Adenosine $A_3$ receptor stimulation reduces muscle injury following physical trauma and is associated with alterations in the MMP/TIMP response

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Urso ML, Wang R, Zambraski EJ, Liang BT. Adenosine $A_3$ receptor stimulation reduces muscle injury following physical trauma and is associated with alterations in the MMP/TIMP response. J Appl Physiol 112: 658–670, 2012. First published November 23, 2011; doi:10.1152/japplphysiol.00809.2011.—We have previously demonstrated that in response to traumatic injury in skeletal muscle, there is a dysregulation of the matrix metalloproteases (MMPs) and their inhibitors (TIMPs), a response hypothesized to interfere with proper skeletal muscle regeneration. Moreover, we have shown that pharmacological activation of the adenosine $A_3$ receptor by CI-IBMECA in skeletal muscle can protect against ischemia-reperfusion and eccentric exercise injury. However, the mechanism by which CI-IBMECA protects muscle tissue is poorly defined. This study investigated the effects of CI-IBMECA on MMP/TIMP expression in skeletal muscle and tested the hypothesis that adenosine $A_3$ receptor-stimulated protection of skeletal muscle following traumatic injury is associated with a blunting of MMPs involved in inflammatory processes and collagen degradation, and an increase in MMPs associated with extracellular matrix remodeling. Sixty C57BL/6J male mice were injected with CI-IBMECA ($n = 30$) or a vehicle ($n = 30$), and Evans blue dye. Injury was induced by applying a cold steel probe (−79°C) to the tibialis anterior (TA) muscle for 10 s. TA muscles from uninjured and injured legs were collected 3, 10, and 24 h postinjury for analysis of muscle injury and MMP/TIMP mRNA and protein levels. Twenty-four hours postinjury, 56.8% of the fibers were damaged in vehicle-treated mice vs. 35.4% in CI-IBMECA-treated mice ($P = 0.02$). CI-IBMECA treatment reduced membrane type 1 (MT1)-MMP, MMP-3, MMP-9, and TIMP-1 mRNA expression 2- to 20-fold compared with vehicle-treated mice ($P < 0.05$). CI-IBMECA decreased protein levels of latent/shed MT1-MMP 23–2,000%, respectively, 3–10 h postinjury. In CI-IBMECA-treated mice, latent MMP-2 was decreased 20% 3 h postinjury, active MMP-3 was decreased 64% 3 h postinjury, and latent/active MMP-9 was decreased 417,631% 3 h postinjury and 20% 10 h postinjury. Protein levels of active MMP-2 and latent MMP-3 were increased 25% and 75% 3 h postinjury, respectively. The present study elucidates a new protective role of adenosine $A_3$ receptor stimulation in posttraumatic skeletal muscle injury.

Traumatic skeletal muscle injury due to extreme cold, toxins, blast, and/or crush affects contractile, molecular, and histopathological properties of the muscle fiber. After injury, tissue matrix metalloproteases (MMPs) degrade the extracellular matrix (33), deposit new extracellular matrix components (2), and regenerate tissue (49). Optimal remodeling of the extracellular matrix is contingent on tightly regulated MMP activity, which is influenced by transcription, proteolysis of the latent forms of the protein, and/or through reversible inhibition by their natural inhibitors, the tissue inhibitors of metalloproteases (TIMPs) (25, 35).

When the magnitude of the inflammatory response exceeds typical levels in response to traumatic injury, the extracellular matrix remodeling process can be disrupted, having a detrimental effect on skeletal muscle. One example is an excessive increase in the activity of degenerative MMPs, which leads to tissue destruction, cell invasion, and impaired tissue regeneration (23). Our previous work characterizing the effects of traumatic freeze injury on skeletal muscle showed dysregulation of the MMP/TIMP system during the days postinjury (3, 51). Specifically, membrane type 1 (MT1)-MMP and MMP-3 increased more than 200% postinjury, whereas zymography analysis revealed increased MMP-2 and MMP-9 activities. We also observed robust decreases in TIMP-1 and TIMP-2 protein levels. Similar phenomena have been observed in aged and diseased cardiac muscle, resulting in adverse extracellular matrix remodeling and increased fibrosis (21, 26). Therefore, on the basis of our observations in skeletal muscle, we concluded that this dysregulation of the MMP/TIMP system has the potential to contribute to impaired extracellular matrix remodeling, improper resolution of the inflammatory response, and in many cases, increased fibrosis in injured tissue.

These data led us to question the feasibility of blocking the MMP response in traumatized skeletal muscle (25, 35). Our group has previously confirmed that the A3 receptor agonist (CI-IBMECA) exhibits superior benefit in skeletal muscle protection (28, 39, 47, 52, 57). The specific mechanism of A3 receptor agonist-mediated protection in skeletal muscle has not yet been delineated. Previous work has documented that A3 receptor agonists function to protect muscle via mechanisms that may involve reducing inflammation, cellular membrane damage, and oxidant stress (6, 7, 11, 52, 54, 57, 59). Because many of these processes influence MMP activity, we suggest that treatment with the adenosine A3 receptor agonist can modulate the MMP/TIMP system (7, 13, 44, 52).

For instance, others have demonstrated that inflammatory infiltrates are predominant sources of MMPs (41, 53), and the...
primary event linking skeletal muscle injury to intracellular proteolytic events is the infiltration of inflammatory cells in the hours postinjury. This inflammatory reaction may produce additional damage to the extracellular matrix, induce MMP activation, and subsequently increase muscle fibrosis, scarring, and consequent injury (50). Likewise, data indicating that treatment with the adenosine A3 receptor agonist decreases serum creatine kinase levels following muscle injury suggests that one mechanism of protection afforded by adenosine A3 receptor agonist treatment is an attenuation of extracellular matrix degradation (35, 52, 57). Both exercise-induced and trauma-induced injury to skeletal muscle fibers is accompanied by increases in serum creatine kinase and histological evidence of sarcolemmal disruption (1, 29, 31, 32).

Therefore, the objectives of this study were to investigate whether the adenosine A3 receptor agonist was able to reduce injury to skeletal muscle following traumatic freeze injury and to identify a potential mechanism of protection of the adenosine A3 receptor agonist, specifically its effect on the MMP/TIMP system (Fig. 1). We hypothesized that in accordance with data from other models of injury in cardiac and skeletal muscle, the adenosine A3 receptor agonist would reduce the number of injured muscle cells following traumatic freeze injury. We also hypothesized that the adenosine A3 receptor agonist treatment would reduce protein levels of MMPs involved in inflammatory processes and collagen degradation and increase protein levels of MMPs associated with extracellular matrix remodeling.

MATERIALS AND METHODS

Animal care. Ten-week-old C57BL/6J male mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Thirty mice were placed in the CI-IBMECA group, and 30 mice were placed in the vehicle treatment group. Four groups were also included (n = 4/group) to serve as controls: 1) CI-IBMECA no traumatic injury; 2) CI-IBMECA sham (skin incision, suture, no traumatic injury); 3) vehicle no traumatic injury; and 4) vehicle sham (skin incision, suture, no traumatic injury).

All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for research and were approved by the Institutional Animal Care and Use Committee of the U.S. Research Institute of Environmental Medicine. After arriving at the facility, mice were given 14 days to acclimate before the initiation of experimentation. Daily measurements of body weight, food intake, and water intake were made. Mice were provided with food and water ad libitum before and after all procedures. Mice were housed on a 12:12-h light-dark cycle at ambient temperature (25 ± 1°C).

Adenosine receptor agonist and Evans blue dye administration. The adenosine receptor agonist [0.07 mg/kg 2-chloro-N6-(3-iodobenzyl) adenosine-5′-N-methyluronamide (CI-IBMECA); Sigma Chemicals, St. Louis, MO] or vehicle [0.1% DMSO in phosphate-buffered saline (PBS)] was administered in a sterile 0.1-ml volume by intraperitoneal injection 2 h before induction of freeze injury. This allowed time for absorption of the adenosine ligand and for presence in the circulation before the skeletal muscle injury (52, 57). Evans blue dye (EBD), prepared as a 1% (wt/vol) solution to yield 1 mg EBD/10 g body wt, was given via a separate intraperitoneal injection 2 h before injury.

Injury model. We chose a freeze injury model because the inflammatory response mimics other traumatic muscle injuries. However, the injury model that we use is unique because it results in a specific, localized injury to the area where the cold probe is applied to the muscle. It affects only structures and tissues that lie within the area of contact of the cold probe. Unlike a blast injury that results in widespread tissue destruction, our injury does not result in damage to the tissues and structures that surround the injury site (50). Under isoflurane anesthesia (1.5%), the hindlimbs of the mice were shaved and the left tibialis anterior (TA) muscle was exposed via a 1-cm-long incision in aseptically prepared skin overlying the muscle. Traumatic

Fig. 1. Hypothetical model of the action of CI-IBMECA treatment on the injury response in skeletal muscle. In response to traumatic injury, inflammatory processes, reactive oxygen species (ROS), and extracellular matrix degradation are activated. As a consequence, inflammatory cytokines (e.g., TNF-α) and signal transduction cascades are induced. These primary cytokines will propagate the inflammatory response by acting on matrix metalloproteases (MMPs). Inflammatory cytokines activate MMP-2 and MMP-9, which then degrade extracellular matrix components (collagen, gelatin, laminin) and activate other MMPs that also promote extracellular matrix degradation (MT1-MMP) and remodeling (MMP-3). This process is followed by matrix breakdown, synthesis, and repair, which is imperfect in our injury model, as is reflected by the robust increase in MMPs and a failure for tissue inhibitor of metalloproteases (TIMP-1 and TIMP-2) to increase. We suggest that CI-IBMECA treatment acts on inflammatory and ROS pathways to mitigate this pathological MMP response. In turn, this promotes a more resilient muscle fiber, which is less susceptible to cellular damage.
freeze injury was induced by applying a 6-mm-diameter steel probe, cooled to the temperature of dry ice (−79°C), to the belly of the TA muscle for 10 s. A similar procedure was performed on the TA muscles of sham animals, except the steel probe was not cooled. All injuries were performed by the same investigator to ensure consistent injury. After injury or sham procedure, the skin incision was closed using 6-0 silk suture (Ethicon, Piscataway, NJ).

After the procedure and before recovery from anesthesia, the analgesic buprenorphine (0.1 mg/kg sc) was administered. Mice recovered on a heating pad to prevent anesthesia-induced hypothermia. Postsurgical body weight measurements were assessed daily. Mice were euthanized at 3, 10, and 24 h postinjury using CO₂ inhalation and cardiac puncture. TA muscles from both hindlimbs (uninjured contralateral TA muscles were used as controls) were excised, and excess connective tissue was trimmed before weighing. Samples were snap frozen in liquid N₂ and stored at −80°C.

Tissue analysis. Before processing, all muscles were positioned ventral side up and split lengthwise with a surgical blade into two equal portions for mRNA and protein analysis. The size of the steel probe was chosen to ensure the site of the injury covered the upper two-thirds of the TA muscle. A subset of muscle samples were preserved whole for histological analysis of muscle injury. The site of injury in the experimental group was visually evident to the naked eye as a line of demarcation around the injured tissue. Because we did not observe any significant changes in genes or proteins of interest between the right and left legs in our four control groups, in our experimental groups we used each animal’s contralateral uninjured leg as an internal control. The rationale for this approach was to tease out the specific effects of the Cl-IBMECA treatment in injured muscle without confounding variation due to interanimal variability.

Quantification of skeletal muscle injury. TA muscles that were preserved for histological muscle injury analysis were 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 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 of 510–560 nm with emission of 590 nm; fluorescein isothiocyanate cells via a DMS10 filter of 450–490 nm with emission at 520 nm). Each field was counted at ×20 magnification and its images captured via the two filters (52, 57). 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 the anti-skeletal muscle actin antibody as previously described (52). The fraction of skeletal muscle staining positive for EBD was used to classify the percentage of muscle that was injured.

**RNA isolation, cDNA synthesis, and quantitative RT-PCR.** Total RNA was isolated from ~10-mg skeletal muscle samples that were excised from the injured portion of the TA muscle, homogenized, and purified according to the manufacturer’s instructions using TRIzol reagent (Sigma). RNA was evaluated for quantity and quality using the optical density (OD) 260/280 ratio with a Nanodrop ND-1000 spectrophotometer (Wilmington DE). cDNA was synthesized from equal amounts of total RNA using a First-Strand cDNA synthesis kit (Fermentas, Hanover, MD). ABgene Absolute qPCR SYBR green master mix (ABgene, Surrey, UK) with ROX dye was used for PCR reactions. Forward and reverse quantitative RT-PCR (qRT-PCR) primers were designed using National Center for Biotechnology Information (NCBI) gene sequences and the Primer Design Platform provided by Integrated DNA Technologies (Coralville, IA). NCBI BLAST searches were performed on primer sequences to ensure specificity. The constitutively expressed gene β-actin was used as a reference standard. Amplification reactions were performed on a Stratagene MX3000P (Agilent Technologies, La Jolla, CA) according to the manufacturer’s instructions with the following primers: MT1-MMP, forward 5′-atggcagcatgaagtgagttg-3′ and reverse 5′-agcttggcagagtggaaagactga-3′; MMP-2, forward 5′-cttcgctgtttctctcacc-3′ and reverse 5′-tagagtgaggagggagacct-3′; MMP-3, forward 5′-tgaggctctttgccaggg-3′ and reverse 5′-gccttggtcttcagagct-3′; MMP-9, forward 5′-ccatgcaaatctcttctgt-3′ and reverse 5′-tagagctctcatgacggatgtaa-3′; TIMP-1, forward 5′-tgaggctcttagcaagttgc-3′ and reverse 5′-ctaggggaaggctgctcaggtc-3′; TIMP-2, forward 5′-ctaggggaaggctgctcaggtc-3′; TIMP-3, forward 5′-ctaggggaaggctgctcaggtc-3′; and reverse 5′-ctaggggaaggctgctcaggtc-3′; and reverse 5′-ctaggggaaggctgctcaggtc-3′; and reverse 5′-ctaggggaaggctgctcaggtc-3′. Data are means ± SE in vehicle-treated (n = 6) and Cl-IBMECA-treated (n = 6) mice. *P < 0.05 vs. vehicle-treated group.
5'- gtatgtcaaggccaaag-3' and reverse 5'-ttctctgtgacccagtccat-3'; and β-actin, forward 5'-ctcttccagccttccttcct-3' and reverse 5'-tgctagggctgtgatctcct-3'. A melting curve was performed at the end of each reaction to detect possible multiple PCR products including primer dimers. Fold changes in gene expression between uninjured and injured legs were determined by utilizing the ΔΔCt method and real-time PCR efficiencies of the target gene normalized to the housekeeping gene β-actin. Expression of β-actin was not affected by the injury (data not shown).

**Immunoblotting.** Approximately one-half of the excised TA muscle (~20 mg/leg) from each mouse was used for protein extraction and downstream analysis. Tissue samples were homogenized in tissue protein extraction reagent (T-PER; Thermo Scientific, Rockford, IL) with a protease inhibitor (HALT cocktail; Pierce Biotech, Rockford, IL). Homogenates were centrifuged at 14,000 g for 30 min at 4°C to remove debris. Total protein was measured via the Bradford assay (Better Bradford Reagent; Thermo Scientific). Equal amounts of supernatant protein (~20 μg) were exposed to SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes, and incubated for 1 h at room temperature in a blocking solution of Tris-buffered saline containing 5% bovine serum albumin (BSA; Sigma). Thereafter, membranes were exposed to primary antibodies for pro/latent 63-kDa MT1-MMP (1:250, biotinylated, AB8104; Chemicon), soluble 50-kDa MT1-MMP (1:2,000, no. 51074; Abcam), MMP-2 (1:350, biotinylated, BAF1488; R&D Systems, Minneapolis, MN), MMP-3 (1:100, goat polyclonal, sc-6839; Santa Cruz Biotechnology, Santa Cruz, CA), MMP-9 (1:350, biotinylated, BA9F909; R&D Systems), TIMP-1 (1:1,000, rabbit polyclonal, no. 38978; Abcam), TIMP-2 (1:1,000, no. 28260; Abcam), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000, no. 8245; Abcam). Our rationale for using GAPDH as an internal loading control vs. β-actin (which was used for qRT-PCR) was that results with β-actin were not consistent and the injury did not change the amount of GAPDH protein. Secondary horseradish peroxidase (HRP; or streptavidin-HRP)-conjugated antibodies were used at 1:10,000 dilution in 5% BSA/TBST (Tris-buffered saline-Tween). Protein was detected using SuperSignal West Pico substrate (Thermo Scientific), and band intensity was quantified using GeneSnap image analysis software (Syngene, Frederick, MD).

Note that due to the inconsistencies that arise when immunoblotting experiments are run on different days under different conditions, we performed experiments for each antibody/protein of interest on the same day. Each 26-well gel was loaded with protein lysate from the injured and uninjured leg of the same animal from Cl-IBMECA- and vehicle-treated groups at each of the time points (3, 10, and 24 h). The gels were then simultaneously run and electrophoretically transferred to PVDF membranes. Membranes were then incubated in blocking solution, primary and secondary antibodies, and SuperSignal enhanced chemiluminescence substrate at the same time. In addition, all
membranes were mounted in a single film cassette so that film exposure times were consistent across the membranes. Densitometry values of the bands of interest were consistent across uninjured legs from different animals.

Zymography. Nonreduced denatured protein (30 µg) was heated and resolved on 10% gelatinase zymogram gels (no. 161-1185; Bio-Rad). After SDS-PAGE, denatured and resolved proteins were renatured by incubating the gels overnight at room temperature in renaturation buffer consisting of 2.5% Triton-X 100 and 5 mM CaCl_2. For development, gelatin degradation was performed via 24-h incubation at 37°C in development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 10 mM CaCl_2). Gels were stained with a 50% MeOH-10% acetic acid solution with 0.5% Coomassie blue R-250 at room temperature for 2 h, followed by destaining with 50% MeOH-10% acetic acid until desired contrast was achieved. Clear bands of lysis representing MMP-2 and MMP-9 zymogen activity were visualized for qualitative comparison.

Statistical analysis. Analysis was conducted using the SPSS statistical package version 16.0 (SPSS, Chicago, IL). A repeated-measures (treatment × time) ANOVA was used to compare mRNA (ΔΔCt values) and protein data from injured vs. uninjured muscle (from the same animal) over time. For qRT-PCR statistical analysis, Ct values were used, rather than fold changes. Immunoblotting results for injured and uninjured tissue were compared back to an internal control for exposure intensity. Thereafter, band intensity from injured vs. uninjured muscle (from the same animal) over time were quantified. P < 0.05 was considered statistically significant. A significant ANOVA finding was followed up with Bonferroni post hoc testing.

RESULTS

Protective role of adenosine A_3 receptor stimulation. Adenosine A_3 receptor stimulation with Cl-IBMECA reduced skeletal muscle injury at the 24-h time point. The percentage of cells staining positive for EBD was reduced ~34% at 24 h postinjury (Fig. 2) in the Cl-IBMECA-treated mice (35.4 ± 16.9%, mean ± SD, n = 10) vs. the vehicle-treated mice (56.8 ± 20.2%, n = 10, P = 0.02).

Adenosine A_3 receptor stimulation modulates mRNA levels of the MMPs and TIMPs. MT1-MMP, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 mRNA levels were measured in injured vs. uninjured TA muscle with comparisons between Cl-IBMECA- and vehicle-treated mice. MT1-MMP mRNA levels decreased within 10 h of injury in the Cl-IBMECA-treated mice with no decreases observed in vehicle-treated mice (Fig. 3A). Cl-IBMECA also modulated the MMP-2...
mRNA response postinjury such that no changes in gene expression were observed in the Cl-IBMECA-treated mice whereas MMP-2 mRNA expression was significantly lower in the vehicle-treated mice postinjury (Fig. 3B). In contrast, MMP-3 mRNA expression was blunted in the Cl-IBMECA-treated mice with significant decreases in MMP-3 expression observed at 3, 10, and 24 h postinjury compared with vehicle-treated mice (Fig. 3C). A similar trend was observed for MMP-9 mRNA expression, with increases in MMP-9 mRNA expression returning to baseline levels in the Cl-IBMECA-treated mice only at 24 h postinjury (Fig. 3D). Cl-IBMECA attenuated TIMP-1 gene expression at 24 h postinjury compared with vehicle-treated mice (Fig. 3E). Alternatively, TIMP-2 gene expression increased ~2-fold in the Cl-IBMECA-treated mice between 10 and 24 h of injury, whereas 4- to 6-fold decreases in TIMP-2 expression were observed in the vehicle-treated mice at similar time points (Fig. 3F).

Adenosine A₃ receptor stimulation blunts translation of MT1-MMP, MMP-2, MMP-3, MMP-9, and TIMP-2. Latent (63 kDa) MT1-MMP protein content was significantly increased by ~23% at 3 h postinjury in vehicle-treated mice, with no changes observed in pro/latent (63 kDa) MT1-MMP protein levels in Cl-IBMECA-treated mice at this time point (Fig. 4A). At 24 h postinjury, protein levels of MT1-MMP decreased below levels in the uninjured leg with no differences between groups. The soluble/active (50 kDa) form of the MT1-MMP protein was significantly increased in both groups of mice postinjury, but Cl-IBMECA treatment abrogated this response (Fig. 4B). Vehicle-treated mice had significant increases in the membrane-tethered/active (57 kDa) form of the MT1-MMP protein 24 h postinjury, with no changes observed at this time point for the Cl-IBMECA-treated animals (Fig. 4C).

Cl-IBMECA treatment affected the pro/latent (72 kDa) and active (62 kDa) forms of the MMP-2 protein, although differences were only apparent 3 h postinjury. Specifically, in the Cl-IBMECA-treated mice, the pro/latent form of the MMP-2 protein was decreased ~19% compared with the vehicle-treated mice (Fig. 5A). In contrast, the active form of the MMP-2 protein was ~22% higher at 3 h postinjury in the Cl-IBMECA-treated mice (Fig. 5B).

The MMP-3 protein can be detected in three forms: one latent (59 kDa) and two active forms (45 and 29 kDa). We observed an effect of Cl-IBMECA treatment on all forms of the MMP-3 protein within 24 h of the injury. The pro/latent form of the MMP-3 protein was increased in the Cl-IBMECA-treated mice compared with the vehicle-treated mice at 3 and 10 h postinjury (Fig. 6A). By 24 h postinjury, latent forms of the MMP-3 protein decreased in the Cl-IBMECA-treated mice only while remaining elevated in the vehicle-treated mice. Alternatively, the active forms of the MMP-3 protein (29 and

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**Fig. 5.** MMP-2 protein levels as a result of freeze-injury and Cl-IBMECA treatment. Skeletal muscle MMP-2 protein. A: latent (72 kDa) MMP-2 protein content. B: active (62 kDa) MMP-2 protein content. Representative immunoblot for latent and active forms is shown at right. Data are means ± SE.; n = 6 mice per treatment group/time point. Values are percent change over uninjured leg. *P < 0.05 vs. vehicle-treated mice; †P < 0.05 vs. uninjured leg.
45 kDa) decreased in Cl-IBMECA-treated mice as early as 3 h postinjury, whereas levels did not decrease to similar magnitudes in vehicle-treated mice until 10–24 h postinjury (Fig. 6, B and C).

Similar to MMP-2, MMP-9 exists in latent (92 kDa) and active (82 kDa) forms. Protein levels of the active form are barely detectable in healthy tissue. Here we show that traumatic injury caused a robust increase in latent MMP-9 at 3 h postinjury in vehicle-treated mice, but this response was blunted with Cl-IBMECA treatment (Fig. 7A). Differences among treatment groups in the active form of the MMP-9 protein were observed at 10 h postinjury, at which time there was a significant increase in the active form of the protein in the vehicle-treated mice compared with Cl-IBMECA-treated mice (Fig. 7B).

TIMP-1 and TIMP-2 protein levels were also assessed in the Cl-IBMECA- and vehicle-treated animals postinjury. Three hours postinjury, TIMP-1 protein levels were decreased ~55% in the injured leg compared with the uninjured leg, with no differences observed between treatment groups. This decrease in TIMP-1 protein levels continued through 10 h postinjury, at which point protein levels began to increase (Fig. 8A).

Regardless of treatment, TIMP-2 protein levels were decreased postinjury, peaking at an ~60% decrease in protein levels in both treatment groups 24 h postinjury. The only difference between treatment groups was observed 3 h postinjury, when TIMP-2 protein in the injured leg of the Cl-IBMECA-treated mice was 22% less than TIMP-2 protein measured in the injured leg of the vehicle-treated mice (Fig. 8B).

Immunoblotting for GAPDH protein levels was performed as a loading control. There was no significant difference in GAPDH protein levels with treatment, injury, or time.

MMP-2 and MMP-9 activity are influenced by adenosine A3 receptor stimulation. Indications of MMP-2 and MMP-9 gelatinase activity were determined via zymography analysis (Fig. 9). Breakdown of blue dye-stained gelatin from Tris-HCl gels by gelatinases were revealed as white zones of lysis. These bands of lysis corresponding to the molecular size of MMP-2 and MMP-9 were visualized and assessed. Zymography revealed a small increase in gelatinase activity of active-MMP-2 (62 kDa) in the injured muscle only, with increased MMP-2 activity at 3 h postinjury in the Cl-IBMECA-treated mice compared with the vehicle-treated mice (Fig. 9). Zymography also
revealed increased lysis activity of MMP-9 (82 kDa) postinjury in the injured legs of both groups. However, substantial lysis activity was observed 10 h postinjury, with increased lysis activity apparent in vehicle-treated mice (Fig. 9).

**DISCUSSION**

Previous investigators have shown that activation of adenosine A3 receptors can protect against ischemia-reperfusion injury in heart and skeletal muscle (11, 30, 57) and eccentric injury of skeletal muscle (52). In regard to traumatic, contusion-type injuries, however, the effect of adenosine A3 receptor activation has not been explored, and effective strategies to protect skeletal muscle against this form of injury are needed. To our knowledge, this is the first study to identify a protective role for CI-IBMECA in skeletal muscle that has sustained a traumatic freeze injury. The study demonstrated that activation of adenosine A3 receptors via administration of CI-IBMECA before traumatic skeletal muscle injury reduced the number of injured muscle cells within 24 h of injury. This study also showed that treatment with CI-IBMECA was associated with decreased protein levels of latent and shed MT1-MMP, latent MMP-2, active MMP-3, and latent and active MMP-9, a response that is thought to indicate a reduction in collagen degradation and activation of the inflammatory response (23, 38, 42, 53). CI-IBMECA treatment was associated with increased active MMP-2 and latent MMP-3 protein, alterations that are thought to attenuate inflammatory processes and facilitate extracellular matrix remodeling (36, 42). A summary of these data is presented in Table 1. Figure 1 provides a summary to depict the suggested mechanism of action of CI-IBMECA treatment on the MMP system in injured skeletal muscle.

**Effects of adenosine A3 receptor stimulation on muscle injury.** Evans blue dye is an effective tool to study cellular membrane permeability and membrane-associated fragility (15). Others before us have shown that the EBD technique is an established method to detect injured skeletal muscle myofibers that may not be detected by standard histological techniques (15, 52, 57). Therefore, to quantify myofibers that were damaged as a result of traumatic freeze injury and to determine whether adenosine A3 receptor activation reduced the number of injured fibers, we injected mice with EBD 2 h before the injury. In vehicle- and CI-IBMECA-treated mice, noninjured muscle showed no evidence of EBD within myofibers. Twenty-four hours postinjury, however, the number of cells stained with EBD was ~34% higher in the vehicle-treated mice compared with CI-IBMECA-treated mice. Our data are in agreement with previous work demonstrating reduced EBD uptake in myofibers in CI-IBMECA-treated mice following ischemia-reperfusion (57) or eccentric-contraction injury (52).

**Effects of activation of the adenosine A3 receptor on MT1-MMP.** Primarily, MT1-MMP serves as a collagenase, promoting cellular invasion of type I collagen (8). Activity of

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**Fig. 7. MMP-9 protein levels as a result of freeze injury and CI-IBMECA treatment.** Skeletal muscle MMP-9 protein. A: latent (92 kDa) MMP-9 protein content. B: active (82 kDa) MMP-9 protein content. Representative immunoblot for latent and active forms is shown at right. Data are means ± SE; n = 6 mice per treatment group/time point. Values are percent change over uninjured leg. *P < 0.05 vs. vehicle-treated mice. ‡P < 0.05 vs. uninjured leg.
MT1-MMP is regulated through posttranslational modification and interactions with its inhibitor, TIMP-2 (38). The MT1-MMP/TIMP-2 complex facilitates extracellular matrix turnover by mediating the activity of other MMPs, including MMP-2 and MMP-9 (17, 18). We report here that MT1-MMP mRNA and protein were decreased in the Cl-IBMECA-treated mice postinjury. The underlying basis for the persistently decreased expression of MT1-MMP in the Cl-IBMECA-treated mice may point to a mechanistic role of Cl-IBMECA in promoting anti-inflammatory effects in injured tissues (6). Reduced inflammation subsequently inhibits ERK, which, through its effects on tumor necrosis factor-α (TNF-α), has
been shown to blunt the transcriptional activation and upregulation of MT1-MMP (45, 46, 56). Observed reductions in the MT1-MMP protein 10 h postinjury may decrease collagen breakdown in the injured muscle, thus representing a secondary mechanism through which A3 receptor stimulation reduces the number of injured skeletal muscle cells.

**Effects of activation of the adenosine A3 receptor on MMP-2, MMP-9, and TIMP-2.** The shed form of the MT1-MMP protein is active and processes latent MMP-2 to active MMP-2 (38). Additionally, the TIMP-2 binding domain is intact, serving as a reservoir for TIMP-2 binding, subsequently removing available TIMP-2 from the cell surface. The formation of the MT1-MMP and TIMP-2 complex inhibits the catalytic activity of MT1-MMP and forms a receptor to bind latent MMP-2 (24, 48). Close proximity of the MT1-MMP, TIMP-2, and latent MMP-2 ternary complex is important, because the proximity of the complex to TIMP-2-free MT1-MMP is necessary for MT1-MMP to activate MMP-2 (18). Moreover, others before us have demonstrated that another potential activator of MMP-2 from the latent to the active form is nitric oxide (NO) (20, 55). Decreased levels of TIMP-2 compared with MT1-MMP may prevent MMP-2 localization to the cell surface, whereas excess TIMP-2 may interfere with the available MT1-MMP needed to activate latent MMP-2 (24).

CI-IBMECA intervention manipulated components of this pathway independently, since the observed changes did not support an interactive relationship between molecules. Because MT1-MMP protein levels decreased postinjury, we expected to observe a decrease in both latent and active MMP-2 and TIMP-2. As expected, the latent form of the MMP-2 protein decreased postinjury, similar to changes observed in MMP-2 mRNA. Increases in TIMP-2 mRNA were modest and likely not sufficient enough to drive increases in TIMP-2 protein levels postinjury.

In contrast, active MMP-2 increased postinjury. Because MMP-2 is a critical molecule that functions to facilitate extracellular remodeling (22, 42, 43), increased activity of the MMP-2 protein immediately postinjury in CI-IBMECA-treated mice suggests that adenosine A3 receptor activation enhances protection of the extracellular matrix, promoting a more rapid turnover of collagen IV in the basal lamina. These events may contribute to the reduced number of injured cells observed via EBD analysis at 24 h postinjury in the CI-IBMECA-treated mice.

Unlike MMP-2, MMP-9 is nearly undetectable in healthy tissue. MMP-9 is sensitive to cytokines and growth factors, however, and in response to their induction, there is an immediate and substantial increase in MMP-9 in affected tissues. In addition, inflammatory cells infiltrating the injured area release MMP-9 (2, 22, 42, 58). Once activated, MMP-9 functions to induce rapid turnover of collagen IV for reparative processes. Conversely, overexpression of MMP-9 has been associated with defective tissue healing, suggesting that high levels of activated MMP-9 lead to excessive degradation of extracellular matrix proteins (5). We suspect that the MMP-9 response to the freeze injury is problematic due to the extremely high increases in MMP-9 in both groups postinjury. Effectiveness of CI-IBMECA in mitigating this response was evident in the profound reduction in MMP-9 mRNA within 24 h of the injury in the CI-IBMECA-treated mice.

In vehicle-treated mice, latent MMP-9 protein increased significantly and remained elevated, whereas in the CI-IBMECA-treated mice, latent MMP-9 returned to uninjured levels within 24 h of the injury. Because MMP-9 production is induced by inflammatory cells and cytokines, and previous interventions using CI-IBMECA in other tissues have shown a reduction in the inflammatory response, we suggest that treatment with the adenosine A3 receptor agonist can also mitigate MMP-9 activation in skeletal muscle (13, 22, 23, 27). This observed reduction in MMP-9 mRNA and protein at 24 h postinjury, coinciding with reduced presence of injury in skeletal muscle cells, points to a possible mechanistic influence of adenosine A3 receptor activation in modulating the inflammatory response and subsequent activation of MMP-9, which is highly dependent on proinflammatory cell induction.
Less TIMP-2 protein may have provided increased binding availability of shed MT1-MMP protein in Cl-IBMECA-treated mice. This may have subsequently increased availability for MMP-2 binding and activation. Because MMP-2 is primarily expressed during the regeneration process, it is plausible that regenerative processes are initiated earlier in Cl-IBMECA-treated mice than in vehicle-treated mice (58).

Our data do not support a role for Cl-IBMECA in driving specific adaptations in TIMP-2 protein levels. TIMP-2 protein levels were decreased postinjury in both treatment groups, with TIMP-2 protein levels significantly lower 3 h postinjury in Cl-IBMECA-treated mice. This may be due to increased binding of the TIMP-2 molecule to the MT1-MMP protein or a direct consequence of reduced MT1-MMP activity. The ramifications of the decrease in TIMP-2 protein are inconclusive, because TIMP-2 interactions depend on many factors such as MT1-MMP isoforms and total protein content.

CI-IBMECA treatment influences MMP-3 and TIMP-1 postinjury. MMP-3 is a significant molecule involved in skeletal muscle injury, since it degrades numerous extracellular matrix proteins and the expression and activation of MMP-3 is a regulatory step in the activation of other MMPs (4, 34, 36). For example, mice lacking the MMP-3 gene have an absence of inflammatory cells at the site of injury, contributing to increased scar tissue and delayed healing (34). In contrast, extreme overexpression of MMP-3 mRNA correlates with a robust inflammatory response and potentiates tissue destruction (40). Therefore, TIMP-1, through its inhibitory regulation of MMP-3, is also an important molecule in controlling extracellular matrix proteolysis and regeneration (12).

We report here that injured skeletal muscle produced increased levels of MMP-3 mRNA and latent MMP-3. However, although MMP-3 mRNA was lower in the CI-IBMECA-treated mice between 3 and 24 h postinjury, this did not translate to an immediate reduction in latent MMP-3 protein levels in the treatment group. Latent MMP-3 protein levels were not significantly lower in the CI-IBMECA-treated mice until 24 h postinjury. The acute induction of latent MMP-3 is critical in mediating the proteolysis of substrates surrounding the extracellular matrix and activating processes necessary for tissue remodeling (9, 10, 36). We suggest that the beneficial effects of adenosine A3 receptor activation on extracellular matrix integrity contribute to the acute modulation of the MMP-3 protein in CI-IBMECA-treated mice, with an earlier induction of processes necessary to initiate matrix remodeling. In the vehicle-treated mice, the increased latent MMP-3 protein that endures postinjury may be problematic, because prolonged activation of MMP-3 breaks down the provisional tissue that is formed in the days postinjury. Further evidence of a benefit of Cl-IBMECA treatment in modulation of the MMP-3 response is our data demonstrating an increase in active MMP-3 in the vehicle-treated mice compared with Cl-IBMECA-treated mice. Reduced inflammation due to Cl-IBMECA treatment may contribute to decreased MMP-3 activation and, subsequently, decreased inflammatory cell infiltration at the site of injury (16, 19). Collectively, these adaptations in MMP-3 signaling in CI-IBMECA-treated mice may enhance extracellular matrix integrity immediately postinjury, contributing to the observed decrease in the number of injured muscle cells via EBD analysis.

Despite this, a limitation of Cl-IBMECA treatment was that we did not observe any changes in TIMP-1 protein levels. It is not unreasonable to suggest that TIMP-1 protein levels are decreased in response to the reduced amount of activated MMP proteins, specifically MMP-3. Many factors influence posttranslational processing of the MMPs, so it is difficult to attribute reductions in their inhibitors to a single mechanism.

Summary and conclusions. This work provides evidence that adenosine A3 receptor stimulation provides protection to skeletal muscle from physical traumatic injury via modulation of MMP/TIMP signaling, resulting in favorable alterations that render protection to the extracellular matrix. Indeed, alterations to MMP proteins are specific and dependent on the mechanism of zymogen activation. In this experiment, the beneficial effects of A3 receptor activation on the MMP/TIMP response were observed as early as 3 h postinjury, translating into a reduction of injured fibers at 24 h postinjury. Considering that this time period coincides with the inflammatory response and that A3 receptor activation minimized damage observed 24 h postinjury, the implication is that the beneficial effects of CI-IBMECA on the MMP/TIMP system are in response to the initial inflammatory response.

In summary, the protective role of adenosine A3 receptor activation demonstrated in this study points to a new therapeutic strategy for this form of muscle injury postinjury. Future work should focus on how activation of the adenosine A3 receptor can be combined with other treatment modalities that address other factors associated with the complex and phasic nature of skeletal muscle injury progression and regeneration.

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AUTHOR CONTRIBUTIONS

REFERENCES


