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Adenosine A_3 receptor stimulation induces protection of skeletal muscle from eccentric exercise-mediated injury

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[1]*Pat and Jim Calhoun Cardiology Center, University of Connecticut Health Center, Farmington, Connecticut;* [2]*U.S. Army Research Institute of Environmental Medicine, Military Performance Division, Natick, Massachusetts; and* [3]*Howard Hughes Medical Institute, Departments of Molecular Physiology and Biophysics, Neurology, and Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, Iowa*

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Wang R, Urso ML, Zambraski EJ, Rader EP, Campbell KP, Liang BT. Adenosine A_3 receptor stimulation induces protection of skeletal muscle from eccentric exercise-mediated injury. *Am J Physiol Regul Integr Comp Physiol* 299: R259–R267, 2010. First published April 28, 2010; doi:10.1152/ajpregu.00060.2010.—Effective therapy to reduce skeletal muscle injury associated with severe or eccentric exercise is needed. The purpose of this study was to determine whether adenosine receptor stimulation can mediate protection from eccentric exercise-induced muscle injury. Downhill treadmill exercise (−15°) was used to induce eccentric exercise-mediated skeletal muscle injury. Experiments were conducted in both normal wild-type (WT) mice and also in β-sarcoglycan knockout dystrophic mice, animals that show an exaggerated muscle damage with the stress of exercise. In the vehicle-treated WT animals, eccentric exercise increased serum creatine kinase (CK) greater than 3-fold to 358.9 ± 62.7 U/l (SE). This increase was totally abolished by stimulation of the A_3 receptor. In the dystrophic β-sarcoglycan-null mice, eccentric exercise caused CK levels to reach 55,124 ± 5,558 U/l. A_3 receptor stimulation in these animals reduced the CK response by nearly 50%. In the dystrophic mice at rest, 10% of the fibers were found to be damaged, as indicated by Evans blue dye staining. While this percentage was doubled after exercise, A_3 receptor stimulation eliminated this increase. Neither the A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (0.05 mg/kg) nor the A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (0.07 mg/kg) protected skeletal muscle from eccentric exercise injury in WT or dystrophic mice. The protective effect of adenosine A_3 receptor stimulation was absent in mice, in which genes for phospholipase C β2/β3 (PLCβ2/β3) and β-sarcoglycan were deleted. The present study elucidates a new protective role of the A_3 receptor and PLCβ2/β3 and points to a potentially effective therapeutic strategy for eccentric exercise-induced skeletal muscle injury.

β-sarcoglycan; muscle force; creatine kinase; inflammation

SKELETAL MUSCLE IS SUSCEPTIBLE to various forms of injury, including ischemia, trauma, and physical exertion (6, 8, 12, 15, 36, 50, 56). Skeletal muscle is one of the most vulnerable tissue in the extremities (8, 24). Developing new methods designed to provide cytoprotection to the skeletal muscle is thus important. Previous studies have demonstrated a potent cytoprotective role of adenosine A_1, A_{2A}, and A_3 receptors in ischemia and reperfusion injury of the skeletal muscle (61). Although all three adenosine receptors could induce protection from ischemia/reperfusion injury in skeletal muscle, the signaling mechanism by which each receptor mediates protection is not the same. The adenosine A_3 receptor, but not the A_1 or A_{2A} receptor subtype, signals selectively via phospholipase Cβ isoform to achieve its anti-ischemic effect. While adenosine and its various receptors are well known to be able to protect cardiac and skeletal muscle from ischemia and reperfusion injury, the ability of adenosine to protect against exertion-related skeletal muscle injury has not been studied.

Our previous study confirmed the selectivity of various adenosine receptor agonists at each respective receptor when they were administered in intact mice (61). Using a downhill exercise treadmill to induce eccentric exertion-related skeletal muscle injury (21, 23, 36, 50), we sought to investigate a potential protective role of adenosine A_1, A_{2A}, and A_3 receptor agonists in both normal and dystrophic skeletal muscles. Because the extent of eccentric exercise-induced skeletal muscle injury in normal wild-type (WT) mice is relatively low, we sought to determine whether adenosine receptors can also protect in animals that are susceptible to exertion-related injury. Muscular dystrophic mice are prone to exercise-related skeletal muscle injury. Using the β-sarcoglycan-deficient mouse as a model for muscular dystrophy (23), we examined a potential protective role of adenosine A_1, A_{2A}, and A_3 receptors in eccentric exercise-induced skeletal muscle injury. Sarcoglycan-null mice were chosen as the dystrophic mice to test because disruptions in the sarcoglycan complex are typically accompanied by increased susceptibility to contraction-induced injury (7, 35, 41). In addition, phospholipase Cβ2/β3-knockout (KO) mice and triple phospholipase Cβ2/β3- and β-sarcoglycan-KO mice were developed and utilized to identify potential mechanisms that might be involved in the cytoprotective action of adenosine receptor subtypes.

MATERIALS AND METHODS

Mouse downhill running model for eccentric exercise. Adult (2 1/2 to 3 mo old) (C57BL6 strain) or PLCβ2/β3 or triple PLCβ2/β3- and β-sarcoglycan-KO mice (in C57BL6 background), each weighing ~23–25 g, were subjected to downhill (−15°) treadmill exercise as previously described (21) using an AccuPacer Treadmill (AccuScan Instrument, Columbus, OH). WT and PLCβ2/β3-KO mice were acclimatized to the treadmill for a 15-min period. During this time, speed was increased until 20 m/min was reached. The duration of exercise at 20 m/min was (±SE) 115 ± 5 min in vehicle-treated WT mice and 110 ± 10 min in vehicle-treated PLCβ2/β3 KO mice. Durations of exercise among adenosine agonist-treated WT and PLCβ2/β3 KO mice were matched to those of corresponding vehicle-treated mice. For the β-sarcoglycan-KO and triple KO mice, animals were acclimatized for 5 min at 5 m/min at 15° downhill. These KO

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mice were then exercised at 10 m/min (15° downhill). The duration of exercise at 10 m/min was kept at 25 min for β-sarcoglycan-KO and triple KO mice for vehicle- and adenosine agonist-treated conditions. All mice were gently hand prodded to continue until fatigue or near fatigue as previously described (21, 23). Evans Blue dye (EBD), prepared as a 1% wt/vol solution to yielding 1 mg of EBD/10 g body wt, was given via a separate intraperitoneal injection 5 h before the onset of exercise. Serum samples were collected for creatine kinase (CK) activity measurement 2 h after the end of exercise. Both the level of serum CK activity and the percent area that stained positive for EBD were used as quantitative indices of skeletal muscle injury (23, 61). The groups of animals studied, both at rest and after eccentric exercise, included the vehicle-treated Sham animals and those treated with the adenosine receptor agonists. The mice were euthanized by anesthetic overdose. Gastrocnemius muscles from both lower limbs were removed following euthanasia at 24 h after the exercise. They were quickly frozen, cut into three slices, separated by 2–3 mm, and embedded in Shandon Cryomatrix (polyvinyl alcohol 10%, polyethylene glycol 4%; Anatomical Pathology U.S.A., Pittsburgh, PA). Each slice was processed as one 10-μm section on a Thermo Electron/Shandon Cryotome (Anatomical Pathology), fixed in ice-cold acetone, air-dried, and washed in PBS. Each 10-μm section had seven fields.

Quantification of skeletal muscle injury. EBD selectively stains only injured muscle, and EBD-positive cells were quantified according to previously described methods (23, 61). The percent EBD-positive cells in each field was averaged with those from all seven fields within one 10-μm section. The averaged fraction of EBD-positive cells in each 10-μm section was similar among the three sections. Each 10-μm section was also stained with rabbit polyclonal anti-skeletal muscle actin antibodies (ab15265; Abcam, Cambridge, MA) and goat polyclonal anti-rabbit IgG conjugated with fluorescein isothiocyanate. Sections were mounted, and cross sections were viewed with fluorescent microscopy (EBD-positive cells via a DM580 band pass filter 510–560 nm with emission of 590 nm; fluorescein isothiocyanate cells via a DM510 filter of 450–490 nm with emission at 520 nm). Each field was counted at ×20 magnification, and their images were captured via the two filters for quantification of muscle injury as previously described (61). Images were acquired, stored, and analyzed as JPEG files with a Macrofire camera (Macrofire 1.0; Optronics, Goleta, CA). The percentage of EBD-positive areas (red) was calculated by dividing the area of EBD staining by the total muscle cells, which was defined as the total area stained by an anti-skeletal muscle actin antibody. Serum CK activity was measured with a previously described procedure (22). The fraction of skeletal muscle staining positive for EBD was used as a direct determination of the muscle that was injured, and the serum CK level provided an indirect circulating index of the extent of skeletal muscle injury.

Measurement of skeletal muscle contractile force in vitro. Adult β-sarcoglycan-null mice were injected with vehicle (0.1% DMSO in PBS) as sham or 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IBMECA) (0.07 mg/kg) 2 h before the removal of the extensor digitorium longus muscle. The muscle was immersed in a bath containing buffered physiological salt solution (in mM): 137 NaCl, 24 $NaHCO_3$, 11 glucose, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, and tubocurarine chloride 0.025 maintained at 25°C with pH of 7.4, while bubbling in 95%O_2 and 5% CO_2. Muscle activation was accomplished by electrical field pulses (200-μs duration per pulse). Muscle length and frequency of activation were adjusted to achieve maximal isometric tetanic force as previously described (7). Susceptibility to contraction-induced inury was then assessed in vitro according to a previously established method (27). The lengthening contraction protocol (LCP) consisted of two lengthening contractions with 30% strain relative to fiber length. After the LCP, isometric tetanic force was measured at 1 min post-LCP and then at each 10-min interval up to 1 h. Force deficit was calculated as the difference between pre- and post-LCP force divided by pre-LCP force. Because no differences in force deficit were observed between groups (Sham vs. C1-IBMECA) at any of time points post-LCP, the force deficit at the final time point (1 h after LCP) was used as the representative value for initial force deficit.

Protocol for administration of adenosine receptor agonist and antagonist. Adenosine receptor agonists [0.07 mg/kg for Cl-IBMECA and 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS21680), 0.05 mg/kg for 2-chloro-N^6-cyclopentyladenosine (CCPA)], or vehicle (0.1% DMSO in PBS, i.e., in Sham group) was administered in a sterile 0.1-ml volume by intraperitoneal injection 2 h before onset of exercise. This protocol allowed time for absorption of adenosine ligands and for their presence in circulation before the beginning of any exertion-related skeletal muscle injury. Previous studies demonstrated that intraperitoneal injection of similar doses of adenosine receptor agonists produced potent pharmacological myocardial protection in the mouse (59–61).

Statistical analysis. Unless otherwise indicated, data are shown as means ± SE. One-way ANOVA followed by the Newman-Keuls comparison post test was used to analyze the statistical significance of differences in more than two groups. $P < 0.05$ was considered statistically significant.

Materials and chemicals. The adenosine receptor ligands CGS21680, CCPA, and Cl-IBMECA were obtained from Sigma Chemicals (St. Louis, MO).

PLC β2/β3-deficient mice. PLCβ2/β3-null mice were generously supplied by Dr. Dan Wu (Yale University School of Medicine, New Haven, CT) and were bred as previously described (32). C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animal experiments were conducted under the guidelines for the use and care of laboratory animals for research and approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.

RESULTS

Effects of exercise and adenosine receptor stimulation in normal WT mice. Downhill eccentric exercise caused skeletal muscle injury, as manifested by an increase in the serum level of CK activity. In the normal WT mice, in the absence of exercise, basal CK activity was extremely low (<100 U/L), and no EBD staining was detectable. With exercise, CK activity increased 4- to 5-fold in the Sham (vehicle-injected) mice (Fig. 1*A*). However, EBD staining remained undetectable. In the animals receiving the selective adenosine A_3 agonist, C1–1BMECA, the exercise induced increase in CK was totally abolished (Fig. 1*A*). In contrast, in the normal WT mice, the adenosine A_1 agonist, CCPA, did not protect against exercise-induced muscle injury. In CCPA-treated animals, serum CK levels were increased with exercise and were not significantly different than what was measured in the Sham (vehicle-injected) mice (Fig. 1*B*). Similar results were obtained with the adenosine A_{2A} receptor agonist CGS21680. Exercise-induced CK activities with CGS21680 were essentially identical to those measured in the Sham (vehicle-injected) mice (Fig. 1*C*).

Protective role of adenosine A_3 receptor stimulation in dystrophic mice. The dystrophic β-sarcoglycan KO mice displayed evidence of skeletal muscle degeneration in the basal rested state (Table 1). CK activity was 33-fold higher than what was measured in the resting normal WT mice. In the absence of exercise stress, ~10% of the fibers stained positive with EBD. In the dystrophic Sham mice, eccentric exercise increased CK activity 20-fold over and above the high basal resting values (Fig. 2*A*) and caused a near doubling of EBD staining (Fig. 2*B*).

Adenosine A_3 receptor stimulation with C1-IBMECA decreased the exercise-induced increase in CK activity (Fig. 2*A*).

The increase in EBD staining with exercise in these dystrophic mice was abrogated by adenosine A_3 receptor stimulation (Fig. 2, *B* and *C*). These results differ from the effects of adenosine A_1 or A_{2A} receptor stimulation. The increase in CK with exercise in these dystrophic mice was not changed by adenosine A_1 receptor stimulation (Fig. 2, *C* and *D*) or A_{2A} stimulation (Fig. 2*E*). The % EBD-positive staining in the dystrophic mice following eccentric exercise was also unaffected by prior A_1 receptor stimulation (19.1% ± 4.2%, $n = 17$, $P > 0.05$ vs. Sham dystrophic mice: 17.6% ± 1.9%, $n = 22$). Prior A_{2A} receptor stimulation with CGS21680 (13.96 ± 2.5%, $n = 20$) also had no effect on eccentric exercise-induced skeletal muscle injury ($P > 0.05$ vs. Sham animals).

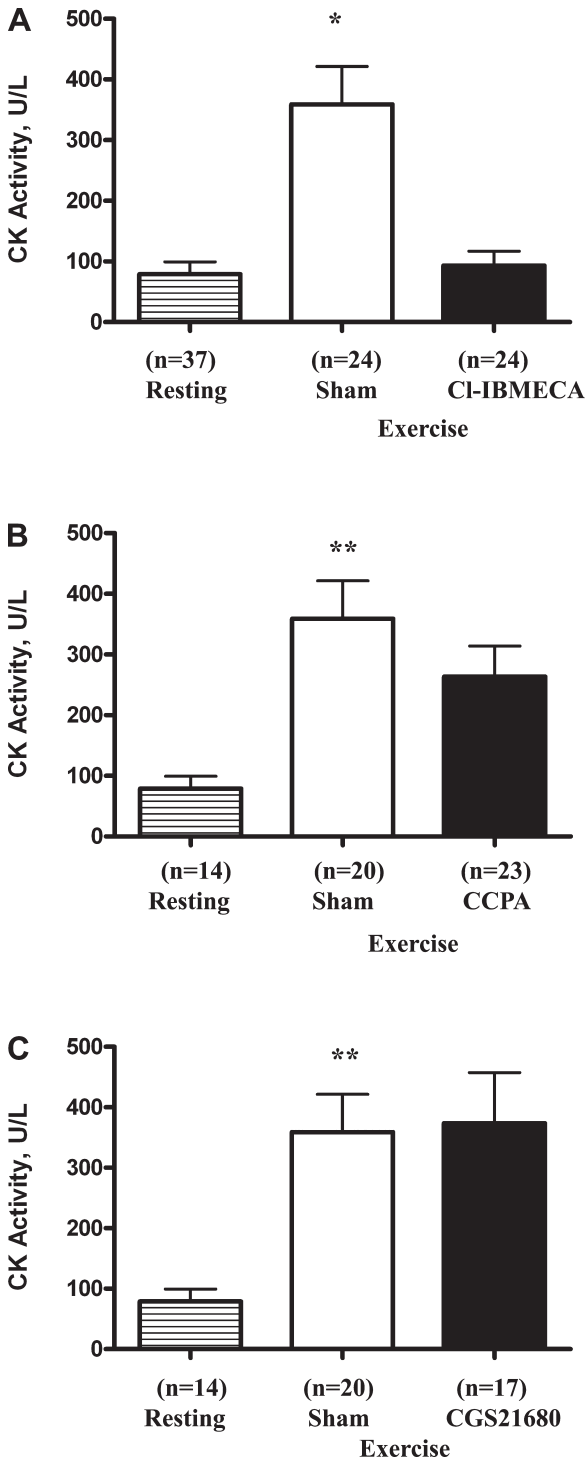


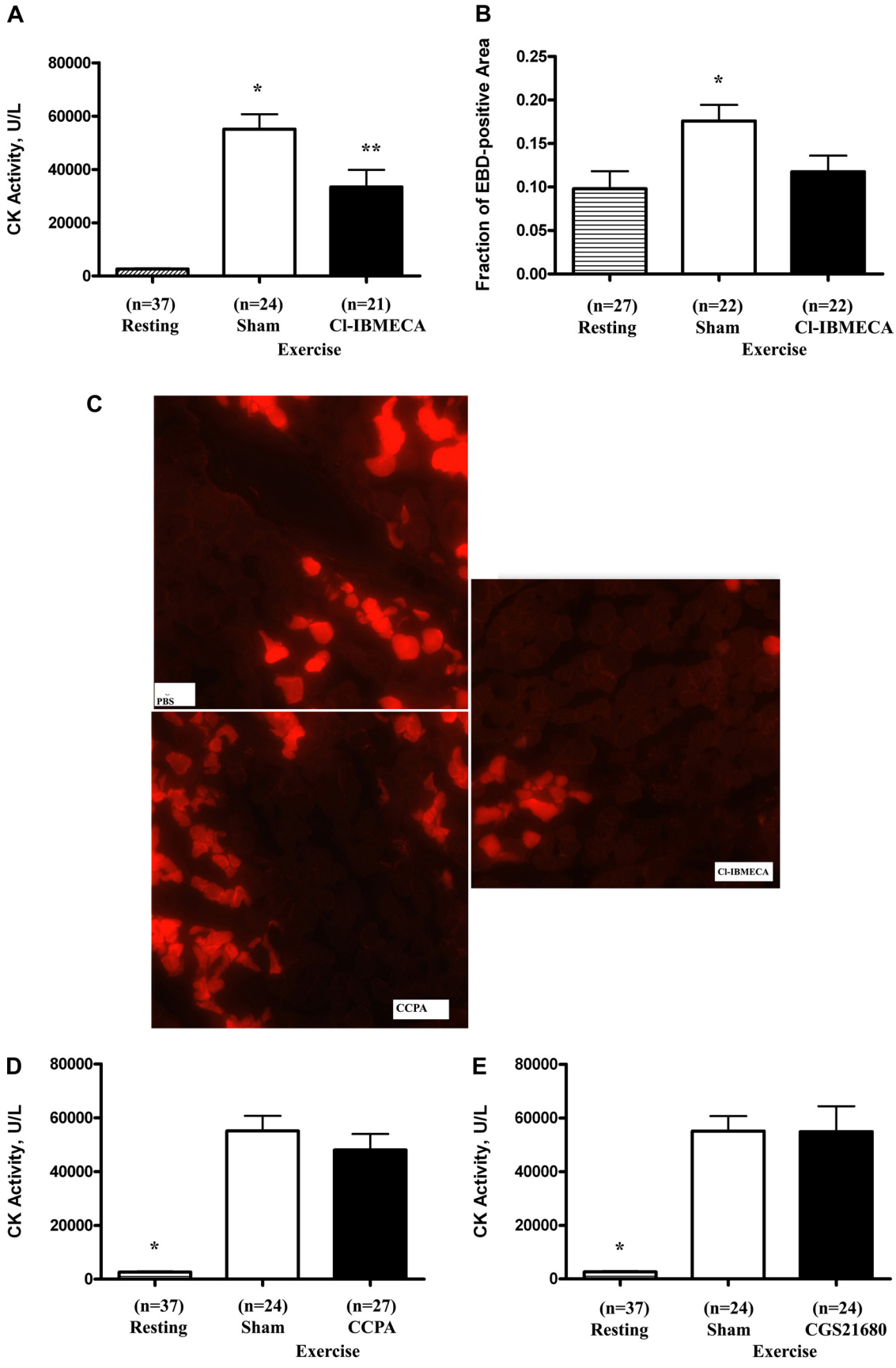
Table 1. *Basal levels of serum CK activity and EBD staining in various genotypes*

Genotype	CK Activity, U/l	EBD, %
WT ($n = 14$)	79.1 ± 20.4**	ND
PLC β2/β3 KO ($n = 5$)	103 ± 31.5**	ND
β-sarcoglycan KO ($n = 37$)	2601 ± 291	9.8 ± 2.0
Triple KO (*$n = 14$ or 9)	3110 ± 479	9.8 ± 2.1

The basal level of serum creatine kinase (CK) activity or Evan's blue dye (EBD) staining was determined as described in Materials and Methods. Data are expressed as means ± SE. Serum CK activity was similar in wild-type (WT) vs. phospholipase C (PLC) β2/β3 knockout (KO) or in β-sarcoglycan KO vs. triple KO ($P > 0.05$). *$n = 14$ mice for CK activity and $n = 9$ mice for EBD staining. **$P < 0.05$ vs. β-sarcoglycan KO or triple KO mice.

Role of phospholipase Cβ. A previous study demonstrated an important role of PLC β2/β3 in mediating the anti-ischemic effect of adenosine A_3 receptors in skeletal muscle (61). To determine whether PLC β2/β3 also mediates the A_3 receptor-induced protection against exertion-related injury, we studied the effect of A_3 receptor agonist in mice deficient in PLC β2/β3. Results summarized in Fig. 3 demonstrated that C1-IBMECA could not reduce the increase in serum CK caused by eccentric exercise-induced damage in mice null for PLC β2/β3. Neither CCPA nor CGS21680 was able to decrease the serum CK rise caused by eccentric exercise in PLC β2/β3 KO mice (data not shown). To test the role of PLC β2/β3 in C1-IBMECA-mediated protection of the dystrophic skeletal muscle, KO of the PLC β2/β3 in β-sarcoglycan-null mice or a triple β-sarcoglycan PLC β2/β3 KO mouse was generated. The basal skeletal muscle injury in the triple KO mice was similar to that in PLC β2/β3 mice (Table 1). Thus, the absence of PLC β2/β3 in the dystrophic mice did not result in greater basal skeletal muscle damage. Eccentric exercise caused a 50% increase in the EBD staining in Sham triple KO animals (Fig. 4*A*). The extent of EBD-positive staining in Sham triple KO mice was similar to that in Sham β-sarcoglycan-deficient animals ($P = 0.30$). Prior treatment with C1-IBMECA did not reduce the increased EBD staining elicited by eccentric exercise-induced skeletal muscle damage. Similar results were obtained using serum CK as a quantitative marker for the dystrophic skeletal muscle injury. Eccentric exercise caused a large increase in the serum CK activity in Sham triple KO mice (Fig. 4*B*). Pretreat-

Fig. 1. Protection of skeletal muscle from eccentric exercise-induced injury by adenosine A_3 receptor agonist in wild-type (WT) mice. Adult WT mice were injected with various adenosine ligands, and then they were subjected to downhill treadmill exercise as described in MATERIALS AND METHODS. Under basal condition without eccentric contraction, serum creatine kinase (CK) activity was low. Eccentric contractions were associated with an increase in serum CK activity in PBS-treated mice. Compared with PBS-treated animals, prior treatment with 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IBMECA) (*A*) lowered the serum CK activity to a level similar to that obtained under basal condition (one-way ANOVA and posttest comparison). Treatment with 2-chloro-N^6-cyclopentyladenosine (CCPA) (*B*) or *p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS21680) (*C*) resulted in a level of serum CK activity similar to that obtained in PBS-treated animals ($P > 0.05$). *$P < 0.05$ vs. basal or Cl-IBMECA; **$P < 0.05$ vs. PBS only. Data were expressed as means ± SE.



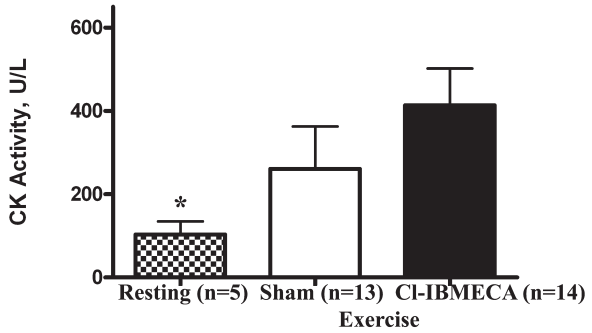


Fig. 3. The adenosine A_3 receptor signals through phospholipase (PLC) β2/β3 to protect against eccentric exercise-induced skeletal muscle injury in PLCβ2/β3 mice. Adult PLCβ2/β3-null mice were injected with PBS or adenosine A_3 receptor agonist and then subjected to eccentrically biased exercise. The extent of skeletal muscle injury was subsequently quantified as described in MATERIALS AND METHODS. In PLCβ2/β3 KO mice not subjected to eccentric exercise, skeletal muscles did not show any Evans Blue dye (EBD) staining and exhibited minimal serum CK activity level. Prior treatment with Cl-IBMECA did not affect skeletal muscle injury. The serum CK activity level was similar to that obtained in PBS-treated mice. $*P < 0.05$ vs. either of the other two groups. Data are expressed as means ± SE.

ment with C1-IBMECA failed to reduce the increased CK level elicited by the eccentric exercise. Adenosine receptor A_1 stimulation with CCPA, which could not protect the WT, PLC β2/β3 KO, or β-sarcoglycan KO mice from eccentric exercise-induced injury, also did not reduce either the increase in EBD staining or in serum CK activity in the triple KO mice (Fig. 4, *A* and *B*). These data were controls, arguing against a change in the A_1 receptor signaling following KO of various genes.

DISCUSSION

Attempts at reducing eccentric contraction-induced skeletal muscle damage have met with variable levels of efficacy. Effective strategies to protect skeletal muscle against this form of injury are needed. Ischemic preconditioning can provide potent protection of the heart (43, 57), as well as the skeletal muscle (13, 14), from ischemia/reperfusion injury. As with cytoprotection of the heart, extracellular adenosine is implicated in mediating the protective effect of ischemic preconditioning in skeletal muscle (13, 14, 46). The present study used an eccentrically biased downhill treadmill running model to test the hypothesis that adenosine receptor stimulation can protect the skeletal muscle from eccentric exercise-induced damage. Agonists selective at the A_1, A_{2A}, and A_3 receptors were utilized to test the potential protective role of each receptor in exertion-related skeletal muscle injury. Prior studies have demonstrated selectivity of these agonists at their respective adenosine receptor subtypes after in vivo administration in intact mice (61).

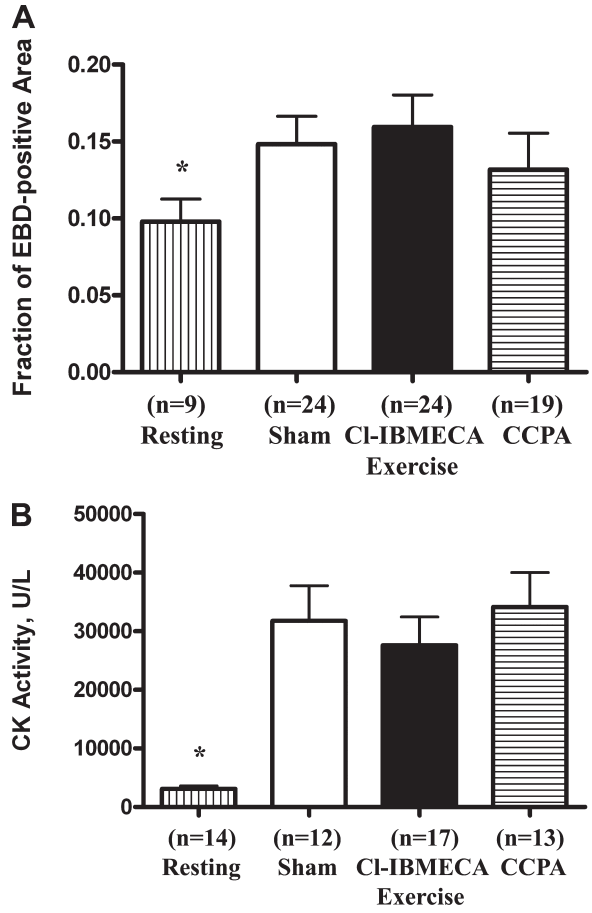


Fig. 4. A_3 receptor agonist Cl-IBMECA does not protect dystrophic mice that lack PLCβ2/β3. Adult β-sarcoglycan-null mice that are deficient for PLCβ2/β3 or triple KO mice were injected with PBS or adenosine receptor agonists, and then subjected to eccentrically biased exercise. In the triple KO mice, there was detectable injury at the basal time point in the absence of exercise. Compared with basal level of injury, eccentric exercise increased the severity of muscle injury in PBS-treated triple KO mice, as evidenced by a further increase in % EBD-positive cells (*A*) and in serum CK level (*B*). Compared with PBS-treated triple KO animals, treatment with Cl-IBMECA or CCPA resulted in a similar level of EBD staining (*A*) and serum CK activity (*B*) (one-way ANOVA and posttest comparison). $*P < 0.05$ vs. any of the other three groups. Data are expressed as means ± SE.

We used the downhill running model to simulate eccentric exercise and to induce skeletal muscle injury. This model, well-characterized in rodents, is associated with inflammation and skeletal muscle damage (29, 47, 49, 54). Downhill running causes greater injury to muscles than level running at the same speed and for the same duration (1). Essentially, with downhill running, high forces generated in the eccentric contractile phase when active tension is used to decelerate the center of

Fig. 2. Adenosine A_3 agonist protected the dystrophic skeletal muscle from eccentric exercise injury in the β-sarcoglycan-null mice. Adult β-sarcoglycan-null mice were injected with various adenosine ligands, subjected to eccentric exercise, and skeletal muscle injuries were quantified by serum CK level and by % EBD-positive muscle cell, as described in MATERIALS AND METHODS. There was detectable basal injury in the dystrophic muscle in the absence of exercise. Eccentric exercise increased the severity of muscle injury in PBS-treated mice, as evidenced by higher serum CK level (*A*) and more EBD staining (*B*). Compared with PBS-treated animals, treatment with Cl-IBMECA decreased the serum CK activity (*A*) and % EBD staining (one-way ANOVA and posttest comparison). Treatment with CCPA did not affect the exertional injury with a level of EBD staining and of serum CK activity similar to those obtained in PBS-treated animals (*C* and *D*). *C*: representative tracings comparing the extent of the EBD-stained area (red) in PBS-, CCPA-, and Cl-IBMECA-treated dystrophic mice are shown. Following treatment with CGS21680, the level of serum CK activity (*E*) or % EBD staining (not shown) was similar to those obtained in PBS-treated animals. $*P < 0.05$ vs. either of the other two groups; $**P < 0.05$ vs. PBS. Data are expressed as means ± SE.

mass, results in damage to skeletal muscle. This model has been used repeatedly to document pathology and physiology of skeletal muscle damage, and more recently, to explore effective treatment strategies to minimize skeletal muscle damage, functional consequences, and systemic effects.

Attempts to ameliorate eccentric exercise-induced damage to skeletal muscle include nonsteroidal anti-inflammatory drugs (NSAIDs), antioxidants/vitamin C, protein, proteases, phosphatidalserine, quercetin, and chondroitin sulfate. The effects of antioxidant supplements are variable and depend largely on the muscle group tested and the form of eccentric-exercise used (4, 31, 39). The limited data available indicate that NSAIDs largely have no influence on inflammatory responses to eccentric exercise and can be potentially deleterious (10, 28, 40, 55). For example, Mikkelsen et al. (40) reported that NSAID infusion during exercise suppressed the natural exercise-induced increase in the number of satellite cells, indicating that NSAIDs negatively affect satellite cell activity after eccentric exercise, possibly impeding skeletal muscle regeneration. Other studies have demonstrated no effect of dietary supplementation on protecting skeletal muscle from damage, or improving muscle function and soreness posteccentric exercise (5, 18–20, 34, 42, 44). In contrast, two dietary supplements have been tested and have been shown to minimize skeletal muscle damage or muscle soreness. One group has explored the benefits of curcumin before a bout of downhill running in rats, and documented a decrease in postexercise creatine kinase levels, as well as, inflammatory cytokines (21). However, the current understanding of the mechanism of curcumin's action is not well defined and is limited to its general antioxidant and anti-inflammatory properties. Likewise, the bioactive herb Honokiol has been shown to have similar anti-inflammatory properties in rat skeletal muscle, ameliorating eccentric exercise-induced damage following downhill running on a treadmill (16). Overall, more effective therapies that can reduce skeletal muscle injury from eccentric contraction are needed. The present data demonstrated for the first time that activation of the adenosine A_3, but not the A_1 or A_{2A} receptors, can protect against mechanisms that contribute to eccentric exercise-induced skeletal muscle injury. This conclusion is based on a number of lines of evidence.

We showed that eccentrically biased exercise caused an increase in serum CK activity in WT mice. We could not detect skeletal muscle EBD uptake after the eccentric exercise, presumably due to limited skeletal muscle injury in the WT mice. Prior treatment of the WT mice with Cl-IBMECA totally abolished the increased serum CK activity caused by the eccentric exercise. The adenosine A_1 receptor agonist CCPA or the A_{2A} agonist CGS21680 did not decrease the exercise-induced rise in serum CK.

To further test this protective effect of adenosine A_3 receptors, β-sarcoglycan-null mice (β-sarcoglycan-null mice) were used as a model of muscular dystrophy. Mutations in the β-sarcoglycan gene cause limb-girdle muscular dystrophy type 2E (LGMD 2E), which is associated with skeletal muscle weakness, moderately elevated serum CK, and often cardiomyopathy (37, 45). Although there may be some variability in onset and progression, patients with LGMD 2E tend to be clinically significant and may be as severely affected as patients with Duchenne muscular dystrophy. β-sarcoglycan-null mice develop severe muscular dystrophy with disruption of sarcoglycan and dystroglycan complexes in skeletal, cardiac, and smooth muscle. The β-sarcoglycan-null mice have been developed as a model to study the pathogenesis and potential treatment of this form of muscular dystrophy. These dystrophic mice showed significant levels of injury in unexercised muscle at baseline. The extent of basal skeletal muscle injury was significant enough to cause an increase in serum CK activity and EBD staining. β-sarcoglycan-null mice developed elevated indices of skeletal muscle damage following downhill running with a large increase in both serum CK level and EBD staining. Prior treatment of the muscular dystrophy mice with Cl-IBMECA reduced the eccentric exercise-mediated increase in serum CK activity and in EBD staining. However, it should be noted that there are several mechanisms that may contribute to elevations in these markers of skeletal muscle damage, including ischemia/reperfusion, and the intensity of the exercise.

CCPA and CGS21680, and adenosine A_1 and A_{2A} receptor agonists respectively, failed to reduce eccentric exercise-mediated skeletal muscle injury. Thus, the adenosine A_3, but not the A_1 or A_{2A} receptor, was able to protect the dystrophic skeletal muscle from physical exertion-mediated damage. The selective protective effect of A_3 receptors was abrogated by the absence of PLCβ2/β3 in both healthy WT mice and β-sarcoglycan-null mice. PLCβ2/β3 mediates the protective effect on eccentric exercise-induced injury by adenosine A_3 receptors, similar to its role in protecting the skeletal muscle from ischemia-reperfusion injury (61). Overall, adenosine receptor stimulation protection is general for both WT and sarcoglycan-deficient muscle. How adenosine receptor stimulation protects in both groups of mice may differ.

The initial damage to muscle induced by a severe series of eccentric contractions consists of disruptions to the sarcolemma (17), individual sarcomeres (38), and excitation-contraction coupling (3). This initial damage causes an initial deficit in force generation capability. For extensor digitorum longus muscle assessed in vitro, injection of C1-IBMECA (0.07 mg/kg ip) in β-sarcoglycan-null mice has no effect on the initial force deficit induced by a protocol of eccentric contractions (Fig. 5). This observation suggests that activation of A_3 receptors does not

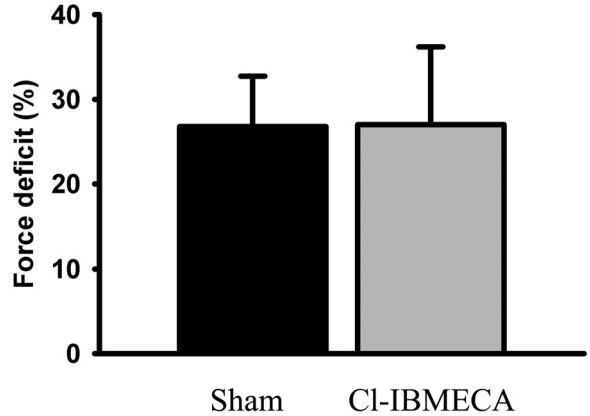


Fig. 5. Cl-IBMECA injection in β-sarcoglycan-null mice did not reduce the initial force deficit caused by in vitro eccentric contractions. Extensor digitorium longus muscles from β-sarcoglycan-null mice injected with vehicle (0.1% DMSO in PBS) as Sham (n = 3) or with Cl-IBMECA (0.07 mg/kg) (n = 3) were isolated and force deficit determined after being subjected to an in vitro lengthening contraction protocol as described in MATERIALS AND METHODS to supplement. Data are expressed as means ± SE.

influence the initial injury to muscle. In the hours to days following initial damage incurred during eccentric exercise, secondary injury occurs by an inflammatory response (29, 47). For the treadmill running experiment, the beneficial effects of A_3 receptor activation were observed hours after running. Considering that this time period coincides with the inflammatory response and that C1-IBMECA had no effect on initial damage observed in vitro, the implication is that the beneficial effects of A_3 receptor activation observed following treadmill running resulted from decreased secondary damage.

Activation of the A_3 receptor in rodent immune cells such as mast cells is proinflammatory (48, 53). However, activation of the adenosine A_3 receptor can block superoxide formation and chemotaxis of murine bone marrow neutrophils (55a) and can also inhibit neutrophil function in canine and rabbit preparations (33). Although activated mast cells and neutrophils mediate skeletal muscle ischemia/reperfusion injury (9, 25, 26), mast cells and neutrophils may also cause eccentric contraction-mediated skeletal muscle injury. Since eccentric contraction is associated with an increase in inflammatory markers and cytokines (29, 47, 54), it is possible that the in vivo administration of A_3 receptor agonist exerted an overall anti-inflammatory effect and reduced the inflammation-mediated skeletal muscle damage during eccentric contraction. PLCβ2/β3 may mediate an anti-inflammatory effect of A_3 receptors on circulating immune cells since the global PLCβ2/β3 gene KO also includes KO of PLCβ2/β3 in skeletal muscle and circulating immune cells. In this scenario, KO of PLCβ2/β3 would eliminate the anti-inflammatory effect of A_3 receptors on immune cells and thus abrogate their cytoprotective effect on skeletal muscles. The data highlight an important role of this enzyme in preventing eccentric contraction-induced damage to skeletal muscle.

Another consideration is the vascular effect of the adenosine A_3 receptor agonist. This is relevant in view of the finding that a preserved blood flow to the exercising muscle via nNOS is important in preventing muscle fatigue (35). Additionally, ischemia is a possible contributor to the damage during downhill running. However, some differences exist in muscle damage markers between eccentric exercise and ischemia/reperfusion (51). Genetic absence or antagonism of adenosine A_3 receptors augmented an increase in coronary flow or hypotension mediated by adenosine or an A_{2A} receptor agonist (52, 58), pointing to a vasoconstrictive role of the vascular A_3 receptor. Activation of the A_3 receptors can increase vascular permeability in mice (48, 53). It is unlikely that the vasoconstrictive and permeability effect of A_3 receptors is beneficial. Differentiating the effect of adenosine A_3 receptors at the levels of vasculature, circulating immune cells, and skeletal muscle deserves further study.

Perspectives and Significance

The study showed for the first time that stimulation of adenosine A_3 receptors in intact animal can protect skeletal muscle from eccentric exercise-induced injury. This protective effect was seen in both normal healthy and abnormal dystrophic skeletal muscles. The present study extended our previous finding of an anti-ischemic effect of adenosine A_3 receptors in skeletal muscle in an intact animal. It further confirms an important role of the PLCβ2/β3 in mediating the cytoprotective effect of adenosine A_3 receptors in that tissue. Currently, interventions to mitigate eccentric exercise-induced skeletal muscle injury have met with either limited or variable levels of efficacy. The A_3 receptor agonist intervention points to a potential means of treatment for such injury, whether it acts through a direct mechanism exerted at the skeletal muscle level or via an indirect mechanism such as minimizing inflammatory processes.

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DISCLOSURES

There is no conflict of interest to disclose by any of the authors. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

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