</tr>
<tr>
<td>n Coefficient</td>
<td>-5.3 ± 0.2</td>
<td>-5.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Minimum and maximum torques measured and estimated represent twitch and peak tetric torques, respectively. EC₅₀ is the stimulation frequency at which half of the rise in amplitude of torque occurred. The n coefficient describes the slope of the steep portion of the torque-frequency curves depicted in Fig. 7. *Different from Ecc Bout 1 Pre; †different from Ecc Bout 1 0d; ‡different from Ecc Bout 2 Pre/14d; §different from Ecc Bout 2 0d (P < 0.05).
Fig. 9. In vitro isometric specific twitch (A) and maximal tetanic (B) force before and after 10 isometric (Iso) or eccentric contractions performed at 35°C or 15°C. Values (means ± SE) with different letters are significantly different (P < 0.05).

Fig. 10. In vitro specific isometric or eccentric force produced during a 10-contraction injury (eccentric) or control (isometric) protocol at 35° or 15°C. Values are means ± SE.

Reduced by ~25% in comparison to unexercised control, 35°C isometric, and 15°C eccentric group values.

Regression analysis of JP content and force deficits. JP content of all TA muscles from studies one and two and EDL muscles from study three that had a corresponding pre- and postexercise functional measurement was included in a single regression analysis (Fig. 12). On the basis of this analysis, JP content and force-producing capacity are significantly associated (Fig. 12).

Study Four: Contribution of Stretch-Activated Channels and SOCE to Force Deficits

Administration of gadolinium or streptomycin did not alter twitch or peak tetanic isometric force before the performance of eccentric contractions in vitro. For control (i.e., Krebs-only), streptomycin, and gadolinium groups, preinjury isometric twitch forces were 3.0 ± 0.3, 3.4 ± 0.5, and 3.0 ± 0.5 N/cm², and peak tetanic forces were 21.0 ± 0.9, 21.3 ± 0.6, and 22.5 ± 1.5 N/cm², respectively. Moreover, these pharmacological agents neither attenuated nor enhanced isometric force deficits or caffeine contracture force following eccentric contractions. Following the eccentric contractions, control, streptomycin, and gadolinium group twitch forces were 1.4 ± 0.6, 1.4 ± 0.9, 1.6 ± 0.1 N/cm²; peak tetanic forces were 13.0 ± 0.3, 12.0 ± 0.9, 13.6 ± 0.5 N/cm²; and caffeine contracture forces were 5.0 ± 0.4, 5.1 ± 0.3, and 5.2 ± 0.4 N/cm², respectively. The injury stimulus was similar among groups, because peak eccentric force throughout the 10 eccentric contractions was similar for control, streptomycin, and gadolinium groups (e.g., first eccentric contraction; 40.3 ± 1.8, 38.3 ± 1.6, and 41.7 ± 2.1 N/cm², respectively).

DISCUSSION

Skeletal muscle can be injured when the contractile stress and/or strain exceeds what the muscle normally accommodates. As seen in this study and numerous others, the functional consequence of this injury is immediate and prolonged impairment in force-producing capacity (Figs. 1–3). Force-producing and transmitting structures are known to fail with eccentric contractions and contribute to the strength deficits (7, 29, 60). However, with our mouse injury model, we have documented that the majority of strength deficits early after injury (0–5 days) stems from failure with EC coupling, specifically with the interaction between Cav1.1 and RyR1 (21). The primary findings of this study are that JP1 and JP2, proteins thought to localize triadic membranes, are damaged after a small number of eccentric contractions, and that the loss of JP1 and JP2 is associated with EC uncoupling and strength deficits.

The magnitude and time course of JP1 and JP2 damage following injury were comparable with force deficits and EC coupling failure. In this study, through the first 3 days after a single bout of eccentric contractions, peak tetanic force was reduced by ~30% to 45% and JP1 and JP2 content was reduced by a similar 33% to 55%. Additionally, in two separate studies that also observed an ~45% isometric tetanic force loss after eccentric contractions, electrically stimulated SR Ca²⁺ release was shown to be reduced by 25% to 40% immediately and 3 days after the injury (5, 21). Thus, during the first 3 days after injury induction, a 40% to 50% loss of JPs appears to result in a slightly lower relative disruption to EC coupling, which is in agreement with the ~50% reduction in electrically stimulated Ca²⁺ release reported after JP1 and JP2 have been knocked down via short hairpin RNA in adult skeletal muscle to ~60% of control values (18). Furthermore, JP1 and JP2 damage during the in vitro injury protocol used in this study induced a much smaller force deficit than observed after in vivo injury (i.e., only a ~15% force deficit), but in concert with this smaller deficit only ~25% of JP1 and JP2 were damaged. Although JPs exhibit a close circumstantial association with
eccentric contraction-induced force deficits and EC coupling failure, regression analysis of JP content and force-producing capacity indicated a significant but modest statistical relationship ($R = 0.486$, $P = 0.001$) (Fig. 12).

It is remarkable that JP1 exhibits only a partial protection from damage immediately after a repeated bout of eccentric contractions, while JP2 appears to be fully protected from damage after a second bout of eccentric contractions. We are not aware of another protein that has been implicated in contributing to early force deficits that does not exhibit significant protection from damage immediately after repeated bouts of eccentric contractions. For example, it has been shown that the cytoskeletal and structural proteins desmin, dystrophin, and titin all exhibit an immediate protective repeated bout effect (29). Immediate force deficits can be only minimally attenuated following repeated bouts of eccentric contractions in humans (9, 40) and in animals (10, 11, 23) because EC coupling failure, in line with serving as a protective mechanism, is not significantly attenuated after injury (23). That JP1 content is significantly reduced after a second injury bout, but then recovers at an enhanced rate, is telling in its role in EC coupling failure and strength deficits after injury.

The obvious means by which JP1 and JP2 damage can reduce force production is through disruption of EC coupling. Cav1.1 physically couples to RyR1 for voltage-induced calcium release to occur in skeletal muscle (6, 8, 15). Either knockdown or genetic ablation of JP1 and JP2 results in triad deformation, EC coupling disruption, and weakness (18, 24, 26). Triad disruption has been observed after eccentric contractions (45), supporting the notion that the JP damage after injury that we have demonstrated in this study may contribute to the dissociation of Cav1.1 and RyR1 and thus EC coupling failure. Alternatively, JP1 and JP2 damage may reduce SR $Ca^{2+}$ release and force output by disrupting SOCE and lowering SR $Ca^{2+}$ levels (13, 14, 18). Although we observe reduced caffeine-induced force output after injury, which may reflect lower SR $Ca^{2+}$ content, pharmacological blockade of SOCE with 20 $\mu$M gadolinium did not affect voltage-gated force or caffeine contracture force after eccentric contraction-induced muscle injury. If JP damage had disrupted SOCE and SR $Ca^{2+}$ content, then blocking store-operated $Ca^{2+}$ channels should have exacerbated force deficits after injury. Therefore, SOCE failure does not appear to contribute to strength deficits immediately after injury in our mouse model.

It is likely that JPs are lost during the performance of eccentric contractions by two possible mechanisms: triad strain and/or proteolysis. Following both in vivo and in vitro eccentric contraction-induced muscle injury, intracellular $Ca^{2+}$ con-

Fig. 11. Temperature dependence of EDL muscle JP1 and JP2 protein loss after performing eccentric contractions in vitro. Normalized JP1 (B) and JP2 (C) content and tubulin (D) content were determined via Western blot (A) in unexercised EDL muscle (C or control) and in muscles that performed 10 isometric contractions at 35°C (I-35) or 10 eccentric contractions at 15° or 35°C (E-15 or E-35, respectively). Values are means ± SE. *Significantly different from control values ($P < 0.05$).
centration increases (5, 21, 34, 43, 51, 59), presumably as a result of activation of stretch-activated channels (SACs) (43, 59). It has been demonstrated in mechanically skinned and intact muscle fibers that elevated intracellular Ca\(^{2+}\) can disrupt depolarization-induced Ca\(^{2+}\) release and may result in triad deformation (28, 59). Recently, pharmacological calpain inhibition has been shown to attenuate EC coupling failure in mechanically skinned muscle fibers with an elevated cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)](i)) (48, 49) and to attenuate a secondary, but not the immediate, deficit in force after injury in isolated EDL muscle (60). Therefore, on the basis of these studies it is possible that Ca\(^{2+}\) entry through SACs during eccentric contractions triggers a calpain-mediated cleavage of JPs. However, in the current study, blocking SACs did not affect voltage-induced force and caffeine contracture force immediately after injury induction. Moreover, our lab group has previously shown that neither calpain inhibition with leupeptin (51) nor extracellular Ca\(^{2+}\) concentration manipulation (32) influences force loss associated with eccentric contraction-induced muscle injury. If elevations of intracellular [Ca\(^{2+}\)](i) (21, 34) and subsequent calpain activation were causing JP damage, then one would expect to see a progressive decline in JP protein content, as is suggested by the trend of force-bearing protein degeneration by calpain during the first hour after injury (60). However, neither JP content nor force decreased during the days after injury in the current study (Figs. 1, 3, and 5). Although we have evidence that force loss immediately after eccentric contractions stems in part from some undefined enzymatic process (51), our data do not implicate calpain as a primary cause of force deficits in our injury model.

JPs may also be disrupted from triad strain resulting from osmotic stress following eccentric contractions. Intracellular Na\(^{+}\) concentrations can increase following eccentric contractions through the activation of SACs (36, 57). Elevations in intracellular Na\(^{+}\) may result in osmotic stress across the T-tubule membrane (1), resulting in vacuole formation (58) and spontaneous SR Ca\(^{2+}\) release events, which are indicative of dissociation of Cav1.1 and RyR1 (4, 31). Interestingly, osmotic stress has been shown to disrupt MORN motif binding to the plasmalemma in plant cells (19), lending support that T-tubule membrane osmotic stress may disturb JP1 and JP2 MORN motif binding after eccentric contractions. However, the fact that we did not observe an attenuation in force deficits with SAC blockade, and presumably blockade of prolonged elevations of intracellular [Na\(^{+}\)](i) (36), suggests that osmotic stress imparted by ion entry through SACs is not involved in the immediate loss of JP1 and JP2.

Alternatively, JP1 and JP2 may be damaged by mechanical stress and/or strain imposed on the tissue by the eccentric contractions themselves (37, 50). Triad deformation has been observed following the performance of eccentric contractions in vivo (45), raising the possibility that JP damage is a mechanically induced event. Additionally, we have shown previously that the temperature dependency of force loss is not entirely explained by enzymatic processes, but may be related to a more readily compromised plasmalemma during eccentric contractions at physiological temperatures (51). Because membranes are known to undergo a phase transition as temperature increases from 20\(^\circ\) to 40\(^\circ\)C (25), JP MORN motif binding to the T-tubule may be more readily mechanically disrupted during eccentric contractions at physiological temperatures. In light of the differential response between JP1 and JP2 following the repeated bout of eccentric contractions, multiple mechanisms of JP1 and JP2 damage may be involved, including mechanical-, proteolytic-, and even posttranslational (e.g., S-nitrosylation) (17, 41) modification-mediated mechanisms.

Given the complexity of Ca\(^{2+}\) handling in skeletal muscle fibers, it is likely that other triadic proteins may also be damaged during eccentric contractions and may therefore contribute to EC coupling failure and strength deficits in injured muscle (55). For example, JP-45 locates to the triad, binds to Cav1.1 in the T-tubule membrane and calsequestrin in the SR, appears to play a role in targeting Cav1.1 to the triad, and is required for normal EC coupling (2, 3, 12). On the basis of these characteristics, it is interesting to postulate that junctophilin and JP-45 damage consequent of performing eccentric contractions results in dissociation of Cav1.1 from RyR1 and then migration of Cav1.1 from the triad. In partial support, we have previously observed that Cav1.1 muscle content is increased by ~20% within an hour after eccentric contraction-induced injury (22), suggesting that Cav1.1 content may be increased in response to this delocalization. Future study should be directed at assessing the contribution of other triadic proteins, especially those that cross the T-tubule membrane gap like JP-45, to EC coupling failure in injured skeletal muscle.

In the current series of studies, we have demonstrated that junctophilins are damaged by the performance of maximal and submaximal eccentric contractions, but not nonfatiguing concentric or isometric contractions, at physiological temperatures. Additionally, we have provided circumstantial evidence indicating that JP1 and potentially JP2 damage is involved in early strength deficits and EC coupling failure. This was shown by first demonstrating that JP1 is damaged to a similar degree following an initial and repeated bout of eccentric contractions, and then by demonstrating that JP damage, force reductions, and EC coupling failure are similarly temperature dependent. Lastly, a combined regression analysis for all three studies of JP1 and JP2 content to muscle force producing capacity indicated that reductions in force-producing capacity after injury are significantly associated with JP content (Fig. 12). While we have not determined the role of other triadic proteins such as JP-45 (12, 55), the results of the current study offer circumstantial support that JP damage is involved in early force deficits due to EC coupling failure following the performance of eccentric contractions.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


