

Stretch-Activated Ion Channel Blockade Attenuates Adaptations to Eccentric Exercise

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ABSTRACT

BUTTERFIELD, T. A., and T. M. BEST. Stretch-Activated Ion Channel Blockade Attenuates Adaptations to Eccentric Exercise. *Med. Sci. Sports Exerc.*, Vol. 41, No. 2, pp. 351–356, 2009. **Purpose:** The purpose of this study was to test the hypothesis that stretch-activated ion channel (SAC) function is essential for the repeated bout effect (RBE) in skeletal muscle. Specifically, we investigated if daily injections of streptomycin (a known SAC blocker) would abrogate the muscle's adaptive resistance to the damaging effects of eccentric exercise over a 4-wk period. Furthermore, we hypothesized that the lack of an RBE would be due to the lack of functional adaptations that typically result from repeated bouts of eccentric exercise, including increased peak isometric torque, muscle hypertrophy, and rightward shift of the torque–angle relationship. **Methods:** Twelve New Zealand white rabbits were each subjected to 12 bouts of eccentric exercise over a 4-wk period while receiving either daily injections of streptomycin or sham injections. **Results:** Although blocking the SAC function completely eliminated the expected adaptive response in biomechanical parameters during the exercise regimen, there remained evidence of an acquired RBE, albeit with an attenuated response when compared with the muscles with intact SAC function. **Conclusion:** Blocking sarcolemmal SAC eliminates functional adaptations of muscle after eccentric exercise. In the absence of SAC function, muscles subjected to chronic eccentric exercise still exhibit some degree of the RBE. As such, it appears that the signaling cascade that results in functional, biomechanical adaptations associated with the RBE during eccentric exercise is dependent upon intact SAC function. **Key Words:** REPEATED BOUT EFFECT, TORQUE–JOINT ANGLE RELATIONSHIP, STRETCH-ACTIVATED CHANNELS, SKELETAL MUSCLE

The recognition of stretch-activated ion channels (SAC) and the ongoing discovery of downstream signaling pathways in skeletal muscle have led to novel hypotheses regarding myocyte homeostasis (22). Specifically, it is becoming apparent that the role of ionic changes and cell signaling during exercise may play a synergistic role in cell damage (39,40) and adaptation (25). Although classic thinking would dictate that mechanical disruption of the sarcolemma is the precipitating event in exercise-induced muscle damage and repair (3), recent histological evidence has shown that membrane disruption is minimal immediately after exercise *in vivo* (5). This observation supports novel hypotheses incorporating the role of inflammation (36) and ionic changes (40) in the latent progression of membrane disruption after repeated loading. Recently, blocking SAC function has been shown to result

in a smaller force deficit after eccentric exercise and an attenuation of membrane disruption in healthy (39) and dystrophic muscle (41).

Although the relationships between SAC, cell damage, and inflammation have yet to be firmly established, reductions in fiber damage have also been observed by reducing both leukocyte infiltration (37) and their activation (7) before eccentric muscle stretch. Interestingly, noninjurious passive stretch of skeletal muscle with intact SAC function results in a neutrophil diapedesis (38) and an influx of extracellular calcium (4). Similarly, inhibition of extracellular calcium influx through blocking of the SAC has been shown to reduce leukocyte infiltration in smooth muscle (1). Ironically, both calcium and leukocytes each have dichotomous functions. For instance, high intracellular calcium concentrations can be destructive to the fiber membrane (18), whereas transient extracellular calcium influx has been shown to promote a mitogenic response (12). Similarly, leukocyte depletion results in both a reduction in cell damage (7) and an attenuated repair response (37).

Although the role of SAC in exercise-induced muscle damage is just beginning to be elucidated, their potential role in exercise-specific adaptations has yet to be investigated. There is evidence that these channels may play a vital role in mechanotransduction: the link between mechanical deformation and cell signaling (8). Blocking SAC function has resulted in attenuated activation of signaling cascades

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such as the mitogen-activated protein kinase (MAPK) and the mammalian target of rapamycin (mTOR) pathways (19,25), both known to influence hypertrophic growth in skeletal muscle. Recently, Butterfield and Herzog (11) showed that long-term eccentric exercise *in vivo* resulted in serial sarcomere number adaptations that were best predicted by fiber strain. This cellular adaptation has been proposed to account for the repeated bout effect (RBE) or the acquired resistance to the damaging effects of eccentric exercise with subsequent bouts (31). Therefore, the purpose of this study was to test the hypothesis that SAC function is essential for the RBE. Specifically, we tested the hypothesis that 4 wk of eccentric exercise training without SAC function would block the muscle's adaptive resistance to the damaging effects of eccentric exercise. Furthermore, we hypothesized that the lack of an RBE would be due to the lack of functional adaptations that typically result from repeated bouts of eccentric exercise, including a rightward shift in the torque–joint angle relationship, muscle hypertrophy, and an increase in peak isometric torque.

METHODS

Surgical implantation and SAC blocker administration. Skeletally mature New Zealand white rabbits ($n = 12$) were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and were housed (singly) in the University Laboratory Animal Resources Facility. All experimental procedures were approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University and were conducted in adherence to the policy statement of the American College of Sports Medicine regarding research with experimental animals. On the day of surgery, custom-made nerve cuff electrodes (20) were secured around the left and the right peroneal nerves and were connected to homemade interfaces as previously described (11). Beginning on the day of surgery, rabbits were divided into two groups: a streptomycin treatment group (strep⁺, $n = 6$) and an injection control group (strep⁻, $n = 6$). The strep⁺ group received intramuscular injections of streptomycin (reconstituted to 800 mg·mL⁻¹; Sigma-Aldrich, St. Louis, MO) per day at 300 mg·kg⁻¹ body weight (39), and the strep⁻ group received a needle stick (27 gauge) only. Injection sites were changed daily, alternating between the left and the right paraspinal muscles and gluteals. For this *in vivo* study, we chose to use streptomycin to block SAC function. It has lower toxicity compared with alternative compounds, is an effective blocker of the SAC, and has a low dissociation rate constant from the channels of interest (26). All injections were administered in three equal volumes each day and continued for the duration of the experimental protocol (40 d) until tissue harvest.

Rabbit preparation and exercise, day 1. All exercise protocols commenced within 7 d after surgery. On day 1 of the exercise protocol, rabbits were tranquilized with a

subcutaneous injection of 0.18 mL (10 mg·mL⁻¹) acepromazine (Vedco Inc., St Joseph, MO) and were anesthetized using isoflurane gas (induction with 5% and held under using 1.5% isoflurane, 1.0 L·min⁻¹ O₂). External connections were made to the peroneal nerves using silicone-insulated 10 strand cables (Crooner Wire Company, Chatsworth, CA) threaded through short beveled 22-gauge needles (Becton–Dickinson and Company, Franklin Lakes, NJ). The ends of the wires were bared and barbed, and the needles were inserted through the skin of the rabbit into the two wells of each interface. The needles were then removed from the interfaces, leaving the wires connected (11). Rabbits were placed supine in a heated, canvas sling, and using a noninvasive stereotaxic frame, the knee joint was held at 90°. The foot was strapped to a servomotor footplate (Parker Hannifin Corporation, Irwin, PA), and ankle movement was controlled via Motion Planner[®] software (Compumotor, Rohnert Park, CA). The medial condyle of the tibia and the medial malleolus of the ankle were marked with ink, and using a small plastic goniometer, the tibiotarsal joint angle was set to 90° (increased tibiotarsal joint angle = increased plantarflexion). This 90° tibiotarsal joint configuration was encoded to the motor controller and served as the reference angle for the remainder of the experiment. The corresponding nerve cuff was then connected to the stimulator, and the α -motoneuron threshold was determined (pulse duration = 0.1 ms, frequency = 150 Hz, train duration = 500 ms).

Immediately preceding the first exercise bout, the first isometric torque–joint angle relationship ($T-\theta_1$) was determined by supramaximally stimulating ($3 \times \alpha$ -motoneuron threshold voltage, pulse duration = 0.1 ms, frequency = 150 Hz, train duration = 1000 ms) the dorsiflexor muscles, beginning at a tibiotarsal angle of 55° and progressing in 5° increments to 155° (i.e., 21 measurements). The foot was returned to a dorsiflexed position (55° tibiotarsal angle) for 2 min of rest between contractions. Once the preexercise torque–joint angle relationship was obtained, eccentric contractions were performed from a tibiotarsal angle of 95° to 145° of plantarflexion at 100°·s⁻¹ for the eccentric exercise groups, with the activation preceding the muscle-tendon unit stretch by 100 ms. This configuration and range of motion and timing of activation result in a reproducible magnitude of fiber strain, muscle damage, and adaptation (9). The duration of each eccentric stretch was 500 ms, followed immediately by a 500-ms passive return to the starting joint angle and the immediate preactivation and subsequent cycle. This active lengthening, passive shortening cycle was repeated with no rest between cycles for ten repetitions, followed by 2 min of rest. The complete exercise regimen consisted of five sets of ten cycles. After exercise on day 1, the second postexercise torque–joint angle relationship ($T-\theta_2$) was obtained to assess the magnitude of muscle damage or alterations in functional mechanical measures after the first exercise bout ($[T-\theta_2] - [T-\theta_1]$). To assess any long-term adaptations due to growth, illness, or drug effects, baseline

mechanical measures of the contralateral control limb were also obtained on day 1. Subsequently, the contralateral limb served as a control for the remainder of the experiment.

Subsequent and final exercise bouts. On subsequent exercise days, methods for administration of tranquilizer and anesthesia were identical with that described above for day 1. The experimental limb of each rabbit was exercised three times per week for 4 wk, with at least 48 h of rest between bouts. Afterward, the rabbits were placed on a heated pad, allowed to recover from the anesthesia, and returned to their cages. One week after the last exercise bout (bout 12), the third torque–joint angle relationship ($T-\theta_3$) was obtained for the 21 tibiotarsal joint angles (55° – 155° in 5° increments) for both the exercised and the control limbs. This method was used to assess the functional adaptation to the 12 bouts of exercise (measured as the change in peak torque and the magnitude of the shift in the torque–joint angle relationship between day 1 and day 12 [$T-\theta_3$] – [$T-\theta_1$]). Immediately after the torque–joint angle evaluation, one more injurious eccentric exercise bout was performed on each hindlimb followed by a fourth and final postexercise torque–joint angle relationship ($T-\theta_4$). This final test was performed to assess the magnitude of damage (reduction in peak isometric torque postexercise when compared with peak isometric torque preexercise) sustained after one last eccentric exercise bout [$T-\theta_4$] – [$T-\theta_3$]. The difference in the reduction in peak isometric torques between day 12 and day 1 was deemed an acquired resistance to exercise-induced damage and was called the RBE.

Statistical analysis. The joint angles for peak isometric torque production were calculated and compared as previously described (10). To measure the differences in mechanical variables between exercised and control limbs, Student's paired *t*-tests were used in both groups. Subsequently, independent sample *t*-tests were used to measure the effects of both chronic exercise on adaptation between the streptomycin- and the sham-injected groups. Student's paired *t*-tests were used for the control hindlimbs as well to measure any differences in unexercised muscles that may be explained by drug treatment in the absence of exercise for the duration of the study. All data are presented as means \pm SD.

RESULTS

Effects of drug administration on muscle function. Over the 6-wk experiment, the change in weights of the strep⁺ rabbits ($-3.2\% \pm 7.0\%$) and strep⁻ rabbits ($-2.6\% \pm 1.1\%$) were not different ($P = 0.178$). In addition, after the first week of injections when no exercise was performed, peak isometric torque production was not different between experimental and control hindlimbs in the strep⁺ group (0.64 ± 0.12 vs 0.60 ± 0.09 N·m, $P = 0.247$) and the strep⁻ group (0.53 ± 0.07 vs 0.48 ± 0.10 N·m, $P = 0.156$). For the control limbs, peak torque produced on the final day of testing (4 wk of rest) was not different when compared with the peak torque produced on day 1 in the strep⁺ ($0.60 \pm$

0.10 vs 0.51 ± 0.22 N·m, $P = 0.087$) and the strep⁻ (0.48 ± 0.10 vs 0.52 ± 0.08 N·m, $P = 0.273$) groups.

Peak torque production. Muscles subjected to 4 wk of eccentric exercise training with intact SAC function (strep⁻ group) showed a marked improvement in peak isometric torque production from 0.53 ± 0.07 N·m on day 1 to 0.71 ± 0.06 N·m on the final day ($P = 0.013$). Conversely, muscles subjected to a 4-wk eccentric exercise regimen with SAC function blocked via streptomycin injections did not show any significant change in peak isometric torque. On day 1, mean peak isometric torque production in the strep⁺ group measured 0.64 ± 0.12 N·m compared with 0.53 ± 0.14 N·m on the final day ($P = 0.832$).

Muscle mass. The mass of the exercised muscles was compared with the unexercised contralateral control muscles to measure the adaptation between the exercised and the unexercised muscles within the same groups. After 4 wk of eccentric exercise, TA mass was increased compared with the unexercised control limbs in the strep⁻ group (4.92 ± 0.32 vs 3.55 ± 0.32 g, $P = 0.001$) as well as the strep⁺ group (4.78 ± 0.22 vs 4.12 ± 0.11 g, $P = 0.021$). However, the increase in TA mass in the strep⁺ group ($12.99\% \pm 8.4\%$) appeared to be an attenuated adaptation, as the increase in TA mass in the strep⁻ group ($28.15\% \pm 11.0\%$) was significantly greater ($P = 0.023$).

Torque–joint angle relationships. The torque–joint angle relationship in the strep⁻ group was shifted to the right after 4 wk of eccentric exercise (Fig. 1). This resulted in maximum peak isometric torque being produced at a longer muscle length (mean shift, $+4.47^\circ \pm 0.61^\circ$). Conversely, the strep⁺ group demonstrated a leftward shift of the torque–joint angle relationship ($-13.89^\circ \pm 5.0^\circ$), whereby peak isometric torque was produced at a shorter muscle length (Fig. 2).

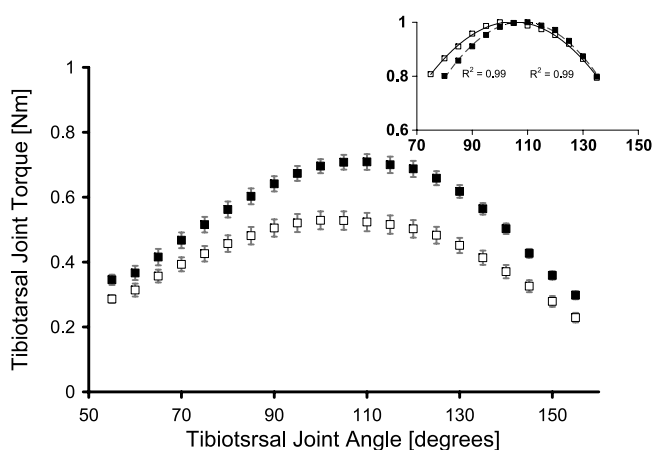


FIGURE 1—Torque–angle relationships depicting muscle function on day 1 (\square) and after 4 wk of eccentric exercise training (\blacksquare) with intact SAC function (strep⁻ group). Note the shift of peak joint torque production to the right after 4 wk of eccentric exercise training (mean shift = $+4.47^\circ \pm 0.61^\circ$), whereby peak isometric torque was produced at a longer muscle length. (Inset panel) Normalized values greater than 75% peak torque for day 1 (\square) and after 4 wk of eccentric exercise training (\blacksquare). Values in each group were fitted with a second order polynomial, depicting the rightward shift in peak joint torque production after exercise.

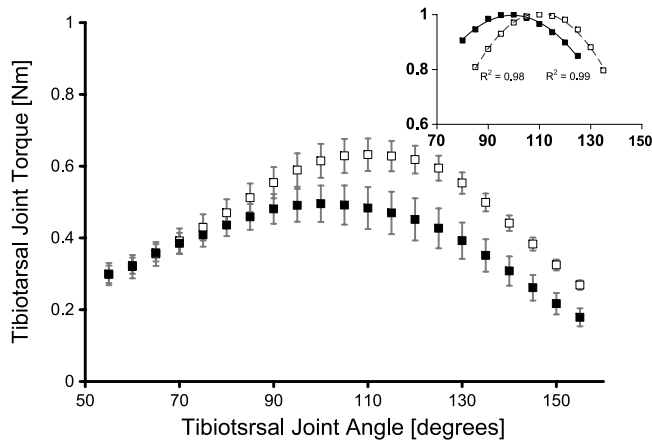


FIGURE 2—Torque–angle relationships depicting muscle function on day 1 (\square) and after 4 wk of eccentric exercise training (\blacksquare) without intact SAC function (strep⁺ group). Note the shift of peak joint torque production to the left after 4 wk of eccentric exercise (mean shift = $-13.89^\circ \pm 5.0^\circ$), whereby peak isometric torque was produced at a shorter muscle length after eccentric exercise training. (Inset panel) Normalized values greater than 75% peak torque for day 1 (\square) and after 4 wk of eccentric exercise training (\blacksquare). Values in each group were fitted with a second order polynomial, depicting the rightward shift in peak joint torque production after exercise.

Measurement of the RBE. As a final measure of adaptation, we measured the reduction in peak isometric torque as an indicator of the trained muscle's resistance to the damaging effects of eccentric exercise. On day 1, the trained limbs of the strep⁻ group sustained a reduction in peak isometric torque of $51.7\% \pm 1.9\%$ compared with preexercise values after eccentric exercise bout number one. On the final day, this value was reduced to $25.6\% \pm 0.8\%$ ($P < 0.001$), illustrating the ability of the muscle to better resist the damaging effects of eccentric exercise. The strep⁺ group sustained similar reductions in peak isometric torque on day 1 ($56.6\% \pm 3.5\%$). However, on the final day, the damage sustained during eccentric exercise in the strep⁺ group was also reduced ($42.8\% \pm 5.3\%$, $P = 0.026$) albeit with an attenuated adaptive response compared with the strep⁻ group ($P = 0.01$).

DISCUSSION

The purpose of this study was to test the hypothesis that intact SAC function is essential for adaptations specific to eccentric exercise associated with the RBE. One limitation to this study was that direct assessment of pharmacological intervention and SAC function was beyond the scope of this study. Therefore, we operated under the assumption that our daily injections effectively blocked the SAC function in experimental animals for the duration of this study. Because this was a long-term study, we used streptomycin for several reasons: 1) due to its lower dissociation constant for SAC compared with other channel blockers; 2) due to its lower toxicity; and 3) it has been used as an effective blocker of SAC function at the dosage applied in our study (26,39). Although streptomycin is less toxic compared with other

channel blockers, it was possible that the measurable effects could have been due to the pharmacological side effects of the drug. Therefore, we compared the control, unexercised limb function to assess any differences in function that could be due to toxicity. As we did not observe any alterations in muscle mechanical function between the control limbs of the injected and the sham-injected animals, we are confident that the differences observed in the two exercised groups are due to the differences in SAC function and the muscle's response to chronic exercise.

Here we have shown the relationship between functional adaptations and RBE to be disproportionate when the SAC were blocked with streptomycin during chronic exercise. A rightward shift in the force–length relationship (or the torque–joint angle relationship) and an increase in force production have long been associated with the RBE, and both have been proposed to reduce the susceptibility of the muscle to damage during exercise (27,28,31). These mechanical adaptations have been proposed to occur through an increase in sarcomere number in series (sarcomerogenesis) and in parallel (myofibrillogenesis), respectively, after long-term eccentric exercise (11,34). By blocking SAC function, the beneficial adaptations in the torque–joint angle relationships and the peak torque were completely abrogated when compared with the sham-injected and exercised muscles. Conversely, the RBE was merely attenuated, as the SAC-blocked muscles still retained the ability to acquire some degree of resistance to the damaging effects of eccentric exercise when compared with unexercised, contralateral control muscles. This finding would suggest to us that intact SAC function influences the rightward shift in the torque–joint angle relationship and the increase in torque production in response to eccentric exercise. However, this does not appear to be the sole pathway for the mechanism of acquired resistance to exercise-induced damage.

Intracellular concentrations of desmin are rapidly increased after eccentric exercise and may serve to protect the subcellular elements from further damage during contraction (29). Similarly, Lapier et al. (23) have shown that intramuscular concentrations of collagen are greater after 3 wk of static stretch and that this adaptation contributed to resistance to the damaging effects of eccentric exercise. Here, we reported a small but significant acquired resistance to eccentric exercise-induced damage in the SAC-blocked group in spite of a leftward shift in the torque–joint angle relationship and no change in peak isometric torque production. This observation, in conjunction with the finding that these muscles exhibited a greater wet weight after the exercise regimen, suggests to us that the RBE in this group may be attributed to an increase in noncontractile proteins. This is supported by the findings of McBride (25), who reported an increase in noncontractile protein in rat skeletal muscle subjected to exercise while blocking SAC function. Overall, this demonstrates that the RBE can be acquired during exercise without adaptations in force production and adds support for alternative mechanisms for its occurrence.

SAC are ubiquitously expressed in the sarcolemma of skeletal muscle and influence intracellular calcium concentrations during modified muscle use. Duan et al. (16) were the first to show that increased intracellular calcium levels after downhill running in rats were due to a calcium influx from a source other than the sarcoplasmic reticulum. Intracellular calcium levels can be elevated within 10 min after the onset of eccentric exercise (2). Recent evidence suggests that calcium influx through activation-dependent (L-type) channels or SAC may play a greater role in membrane disruption compared with mechanical factors during exercise (39,41). Blocking L-type channels and SAC function results in a significant reduction in cell damage, albeit through apparently different mechanisms. For instance, inhibiting the influx of extracellular calcium into the muscle cell through SAC during eccentric exercise attenuates membrane disruption (41) and may abrogate inflammation (39). This finding is in agreement with a proposed mechanism whereby inflammatory cell accumulation in exercised muscle is mediated through a calcium dependent pathway (32).

Although we are uncertain of the mechanisms involved, it is possible that calcium influx into the myocyte through SAC is essential for muscle injury and repair during and after exercise. Lengthening contractions within a physiologic range result in the progressive accumulation of subcellular disruption to the contractile apparatus or cytoskeleton (17). The repair of contractile proteins is essential to recovery of muscle function, and the addition of contractile proteins is essential for the functional adaptations observed in the control muscles: increased torque at a greater muscle length. Extracellular calcium influx into the cell has been shown to result in hypertrophic growth in developing myotubes through activation of the calcineurin pathway (15). Recent evidence suggests that SAC function is essential to the activation of mitogen-activated protein kinases (MAPK) within skeletal muscle during muscle loading (25), and this pathway is a regulator of protein synthesis in skeletal muscle. In addition, mRNA levels of the downstream transcription factor *c-fos* have been shown to correlate with stretch magnitude in protocols designed to induce myofibrillogenesis and sarcomerogenesis (14). Recently, by blocking SAC function, McBride (25) showed a reduction in the expression of *c-fos* during and after eccentric exercise and attenuated muscle hypertrophy when compared with controls with functioning SAC. Subsequently, additional work from the same laboratory has revealed a link between the SAC function and the mammalian target of rapamycin (mTOR) pathway, a key regulatory pathway of protein synthesis in skeletal muscle (33).

Blockade of L-type calcium channels has been shown to reduce intracellular calcium concentration by reducing cal-

cium influx from the T tubules (5). Interestingly, this approach does not affect leukocyte infiltration (6), illustrating that the source of calcium influx to the cell may influence the extent of inflammation observed during exercise. It is notable that nonsarcoplasmic reticulum calcium is required for transcriptional-dependent gene expression (12) and that passive stretch also results in an influx of calcium from the extracellular space (4), subsequent inflammatory cell infiltration (30), and the RBE (21) in skeletal muscle. Muscle use through small excursions results in neutrophil recruitment and diapedesis to the muscle ECM in the absence of cell damage (30). Altering or increasing the magnitude of the stimuli (through altered activation or loading) appears to result in greater neutrophil infiltration to stretched muscle fibers (30). There are several possibilities for upstream regulators of neutrophil diapedesis in the absence of membrane disruption, including the expression and release of cytokines that may be related to membrane SAC (13). Although it has been accepted that neutrophils function in the repair processes of skeletal muscle (35), it has recently been shown that neutrophils are necessary for the RBE after eccentric exercise (24). It is therefore possible that blocking SAC function inhibits neutrophilia during exercise, and investigations into this potential mechanism are currently underway in our laboratory.

In summary, functional adaptations such as the rightward shift in the force-length relationship and the increased torque production have been proposed to account for the RBE after long-term exercise in skeletal muscle, although the exact mechanisms remain unknown. Herein, we have demonstrated that muscles subjected to eccentric exercise in the presence of a SAC blocker do not acquire these adaptations. However, in the absence of these biomechanical adaptations and SAC function, muscles subjected to chronic eccentric exercise still exhibit some degree of the RBE. This acquired resistance may be attributed to an increase in intermediate filament or collagen concentrations. As such, it appears that the signaling cascade that results in the rightward shift of the FLR and increased peak isometric torque during eccentric exercise is dependent upon intact SAC function. However, this pathway is likely not the sole mechanism for skeletal muscle to acquire the RBE. Further work is ongoing in our laboratory to uncover the relationships between SAC function and cell signaling.

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