

ASIC3 Is Required for Development of Fatigue-Induced Hyperalgesia

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Abstract An acute bout of exercise can exacerbate pain, hindering participation in regular exercise and daily activities. The mechanisms underlying pain in response to acute exercise are poorly understood. We hypothesized that proton accumulation during muscle fatigue activates acid-sensing ion channel 3 (ASIC3) on muscle nociceptors to produce hyperalgesia. We investigated the role of ASIC3 using genetic and pharmacological approaches in a model of fatigue-enhanced hyperalgesia. This model uses two injections of pH 5.0 saline into muscle in combination with an electrically induced fatigue of the same muscle just prior to the second injection of acid to induce mechanical hyperalgesia. We show a significant decrease in muscle force and decrease in muscle pH after 6 min of electrical stimulation. Genetic deletion of ASIC3 using knockout mice and pharmacological blockade of ASIC3 with APETx2 in muscle prevents the fatigue-enhanced hyperalgesia. However, ASIC3^{-/-} mice and APETx2 have no effect on the fatigue response. Genetic deletion of ASIC3 in primary afferents innervating muscle using an HSV-1 expressing microRNA (miRNA) to ASIC3 surprisingly had no effect on the development of the hyperalgesia. Muscle fatigue increased the number of macrophages in muscle, and removal of macrophages from muscle with clodronate liposomes prevented the development of fatigue-enhanced hyperalgesia.

Thus, these data suggest that fatigue reduces pH in muscle that subsequently activates ASIC3 on macrophages to enhance hyperalgesia to muscle insult.

Keywords Pain · Exercise · Proton · Acid · ASIC · Hyperalgesia · Macrophage

Introduction

Regular exercise remains one of the more effective treatments for chronic pain conditions such as fibromyalgia and low back pain [1, 2], but exercise can acutely exacerbate pain, hindering participation in regular exercise and daily activities [3–6]. Indeed, people with chronic pain show significant reduction in physical activity levels when compared to healthy controls and poor compliance with regular exercise [7, 8]. Therefore, reducing pain during exercise and activity is critical for promoting participation in regular daily activities and effective exercise programs.

The mechanisms that produce an acute exacerbation of muscle pain with exercise in people with chronic pain are not well understood. The enhanced pain response occurs with levels of activity that do not typically produce tissue damage or pain in healthy subjects [9, 10]. Animal studies similarly show an enhanced hyperalgesia when combining fatiguing, nondamaging exercise with a nonpainful low-dose muscle insult [11–13]. We previously show that whole-body fatiguing exercise enhances hyperalgesia to muscle insult and is associated with increased activity in brainstem neurons without significant changes in the muscle metabolites [11, 14, 15]. On the other hand, enhanced hyperalgesia to muscle insult after fatiguing a single muscle was not associated with changes in brainstem neurons [13].

Muscle fatigue releases a wide range of by-products, including protons (decreasing pH) and lactate, which could

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subsequently activate nociceptors to produce pain. Decreasing pH in muscle produces pain and hyperalgesia in healthy human subjects [16, 17], produces hyperalgesia in animals [18], and activates acid-sensing ion channels (ASICs) [19, 20]. During moderate fatiguing exercise, intramuscular pH decreases to approximately 6.6 [21, 22], values that would activate ASICs in muscle [23]. In fact, both protons and lactate produce ASIC-like currents and enhanced intracellular calcium when applied to dorsal root ganglia neurons including those innervating muscle [19, 20, 24–26]. ASIC3, in particular, has been implicated in the development of hyperalgesia in models of muscle pain, both inflammatory and noninflammatory [27–29]. Muscle nociceptors express ASIC3 messenger RNA (mRNA) and protein, and ASIC3 is found in higher quantities in afferents innervating muscle when compared to skin [26, 27, 30]. We therefore hypothesized that decreases in pH during muscle fatigue activate ASIC3 on nociceptors to enhance the hyperalgesia to a low-dose muscle insult.

Materials and Methods

Animals All experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and IASP Ethical Guidelines for the Use of Animals in Research. Male and female mice (6–10 weeks old) were bred at the University of Iowa. Mice on C57BL/6J background lack the ASIC3 gene (ASIC3^{-/-}, $n=7$ male, 8 female) and C57BL/6J mice ($n=97$ male, 22 female). ASIC3^{-/-} mice have been previously characterized [31, 32] and show similar results when compared against wild-type littermates and C57/BL6 in study of muscle pain [27, 28].

Fatigue Paradigm Muscle fatigue was induced using a modified Burke protocol that produces rapidly recovering single muscle fatigue as previously described [13, 33, 34]. Briefly, mice were deeply anesthetized using 2–4 % isoflurane. Needle electrodes connected to a Grass S88 solid-state square waveform generator (Grass Technologies, West Warwick, RI) were inserted into the belly of the gastrocnemius. Baseline maximum force was established by applying three 100-Hz trains at 7 V. To induce fatigue, mice were given 6 min of submaximal contractions using 7-V stimulations at 40 Hz for 3.75 s with 4.25 s of rest between contractions. Three additional maximum force contractions were then elicited to determine the decline in force after fatiguing contractions. Force was measured by attaching the plantar surface of the foot to a force plate connected to an iWORX FT-302 force transducer (iWorx, Dover, NH). Data was collected using LabVIEW software and analyzed using FreeMat and Python scripts. Force transducer data was converted to millinewton using a

standard curve of 1-g weights applied to the apparatus. Fatigue was operationally defined as a decline in force between baseline and final maximum force contractions.

ASIC3^{-/-} ($n=7$ male, $n=7$ female) mice were compared to wild-type mice (male $n=6$, $n=6$ female) for initial maximum contraction force, final maximum contraction force, and at each of the submaximal fatiguing contractions. Additionally, muscle fatigue was measured in mice pretreated with the ASIC3 antagonist APETx2 at low (20 μ M, $n=8$) and high (200 μ M, $n=7$) doses or saline control ($n=9$). Animals missing 20 or more data points (≥ 45 %) in a fatigue recording were excluded from the fatigue analysis (males $n=2$ ASIC3^{-/-}, $n=2$ wild type, saline control $n=1$, low-dose APETx2 $n=2$, high-dose APETx2 $n=2$; females none excluded).

Metabolite Recording Muscle pH was measured in deeply anesthetized animals using a pH probe inserted into the belly of gastrocnemius before and after electrically stimulated muscle contractions. After shaving the overlying hair, an incision was made in the skin to open an 8 mm \times 8 mm square over the center of the gastrocnemius muscle. Muscle fibers were gently teased apart, and the tip of the probe was inserted into the muscle.

For the measurement of pH, a micro-pH probe (Lazar Research Laboratories, Los Angeles) connected to a JENCO 6230N (Jenco Instruments, San Diego) pH meter was calibrated at pH 7.0 and 4.0 before insertion into the muscle of male wild-type mice. In the control group ($n=4$ male, $n=4$ female), the muscle was exposed and measurements were taken 6 m apart. For the experimental group ($n=5$ male, $n=4$ female), the muscle was exposed and then measurements were taken before and after the 6-m fatigue treatment. The pH and force data were sampled continuously at 30 Hz using a Python script to record data from the JENCO 6230N pH meter. Though the sampling was continuous, the pH probe was applied to the muscle for only 30 s at a time to avoid loss of pH sensitivity. When not measuring muscle pH, the sensor was stored in a pH 6.8 phosphate buffer containing heparin, which served as both a reference to ensure sensitivity was maintained and as a cleaning solution. After each contact with the muscle, the tip was cleaned briefly with a cotton swab soaked in acetone.

In order to avoid electrical interference caused by unwanted circuits forming between the main power supply, electrical stimulator, and measurement probes, a wirelessly controlled battery-powered electrical stimulator was developed. An Arduino Uno R3 microcontroller was programmed with the electrically stimulated muscle fatigue protocol. A digital output pin was used to control a transistor that gated a 7-V current through needle electrodes. An RN-42 Bluetooth antenna (Sparkfun Electronics, Boulder, CO) transmitted serial input to and from an Android device in order to receive and confirm instructions from the user. This device was validated against

the Grass S88 solid-state square waveform generator and produced identical contractions at 40 and 100 Hz.

Fatigue-Enhanced Pain Model Muscle pain was induced by combining low-intensity muscle insult with 6 min of fatiguing contractions. On day 1, mice were anesthetized with 2–4 % isoflurane and given an intramuscular (i.m.) injection of 20- μ l normal saline adjusted to pH 5.0. On day 5, mice were anesthetized with 2–4 % isoflurane and underwent 6 min of fatiguing contractions. Immediately after completing fatiguing muscle contractions, the mice were given a second i.m. injection of 20 μ l pH 5.0 normal saline. The pH of the normal saline was adjusted with HCl to pH 5.0 \pm 0.1. Control injections consisted of two injections of normal saline (pH 7.2 \pm 0.1) 5 days apart without the 6 min of fatiguing contractions. The unbuffered pH 5 saline injections reduce muscle pH to approximately 6.9 [18], which is comparable to decreases seen after intense exercise [35, 36]. Previous studies show that the combination of pH 5.0 saline with muscle fatigue is critical for the development of mechanical hyperalgesia, as neither two injections of pH 5.0 alone nor two injections of pH 7.2 saline combined with fatigue produce hyperalgesia [11–13]. On the other hand, two injections of pH 4.0 produce decreases in muscle pH to an average of pH 6.5 and results in long-lasting hyperalgesia [18].

Muscle Withdrawal Thresholds Muscle withdrawal thresholds (MWT) were measured by applying force-sensitive tweezers to the belly of the gastrocnemius muscle as previously described [11], where lower thresholds indicate greater sensitivity. Mice were acclimated to this behavioral paradigm in two 5-min sessions over a 2-day period prior to the first injection. Briefly, mice were placed in a gardener's glove, the hindlimb was held in extension, and the muscle was squeezed with force-sensitive tweezers until the animal withdrew its hindlimb. An average of three trials per animal was taken at each time period. A decrease in withdrawal thresholds was interpreted as muscle hyperalgesia. Our prior work shows that anesthetizing the skin during this test does not change withdrawal thresholds, but anesthetizing the deep tissue increases the threshold thus validating this as a measure of muscle hyperalgesia [37].

Genetic Deletion of ASIC3 in the Exercise-Enhanced Pain Model The role of ASIC3 was assessed using genetic and pharmacological approaches. ASIC3^{-/-} ($n=8$ male, 4 female) were compared side-by-side with C57BL/6 mice ($n=7$ male, $n=4$ female) in the exercise-enhanced pain model. Muscle withdrawal thresholds were measured at baseline and 24 h after the induction of the pain model, i.e., after the second injection of pH 5.0 saline.

Antagonism of ASIC3 in the Exercise-Enhanced Pain Model For the behavioral pharmacology studies, male and

female C57BL/6 mice were pretreated with i.m. injection of APETx2 (males 20 μ M, $n=14$; 70 μ M, $n=8$; 200 μ M, $n=7$, females 200 μ M, $n=4$) or vehicle (0.9 % saline, males $n=16$, females $n=4$) 5 min before muscle fatigue on day 5. 24 h after the second pH 5 saline injection, animals were tested for muscle withdrawal threshold. Animals were divided into multiple groups tested across several weeks. Each testing group consisted of vehicle control and multiple doses of both APETx2.

Neuron-Selective Knockdown of ASIC3 in the Fatigue-Enhanced Pain Model To test the role of neuronal ASIC3 in fatigue-enhanced muscle pain, ASIC3 was selectively down-regulated using herpes simplex virus 1 (HSV-1) expressing an artificial microRNA (miRNA) against ASIC3 (HSV-miR844). Our laboratory developed and characterized this construct showing efficient and functional knockdown of ASIC3 in dorsal root ganglia cells after injection of HSV-miR844 into the gastrocnemius muscle [38]. Further, this strain of HSV selectively targets neuronal cells [39, 40]. In the present study, male C57/BL6 were assessed for muscle withdrawal threshold at three time points: prior to any manipulation (baseline 1), 4 weeks after HSV-1 treatment (baseline 2), and after exposure to the exercise-enhanced pain model. After the first baseline, mice were injected with either HSV-1 expressing miR844 (active, $n=7$) or HSV-1 expressing eGFP (control, $n=7$). Specifically, an incision was made into the skin, and the needle was passed into the belly of the gastrocnemius muscle through that opening. Twenty microliters of solution was injected over the course of 1 min. After injection, the wound was covered by sterile gauze, and the tissue was allowed to absorb the solution for 15 min before closure with a subcuticular suture. The mice were then allowed to recover for 4 weeks. Following incubation, a second baseline muscle withdrawal threshold was taken to assess for potential effects of viral infection on pain behavior. Then, mice were exposed to the fatigue-enhanced pain model, and muscle withdrawal thresholds were repeated for a final time.

The same animals used in the behavioral experiment were then sacrificed, and L4–L6 dorsal root ganglia (DRG) were then sacrificed, and L4–L6 dorsal root ganglia (DRG) were tested for ASIC3 mRNA using quantitative PCR (qPCR) as previously described [29, 38]. Briefly, the DRGs were removed from the animal and stored in RNALater (Qiagen, Valencia, CA). DRGs were transferred to TRIzol (Life Technologies, Carlsbad, CA) and homogenized, and RNA was then isolated using chloroform extraction, glycogen, and isopropanol. First-strand complementary DNA (cDNA) was synthesized from 0.2 to 1 μ g RNA using the SuperScript VILO protocol (Life Technologies). qPCR using validated, predesigned TaqMan assays for ASIC3 (Mm00805460_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out using an ABI prism 7900HT sequence

detector (Applied Biosystems, Foster City, CA). Each cDNA sample was run in triplicate, and expression of ASIC3 was normalized to the expression of GAPDH based on quantification cycle (C_q). The results are expressed as means and SEM of the relative abundances ($2^{-\Delta C_q}$) for the DRG ipsilateral to virus injection.

Quantification of Macrophages in the Fatigue-Enhanced Pain Model To determine if electrical stimulation with or without pH 5.0 saline injections increases the number of macrophages in gastrocnemius muscle tissue, male and female C57BL/6 mice ($n=2$ of each sex per condition) were left untreated, exposed to electrical stimulation alone (immediately after fatigue task), or exposed to electrical stimulation with pH 5.0 saline injections (24 h after second acid injection). Mice were then anesthetized (sodium pentobarbital, 100 mg/kg, i.p.) and transcardially perfused with 4 % paraformaldehyde. The gastrocnemius muscle was removed, stored in 30 % sucrose, and frozen. The muscle was cut with a cryostat onto slides at 20 μ m. Standard immunohistochemical techniques were used. Sections were incubated in the primary antibody, rat anti-mouse F4/80 (1:500, AbD Serotec, Raleigh, NC), overnight at room temperature. The next day, sections were rinsed and incubated in the secondary antibody, goat anti-rat Alexa 488 (Invitrogen, Grand Island, NY), for 1 h at room temperature. Sections were coverslipped with Vectashield and imaged on an Olympus BX-51 light microscope. The total number of F4/80+ cells in five muscle sections was counted from each animal off line using ImageJ software.

Depletion of Macrophages in the Fatigue-Enhanced Pain Model To determine if macrophages contribute to the development of mechanical hyperalgesia in the fatigue-enhanced pain model, macrophages were depleted in the muscle using local injection of liposomes containing clodronate into the muscle of male C57/BL6 mice [41]. Muscle withdrawal thresholds were assessed prior to liposome injection and after exposure to the fatigue-enhanced pain model. Mice were injected with either active liposomes containing clodronate ($n=4$) or inactive liposomes containing PBS ($n=4$). Twenty-four hours after liposome injection, mice were treated with the fatigue-enhanced pain model and withdrawal threshold measured. Depletion of macrophages was confirmed by F4/80 immunohistochemical staining cross sections of treated gastrocnemius muscle ($n=4$ clodronate liposomes; $n=3$ inactive liposomes) using the same approach detailed above.

Statistical Analysis Data are reported as means \pm S.E.M. Muscle force, withdrawal thresholds, and pH measurements were analyzed with repeated measures ANOVA followed by post hoc with a Tukey's test. For pH measurements, differences between groups at baseline and after treatment were assessed by independent t tests, and differences within groups from

baseline to posttreatment were assessed by paired t tests. Macrophage quantification was assessed by one-way ANOVA followed by Tukey's test. Significance for the repeated measures ANOVA and Tukey's test was set at 0.05. To control for multiple comparisons, Bonferroni correction was applied to all of the t tests for differences in pH, adjusting the level of significance to 0.0125.

Results

Muscle Fatigue Is Not Changed by Genetic Deletion or Blockade of ASIC3

The force of muscle contractions across fatiguing stimuli was recorded for ASIC3^{-/-} and compared to wild-type mice (Fig. 1a, b). The initial force of contraction, i.e., the first fatiguing stimuli, was significantly greater in the ASIC3^{-/-} mice (female 6.3 \pm 0.59 mN; male 5.5 \pm 0.78 mN) when compared to wild-type mice (female 4.6 \pm 0.35 mN; male 3.4 \pm 0.59 mN) (univariate ANOVA $F_{1,25}=22.8$, $p=0.005$). There were no sex differences for the initial force of contraction. The force of contraction decreased significantly across time in all three groups. However, there were no significant differences between groups in the postfatigue force when data were controlled with the initial force as a covariate. There were also no differences in the time to peak fatigue, defined as peak decrease+10 % between wild type and knockouts or between sexes (wild-type, female 269 \pm 48 s, male 268 \pm 30s; ASIC3^{-/-}, female 276 \pm 26 s, male 237 \pm 75 s). In the animals treated with APETx2, there were no differences in the initial force, rate of fatigue, or postfatigue force when compared to those treated with vehicle in either male or female mice (Fig. 1c, d).

Muscle Fatigue Decreases Muscle pH in Males but Not Females

The effect of muscle fatigue on muscle pH was assessed by inserting a pH probe into the belly of the gastrocnemius muscle before and after either 6 min of fatiguing muscle contractions or 6 min of inactivity. For males, the initial muscle pH of both groups was comparable: 7.14 (\pm 0.04 SEM) and 7.09 (\pm 0.01 SEM) for the fatigued and inactive groups, respectively. After fatiguing muscle contractions, muscle pH dropped to 6.86 (\pm 0.05 SEM), while the inactive group remained relatively unchanged at 7.15 (\pm 0.04 SEM) (Fig. 2a). Muscle fatigue had a significant effect on muscle pH (repeated measures ANOVA, $F_{1,7}=26.505$, $p<0.05$). At baseline, there were no significant differences between groups (Student's t test, $t_7=1.230$, $p=0.258$). The pH of muscles exposed to fatiguing muscle contractions decreased significantly from baseline (paired t test, $t_4=5.778$, $p=0.004$) and

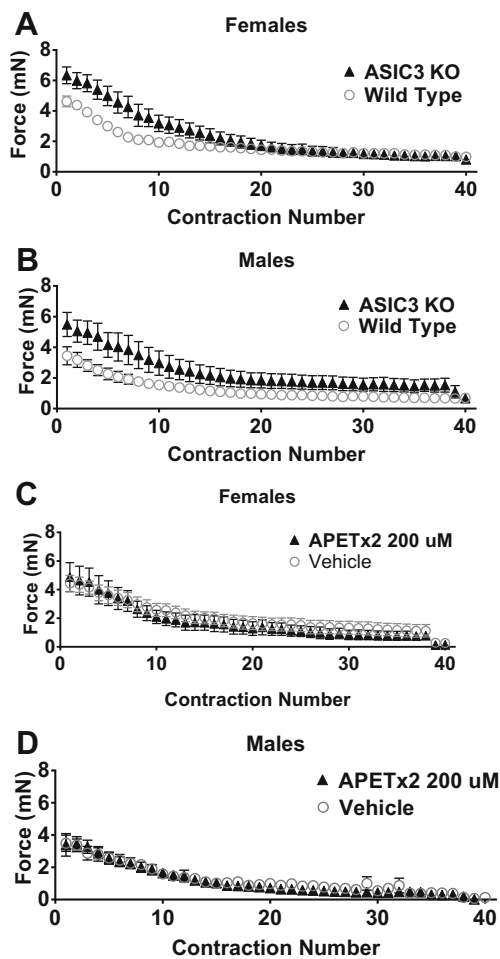


Fig. 1 ASIC3 does not contribute to the development of muscle fatigue. **a** Female and **b** male C57/BL6 mice develop muscle fatigue at similar rates. Initial force on the first contraction was significantly greater in ASIC3^{-/-} mice compared to C57/BL6 mice ($p < 0.05$), but not different between sexes. However, there were no significant differences between wild-type (males $n = 7$, females $n = 7$) and ASIC3^{-/-} (males $n = 6$, females $n = 6$) mice for force of contraction across the 6-min period when normalized for baseline and no difference in force between sexes. In **c** female and **d** male mice, pretreatment with the ASIC3 antagonist APETx2 (200 μ M, males $n = 7$, females $n = 4$) doses had no effect on the rate at which muscle fatigue developed as compared to controls (males $n = 9$, females $n = 4$)

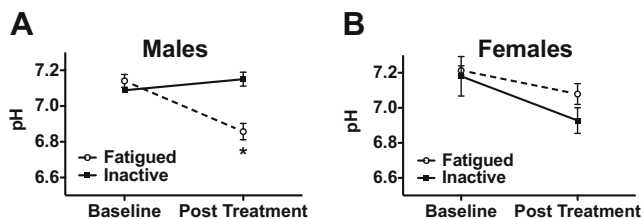


Fig. 2 Electrically stimulated muscle contractions produce significant decreases in interstitial muscle pH in male but not female mice. **a** Male mice treated with 6 min of fatiguing muscle contraction ($n = 4$) showed a significant decrease in interstitial muscle pH as compared to inactive controls ($n = 5$). $*p < 0.05$. **b** Female mice showed no significant difference between fatigued animals ($n = 4$) and inactive controls ($n = 4$)

was significantly lower than the inactive controls (Student's t test, $t_7 = -4.736$, $p = 0.002$). The pH of inactive control muscles did not change from baseline (Fig. 2a, paired t test, $t_3 = -1.452$, $p = 0.243$). In contrast, in female mice, electrically stimulated muscle contractions had no effect on muscle pH (Fig. 2b, repeated measures ANOVA, $F_{1,4} = 2.280$, $p = 0.206$). In contrast to males, for females, muscle fatigue did not result in a decrease in pH from baseline, and there were no differences between inactive and fatigued muscles

Genetic Deletion of ASIC3 Prevents Development of Fatigue-Enhanced Hyperalgesia

The role of ASIC3 in the development of exercise-enhanced muscle pain was tested by comparing muscle withdrawal thresholds in ASIC3^{-/-} animals after exposure to acidic saline and fatiguing muscle contractions and compared to wild-type controls. As previously shown [13], wild-type mice showed a significant decrease in the muscle withdrawal threshold. ASIC3^{-/-} mice showed no decrease and were significantly different from both wild-type mice (Fig. 3a, b, repeated measures ANOVA with post hoc Tukey's test, $F_{2,18} = 20.614$, $p < 0.001$). No sex differences were observed.

Local Blockade of ASIC3 Prevents Development of Fatigue-Enhanced Hyperalgesia

To confirm the results from ASIC3^{-/-} mice and to test if ASIC3 activation at the site of fatigue was necessary, we injected the ASIC3-antagonist ApeTx2 directly into the muscle prior to the fatigue task. As previously shown, mice treated with vehicle (saline) showed a significant decrease in muscle withdrawal threshold. In contrast, APETx2 injected into muscle prevented the decrease in muscle withdrawal thresholds in both male and female mice. In males, the effect of APETx2 occurred in a dose-dependent manner—muscle withdrawal thresholds from mice injected with 70- and 200- μ M doses were significantly higher than vehicle (Fig. 3c, repeated measures ANOVA with post hoc Tukey's test, APETx2 $F_{4,39} = 10.643$, $p < 0.001$). In females, only the highest dose of APETx2 was tested since no sex differences were observed. Females treated with 200 μ M APETx2 had significantly higher muscle withdrawal thresholds than those given vehicle control (Fig. 3d, repeated measures ANOVA, $F_{1,6} = 258.492$, $p < 0.001$).

Downregulation of ASIC3 in Muscle Afferents Has No Effect on Fatigue-Enhanced Hyperalgesia

To test if ASIC3 in neurons innervating the fatigued muscle is required for the development of hyperalgesia in the fatigue-enhanced hyperalgesia, we downregulated ASIC3 by infecting DRGs with HSV-1 expressing a miRNA against

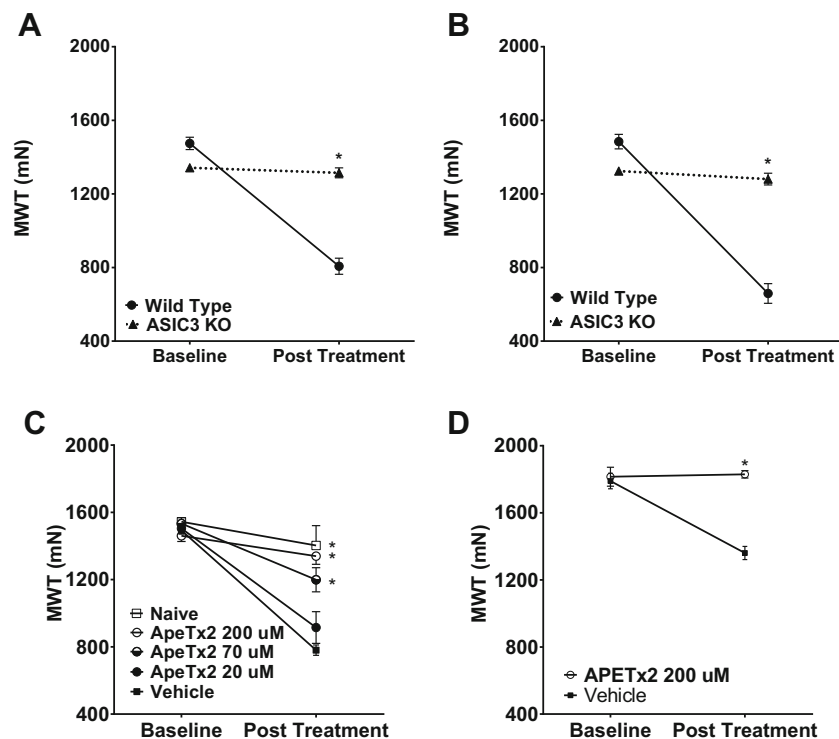


Fig. 3 **a, b** ASIC3^{-/-} mice do not develop mechanical hyperalgesia in the fatigue-enhanced pain model. **a** Male wild-type mice ($n=7$) show significant decreases in muscle withdrawal threshold, while male ASIC3^{-/-} mice ($n=8$) were significantly different, showing no change in muscle withdrawal threshold from baseline. $*p<0.05$. **b** Female wild-type mice ($n=4$) show significant decreases in muscle withdrawal threshold, while female ASIC3^{-/-} mice ($n=4$) were also significantly different, showing no change in muscle withdrawal from baseline. $*p<0.05$. **c, d** Antagonism of ASIC3 during the fatigue task prevents the development of mechanical hyperalgesia in the fatigue-enhanced pain model. **c** Male

C57BL/6 mice were pretreated with 20 μM ($n=14$), 70 μM ($n=8$), or 200 μM ($n=7$) of the ASIC3 antagonist APETx2 or vehicle ($n=16$) during the fatigue task. As a negative control, one group received no treatment ($n=4$). Mice treated with 70 or 200 μM ApeTx2 had significantly higher posttreatment muscle withdrawal thresholds as compared to the vehicle control. **d** Female C57BL/6 mice were pretreated with either 200 μM APETx2 ($n=4$) or vehicle ($n=4$) during the fatigue task. Mice treated with APETx2 had significantly higher posttreatment muscle withdrawal thresholds as compared to vehicle control. $*p<0.05$

ASIC3 [38]. DRGs from mice injected with the HSV-miR844 showed a significant decrease in ASIC3 when compared to DRGs from mice injected with HSV-GFP as a control (Fig. 4a, Student's t test, $p=0.0139$). Despite a significant decrease in ASIC3 in DRG innervating muscle, muscle withdrawal thresholds decreased significantly and, to the same extent, in animals injected with HSV-miR844 and HSV-GFP (Fig. 4b, repeated measures ANOVA, $F_{1,14}=0.325$, $p=0.578$).

Treatment with Electrical Stimulation With or Without pH 5.0 Saline Increases the Number of Macrophages in Muscle Tissue

While previous studies have shown that neutrophils are not changed by electrical stimulation or pH 5.0 saline injection [13], it is unknown if the number of macrophages in muscle tissue changes under these conditions. To test this, C57BL/6 mice were exposed to either electrical stimulation, electrical stimulation with pH 5.0 saline, or left untreated. Immunohistochemical staining with the macrophage marker F4/80 showed a significantly greater

number of cells in the electrically stimulated (mean 381 ± 20 , Tukey's test, $p<0.001$) or electrically stimulated and pH 5.0 saline-treated animals (mean 418 ± 39 , Tukey's test, $p<0.001$) when compared to naïve animals (mean 133 ± 7) (Fig. 5a, one-way ANOVA $F_{2,9}=36.97$, $p<0.001$).

Depletion of Macrophages Prevents Mechanical Hyperalgesia in the Fatigue-Enhanced Pain Mode

Since immune cells, including macrophages, respond to decreases in pH and express ASIC3 [42, 43], we tested if resident macrophages in muscle were necessary for the development of mechanical hyperalgesia in the fatigue-enhanced pain model. Macrophages were depleted locally by intramuscular clodronate prior to induction of the model. Immunohistochemical staining for the macrophage marker F4/80 shows staining of macrophages in muscle from animals that received control liposomes (Fig. 5b) and significantly less from animals that received clodronate liposomes (Fig. 5c). Counting of positively stained macrophages from five sections showed a significant decrease in the number of macrophages from those

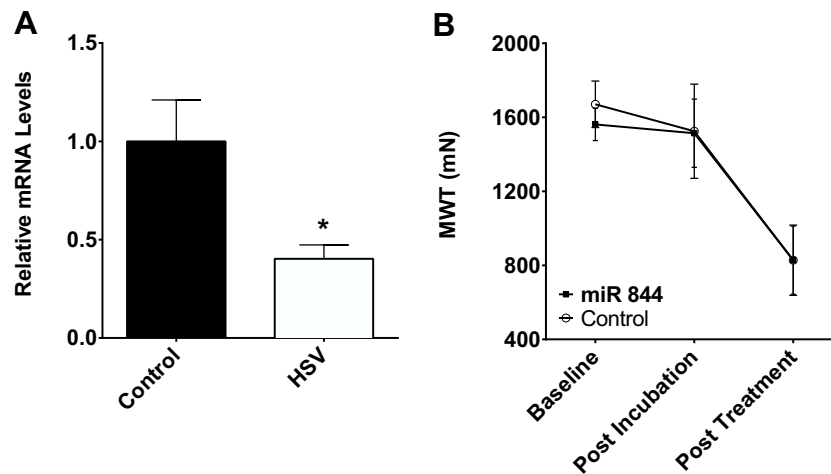


Fig. 4 Neuron-selective knockdown of ASIC3 does not prevent the development of mechanical hyperalgesia in the fatigue-enhanced pain model. **a** Intramuscular injection of herpes simplex virus-1 (HSV-1) expressing miRNA against ASIC3 ($n=8$) into the gastrocnemius muscle produces a twofold decrease in ASIC3 expression in the DRG innervating the gastrocnemius muscle as compared to injection of a control HSV-1 expressing GFP ($n=8$). * $p<0.05$. **b** Mice treated with HSV-1 expressing

miRNA against ASIC3 ($n=8$) 4 weeks prior to exposure to the fatigue-enhanced pain model were similar to those treated with control HSV-1 ($n=8$) at the pretreatment baseline and after 4 weeks of incubation with HSV-1. After treatment with the fatigue-enhanced pain model, mice treated with HSV-1 expressing miRNA against ASIC3 developed hyperalgesia similar to those mice treated with control HSV-1 ($n=8$)

that received clodronate liposomes ($18+6.7$, $n=4$) when compared to those that received inactive liposomes ($66+16$, $n=3$) ($p=0.03$, one-way ANOVA). Mice treated with control liposomes showed significant decreases in muscle withdrawal threshold, while those treated with clodronate liposomes showed no change in muscle withdrawal threshold and were different from controls (Fig. 5d, repeated measures ANOVA, $F_{1,6}=117.16$, $p<0.001$).

Discussion

The current study shows that 6 min of electrically stimulated muscle contractions results not only in substantial fatigue but also in physiologically relevant accumulation of protons, in the detectable range for ASICs. While a number of channels can detect protons, heteromeric channels expressing ASIC3 are necessary for fatigue-enhanced hyperalgesia since genetic deletion and local blockade of ASIC3 prevent development of hyperalgesia. However, our data indicate that ASIC3 on primary afferent fibers innervating muscle is not required for development of hyperalgesia, suggesting that ASIC3 on nonneuronal cells mediates the development of hyperalgesia in this particular model. ASIC3 is expressed in macrophages [43], and we show that muscle fatigue increases the number of macrophages in muscle and removal of macrophages from muscle prevents the development of fatigue-induced hyperalgesia. Therefore, we suggest that decreases in pH from fatiguing muscle activate ASIC3 on resident macrophages that subsequently release algogenic chemicals to produce hyperalgesia.

ASIC3 is critical for the development of hyperalgesia after muscle insult. ASIC3^{-/-} mice do not develop hyperalgesia after repeated acid injections into muscle, inflammation of muscle, or inflammation of joint [27–29, 44–47]. Further, prior studies also show that blockade of ASICs locally in muscle both prevents and reverses muscle hyperalgesia associated with either repeated acid injections into muscle or muscle inflammation [29, 47, 48]. The current study is consistent with these prior studies and shows that fatigue-enhanced hyperalgesia does not occur in ASIC3^{-/-} mice or after pharmacological blockade of ASIC3 in muscle. Muscle afferents express ASIC3, form heteromeric channels, and respond to pH within the ranges produced by fatiguing muscle contractions [25–27, 30, 45]. Muscle afferents from ASIC3^{-/-} mice show no change in pH sensitivity but have a slower rate of desensitization and slower recovery from desensitization [26]. However, the current study shows that depletion of ASIC3 in muscle afferents, with HSV-miR844, has no effect on the development of hyperalgesia. This is in direct contrast to prior studies using the same HSV-miR844 in animals with carrageenan-induced muscle inflammation; hyperalgesia was prevented in these mice [38]. Further, reexpression of ASIC3 in muscle afferents from ASIC3^{-/-} mice rescued the hyperalgesia to muscle inflammation [28]. Together, these data suggest that the fatigue-induced hyperalgesia model is uniquely different from the muscle inflammation model. Indeed, the fatigue-induced hyperalgesia model is not associated with an acute neutrophil response in the muscle [13], while carrageenan muscle inflammation produces a robust neutrophilic inflammation [49]. Further, inflammatory models routinely show upregulation of ASICs and change in sensitivity

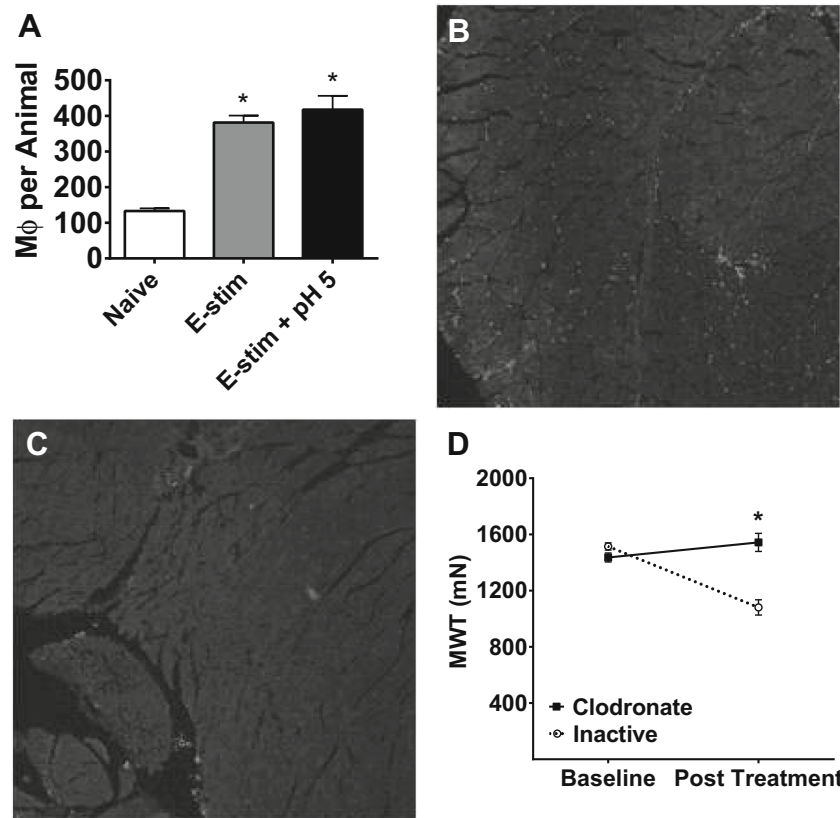


Fig. 5 Fatigue increases macrophages in muscle, and depletion of muscle macrophages prevents fatigue-enhanced hyperalgesia. **a** The total number of F4/80+ macrophages from five sections of muscle was increased immediately after the fatigue task and 24 h after the second pH 5.0 injection in combination with the fatigue task when compared to controls (male $n=2$, female $n=2$ /group). $*p<0.05$. **b** F4/80 immunostain from a gastrocnemius muscle section from a mouse injected with inactive

liposomes. **c** F4/80 immunostain from a gastrocnemius muscle section from a mouse injected with clodronate liposomes. **d** Male C57BL/6 mice treated with intramuscular clodronate ($n=4$) showed significantly higher posttreatment muscle withdrawal thresholds as compared to mice treated with inactive liposomes ($n=4$) prior to exposure to the fatigue-enhanced pain model. $*p<0.05$

to pH in afferent fibers innervating the inflamed tissue and in DRG [38, 44, 50], while repeated acid injections show no such changes in DRGs [25].

Nonneuronal cells express ASIC3 and can mediate the effects of decreases in pH. Prior studies show expression of ASIC3 on synoviocytes, osteocytes, and immune cells [42, 43, 46, 51–53]. Macrophages are distributed throughout the body in nearly every tissue type, including skeletal muscle. They play a role in innate immunity, as well as tissue remodeling, endocrine function, and tumor biology [54]. Macrophages can secrete inflammatory cytokines like interleukin-6 (IL-6), IL-1 β , or TNF which are all known to sensitize nociceptors and produce pain [54–56]. Furthermore, macrophages express a number of receptors and ion channels, including ASIC3, which are capable of monitoring the external environment. Acidic pH (pH 6.5) improves antigen internalization, enhances phagocytosis, and modulates production of cytokines in macrophages [43, 57–59]. Lactate, a known activator of ASIC3, can also activate macrophages [60] and enhances the LPS-induced release of IL-6 from macrophages [61]. This suggests that macrophages may be well suited to

detecting and releasing pro-algesic factors in response to decreases in extracellular pH and these pro-algesic factors (i.e., cytokines, ATP) can subsequently activate receptors located on nociceptors. The current study is consistent with function of ASIC3 on macrophages since macrophages are elevated under conditions which produce fatigue-induced hyperalgesia and depletion of macrophages from muscle prevents the development of fatigue-induced hyperalgesia. Thus, we propose that decreases in pH that occur during fatigue activate ASIC3 on macrophages to release algesic chemical that can subsequently activate nociceptors to produce pain.

Surprisingly, we show sex differences in muscle pH after 6 min of fatiguing isometric contractions, without sex differences in the degree of fatigue. In male mice, there is a decrease in the intramuscular pH to 6.8 with fatiguing contractions of the gastrocnemius muscle; there is no change in females. While the basis for this sex difference in muscle pH is not explored here, a previous study of human subjects performing 8 min of fatiguing isometric exercise showed a similarly greater decrease in muscle pH in male subjects as compared to females [62]. The reason for this may be related to sex

differences in muscle metabolism [63]. Males have a greater proportion of the type II fibers that rapidly generate force [64, 65]. The predominant fibers in female muscle have lower Ca^{2+} -ATPase activity and subsequently slower rates of contraction [66, 67]. Further, muscle in males relies more heavily on glycolysis for energy [68], while females make use of oxidative phosphorylation and lipid metabolism [69–71]. Sex differences in muscle metabolism have a significant effect on the production of lactic acid—after a short sprinting task, males develop significantly higher concentrations of lactic acid than females [72]. This greater accumulation of lactic acid in males may explain the difference in muscle pH between sexes.

The magnitude of pH decrease for males in the present study is consistent with prior studies that show pH values 6.4–6.85 with muscle fatigue [21, 22, 73, 74]. Venous effluent from muscle decreases to a similar degree with exercise (0.3 pH units to pH 7.1) but starts at a higher resting pH (7.4) [75, 76]. Sustained exposure to pH in the 7.0–7.1 range could lead to steady-state desensitization of ASIC channels [77]. Since muscle nociceptors are located in the adventitia of the arteries [30], they are in close proximity to both blood and intramuscular fluid. While the milieu surrounding these nerve terminals is not well understood, it may be the case that the terminals are typically surrounded by pH 7.4 serum and only transiently exposed to the more acidic intramuscular fluid, such as during muscle contractions. In this way, decrease in intramuscular pH may contribute to the fatigue-enhanced pain effect, at least in males. However, the role of pH in the fatigue-enhanced pain effect in females is less clear. It is possible that other metabolites released during muscle fatigue, such as ATP, may play a more significant role in females.

The role of ASIC3 in muscle fatigue is mixed. While the current study showed no difference in fatigue response to electrical stimulation in ASIC3^{-/-} mice or after blockade of ASIC3, we previously show a role for ASIC3 in muscle fatigue. Specifically, male ASIC3^{+/+} mice muscle showed less fatigue than male ASIC3^{-/-} mice and female ASIC3^{+/+} mice (1 h-Rota-Rod task); this difference did not occur in female mice [78]. The discrepancy between studies may be explained by several factors, including the longer duration of exercise, the task-specific nature of muscle fatigue, the role of voluntary effort in task failure, or the muscle fatigued. For example, the current study used a short fatigue task (6 m) resulting in fatigue lasting 10 m in a single muscle [13], as compared to a much longer (3 h) and widespread protocol that produced fatigue lasting (2 h) in our prior study [78]. Notably, when the duration of fatigue task was reduced in our prior study (1.5 h), there was no difference in fatigue between ASIC3^{-/-} and WT mice in males or females [78].

Worsening of pain after exercise remains a significant barrier to adherence to an exercise regimen that is crucial for treating diseases like myofascial pain syndrome, fibromyalgia,

chronic fatigue syndrome, and low back pain [5, 79, 80]. Many mechanisms likely contribute to the development of pain after exercise, including tissue damage and inflammation, but for fibromyalgia patients whose symptoms acutely worsen with even light or moderate exercise that does not damage muscle, targeting ASICs may provide symptomatic relief [81, 82]. Studies show that consistent exercise improves the symptoms in people with fibromyalgia [83] and prevents the development of chronic and exercise-induced pain in animals [84]. Further, patients with chronic fatigue syndrome and fibromyalgia report worsening of their fatigue symptoms with exercise, which is correlated with significant upregulation of ASIC3 mRNA in the blood, suggesting that ASIC3 may contribute to the subjective feeling of fatigue [6]. By inhibiting ASICs prior to exercise in the early period of an exercise regimen, it may be possible to address the acute exacerbation of pain and fatigue with exercise, leading to greater adherence to a regular exercise program.

In conclusion, these data implicate ASIC3 in the development of exercise-enhanced pain through a decrease in pH from the fatigued muscle. Muscle fatigue reduces intramuscular pH which is in the range for activating ASICs. Studies in knockout mice indicate that ASIC3 is the essential subunit within the wild-type channel on muscle DRG but that ASIC3 effects are not due to its location on muscle primary afferent fibers. We further show that depletion of macrophages prevents the fatigue-induced hyperalgesia and suggest that ASIC3 on macrophages mediated the fatigue-enhanced pain response.

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